Inhibition of Type A and Type B (Proteolytic) *Clostridium botulinum* by Sorbic Acid

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The effect of sorbic acid in the pH range 4.9 to 7.0 on the probability P of growth of a single vegetative bacterium of proteolytic strains of *Clostridium botulinum* has been determined by comparison of the most probable number count of the bacteria in media at pH 4.9 to 7.0 containing a series of concentrations of potassium sorbate and in a nutrient medium at pH 6.8 to 7.0. The media were maintained under strictly anaerobic conditions at a redox potential equivalent to lower than -350 mV at pH 7. In medium adjusted to the required pH with HCl, P for strain ZK3 (type A) at pH 5.1 or 5.5 after 2 days at 30°C was similar to that at pH 6.8 to 7.0 but was slightly lower at pH 4.9. Potassium sorbate inhibited growth, the inhibition being a function of the concentration of undissociated sorbic acid. A calculated undissociated sorbic acid concentration of 156 mg/liter delayed growth of strain ZK3 (type A) but did not result in a significant decrease in P after an incubation time of 14 days. Higher concentrations of undissociated sorbic acid caused longer delays before maximum most probable number counts developed, and a calculated undissociated sorbic acid concentration of 282 mg/liter decreased log P for strain ZK3 after an incubation time of 14 days by a factor of 5.5 to 7.5. Four additional type A strains and five type B strains were inhibited to an extent comparable to inhibition of strain ZK3. A mathematical model was developed that related the effect of undissociated sorbic acid and incubation time to the probability of growth of strain ZK3. A total sorbic acid concentration of 1,000 mg/liter would reduce the probability of growth at 30°C in 14 days by a factor of the order of 10^8 at pH 5 and by a significantly higher factor at pH values below this.

Sorbic acid and its salts are widely used as preservatives in food (19, 28, 29), and although they have been used mainly to inhibit the growth of yeasts and molds, they are also valuable in some foods as antibacterial agents. The effect of sorbic acid and sorbates is greater at low pH than at pH 6 to 7. and there is evidence that the major inhibitory effect is due to the undissociated acid, although inhibition by the dissociated acid at pH values above 6 has been reported (7). Species of clostridia were reported to be relatively resistant to sorbic acid (8). A culture medium containing sorbic acid, 1.2 g/liter, was found to be useful for enrichment and isolation of clostridia, and the limiting pH for growth of *Clostridium botulinum* was not appreciably affected by the presence of this concentration; moreover, sorbic acid was utilized as a carbon source by proteolytic strains of this bacterium (35, 36). Despite these reports, work between 1974 and 1978 indicated that potassium sorbate at a concentration of 2,600 mg/kg could be used to replace a high proportion of the nitrite added to cured meat products as an inhibitor of *C. botulinum* (30, 33). Subsequent studies have provided further evidence that the addition of potassium sorbate to poultry products (10, 13), cured meats (25), ham and bacon (14), and cheese spread (4) delayed growth of and toxin formation by *C. botulinum*.

Potassium sorbate at concentrations sufficient to give a calculated undissociated sorbic acid concentration of 250 mg/liter in culture media at pH 5.5 to 7.0 retarded the growth of proteolytic strains of *C. botulinum* from spores and from vegetative bacteria (1, 3). There appears, however, to be no published systematic work than enables assessments to be made of the effect of potassium sorbate or sorbic acid on the

probability of growth of *C. botulinum* at pH 5.5 to 7.0 or under more acidic conditions.

The purpose of the work described in this report was to determine the effect of sorbic acid and pH on the number of vegetative *C. botulinum* cells capable of growth, compared with the number capable of growth under optimum conditions. The inhibitory effect could therefore be expressed in a manner similar to the use of the pseudo-D-value (17) and the decimal reduction in viable count (9) or as an effect on the probability of growth (9, 11).

MATERIALS AND METHODS

Chemicals. Potassium sorbate was obtained from Sigma Chemical Co., Poole, England. Other chemicals were obtained from BDH Chemicals Ltd., Poole, England, and were Analar grade unless specified otherwise.

Bacteria. The cultures used were *C. botulinum* type A strains ZK3, 62A, VL1, 16037, and NCTC 3805 and proteolytic type B strains 2345, B6, NCIB 10657, 3262, and 3266.

Strain 16037 was obtained from the Leatherhead Food Research Association, Leatherhead, England; strain NCIB 10657 was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland; and the remaining strains were obtained from J. S. Crowther, Unilever Research, Sharnbrook, Bedford, England. The cultures were maintained as described previously (22).

Media. Cooked meat medium was used to maintain cultures, and VL layered blood agar and heart infusion agar (Difco Laboratories, Detroit, Mich.) were used to check cultures for purity (22).

The basal medium used for tests of the effect of sorbic acid was peptone-yeast-glucose-starch medium (PYGS medium)

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(22) modified by lowering the concentration of glucose from 5 to 2 g/liter and by either omitting cysteine hydrochloride or lowering its concentration to 0.2 g/liter.

The medium was prepared under strictly anaerobic conditions with a headspace of oxygen-free N_2-H_2-CO_2 (85:10:5), adjusted to the required pH with 1 M HCl, and sterilized by autoclaving. Potassium sorbate solutions were prepared by using a known weight of compound that had been maintained in an anaerobic cabinet overnight. The potassium sorbate was dissolved, to give the required concentration, in distilled water that had been boiled to remove dissolved oxygen, cooled, and maintained under a headspace of oxygen-free N_2-H_2 (80:10). The solution was sterilized by filtration through a membrane filter (pore diameter, 0.22 μm; Millipore U.K., Ltd.) in an anaerobic cabinet, and a 10-ml volume was added aseptically to the sterile basal medium (290 ml). The pH was measured and adjusted, if necessary, with 1 M HCl or 1 M KOH under strictly anaerobic conditions. Portions of medium (9 ml) were then distributed aseptically under oxygen-free N_2-H_2-CO_2 (85:10:5) into 15-ml glass vials (21).

The concentrations of potassium sorbate added to the media were chosen to give the same calculated concentrations of undissociated sorbic acid at each pH, assuming that the pK_a of sorbic acid in water was 4.76 (23, 34). Storage of medium at ambient temperature for 36 days resulted in no significant decrease in the concentration of sorbic acid. The potassium sorbate was added to the medium as a solution sterilized by filtration, because of its possible destruction during autoclaving. When medium containing potassium sorbate was sterilized by heating at 121°C for 15 min, a 10% decrease in concentration of sorbic acid occurred.

Measurement of pH and redox potential. The pH of three vials from each batch of medium was measured on the day before use. Measurements were made with Philips digital pH meters (PW 9409) and combined glass and reference electrodes (type 401-M5) (both from Pye-Unicam Ltd.) at 20 to 25°C. The electrodes were calibrated with freshly prepared buffers (pH 4 and pH 7 buffer powders; Pye-Unicam Ltd.) immediately before each set of measurements.

Redox potentials were measured with the same meters, in the millivolt measuring mode, and combined platinum and reference microelectrodes (type 4800-M5; Pye-Unicam Ltd.) as described by Lund and Wyatt (21). The E_h of the silver/silver chloride electrode 3 M KCl half-cell was taken as +210.5 mV at 20°C (5).

The redox potential measurements based on the standard hydrogen electrode (E_h measurements) were expressed in terms of the equivalent E_h at pH 7 (E_h7) by using the following equation (18) and assuming $n = 1$:

$$E_h7 = E_h \text{ at pH } x + 2.303 \ (RT/nF)(\text{pH } x - \text{pH } 7)$$

where R is the gas constant (8.314 J/mol per K), T is temperature (in kelvin), F is the Faraday constant (96,487 J/V per mol), n is the number of electrons liberated from one molecule of reducant, and pH x is the measured pH of the system. At 25°C, 2.303 $RT/F = 0.0591$ V.

Determination of the effect of pH and potassium sorbate on the probability of growth of single vegetative bacteria of *C. botulinum*. A culture grown in modified PYGS medium at 30°C for 24 h was used to inoculate a further 9-ml volume of the same medium in an anaerobic culture tube (Hungate-type, 16 by 125 mm; A. R. Horwell, Ltd., London, England); this culture was incubated at 30°C for 48 h. After incubation the turbidity of the culture was measured by

using a nephelometer (Evans Electroselenium Ltd., Essex, England); the approximate number of viable bacteria present was estimated from a prior determination, in modified PYGS medium, of the relationship between turbidity and viable count by the most probable number (MPN) method, and this culture was used to prepare inocula. When an inoculum contained a mixture of strains, it was prepared by combining appropriate volumes of several cultures.

Serial 10-fold dilutions of the culture were prepared in modified PYGS medium adjusted to pH 5.2 with HCl under N_2-H_2 (90:10). By using sterile disposable syringes and 26-gauge needles, 0.2 ml of a range of dilutions was inoculated into each of five replicate vials of the test medium and into five replicate vials of modified PYGS medium (pH 6.8 to 7.0); the vials were then incubated at 30°C and examined at regular intervals for up to 4 weeks, and the number that showed growth, evident as a visible increase in turbidity, was recorded. Samples of cultures grown from the lowest inocula were examined by phase-contrast microscopy and plated onto heart infusion agar, incubated in air, and onto VL layered blood agar, incubated under H_2-CO_2 (90:10), to test for purity.

The MPN count of bacteria capable of growth under the test conditions after the stated incubation time was calculated by the method of Hurley and Roscoe (15).

The probability of growth of a single bacterium (P) (11) under the test conditions after the stated time was calculated as: P = (MPN of bacteria that resulted in growth under the test conditions)/(MPN of bacteria inoculated into the test conditions, determined in the basal PYGS medium [pH 6.8 to 7.0]). The 95% confidence limits were calculated as previously described (20).

Assay of sorbic acid in media and in cultures after growth of *C. botulinum*. Samples of sterile media were filtered through a 0.45-μm (pore size) filter (Millipore); samples from cultures were heated at 121°C for 15 min and cooled before filtration.

The samples were diluted, and the concentration of sorbic acid was determined by high-pressure liquid chromatography. The apparatus consisted of a model 420 B autosampler fitted with a 20-μl injection loop, a series 2 pump (The Perkin Elmer Corp.), and a stainless steel column (250 mm by 4.6 mm [inner diameter]) packed with 5-μm Zorbax octadecylsilyl silica (ODS) (Dupont, U.K., Ltd.) protected by an RP18 guard column (Brownlee Labs, Santa Clara, Calif.) packed with 5-μm ODS. The eluant flow rate was 2 ml/min, and sorbic acid was detected by A_{260} with a model LC-75 variable-wavelength UV detector (Perkin Elmer). The detection time was ca. 10.4 min.

RESULTS

Redox potential of culture media. The mean E_h of the basal modified PYGS medium at pH 6.8 to 7.0 was −353 mV (n = 4). The mean E_h values of medium at pH 5.5, 5.1, and 4.9 were −285, −258, and −258 mV, respectively. On the basis of the assumption that one electron was liberated from one molecule of reductant in the dominant redox reaction (22) the calculated E_h7 values of the media at pH 4.9 to 5.5 were between −374 and −381 mV. The presence of potassium sorbate at the concentrations used and of cysteine hydrochloride (0.2 g/liter), had no significant effect on the E_h. After incubation of medium at 30°C for 4 weeks, no significant change in E_h occurred.

Effect of pH and potassium sorbate on the probability of growth of a single vegetative bacterium of *C. botulinum* ZK3.

TABLE 1. Effect of sorbic acid and pH on the time required for growth and on the probability (P) of growth from a single vegetative bacterium of *C. botulinum* ZK3 in PYGS medium at 30°C[a]

Medium[b] parameter			$-\text{Log } P^d$ of growth in following time (days):						
pH	Total sorbic acid (mg/liter)	Undissociated sorbic acid (mg/liter)[c]	2	3	4	5	7	14	21
7.0	0			0	0	0	0	0	0
	2,000	11	−0.12	−0.12	−0.12	−0.12	−0.12	−0.12	−0.12
6.0	2,000	109	3.0	1.28	0.03	0.03	0.03	0.03	0.03
5.5	0	0		0.15	0.15	0.15	0.15	0.15	0.15
	1,015	156		3.56	2.53	0.51	0.28	0.28	0.28
	1,219	188		4.69	3.65	2.92	1.48	1.08	1.08
	1,421	219		6.65	5.65	5.55	5.17	3.34	3.14
	1,828	281		7.65	6.65	6.65	6.53	5.65	5.65
5.1	0	0	0.76	0.76	0.76	0.76	0.76	0.76	0.76
	500	156	>6.80	6.80	5.18	4.50	3.08	1.53	1.53
	600	188		6.15	4.69	4.00	3.94	1.79	1.79
	700	219	>6.8	5.72	5.72	4.76	4.20	1.58	1.58
	900	282	6.72	6.72	6.72	6.72	6.72	6.08	5.42
4.9	0	0	1.6	1.2	1.2	1.2	1.2	1.2	
	374	157	>6.08	>6.08	4.72	4.08	1.49	1.49	
	449	189	7.00	6.00	5.00	4.87	2.66	1.82	
	524	220	7.04	7.04	7.04	7.04	6.00	6.00	
	674	283	>8.08	>8.08	>8.08	>8.08	7.49	7.49	

[a] The results given are those from experiment 3 in Table 2. Similar results were obtained in experiments 1 and 2.
[b] The basal medium was modified PYGS (see Materials and Methods) under a headspace of oxygen-free N_2-H_2-CO_2 (85:10:5).
[c] Calculated from $pK_a = 4.76$.
[d] 95% confidence limits, ca. $\pm$ 0.72.

In the basal PYGS medium at pH 6.8 to 7.0, the maximum MPN count was obtained after incubation for 1 to 2 days at 30°C. In medium adjusted to pH 4.9, 5.1, and 5.5 with HCl, growth of the maximum MPN count usually required incubation for 2 days (Table 1). At pH 5.1 and 5.5, the probability of growth of bacteria in 2 days was not significantly lower than that at pH 6.8 to 7.0, while at pH 4.9, the probability was slightly lower (Table 1).

Potassium sorbate equivalent to a calculated undissociated sorbic acid concentration of 156 mg/liter delayed growth, and incubation for 5 days or longer was required before the maximum MPN counts were obtained, but the probability of growth in 14 days was not significantly lower than that in the absence of sorbate (Tables 1 and 2).

Increased concentrations of potassium sorbate caused greater delays before maximum MPN counts developed, and concentrations giving a calculated undissociated sorbic acid concentration of 282 mg/liter reduced the probability of growth within 14 days at 30°C by a factor of between ca. $10^{5.5}$ and $10^{7.5}$ (Tables 1 and 2). Any increases in MPN counts after longer incubation were relatively small. The inclusion of cysteine hydrochloride (0.2 g/liter) in the medium did not significantly affect the inhibition by sorbic acid (results not shown).

Potassium sorbate equivalent to a total sorbic acid concentration of 2,000 mg/liter caused no detectable inhibition at pH 7.0, at which the calculated concentration of undissociated sorbic acid was 11 mg/liter, and only a very slight delay in growth at pH 6.0, at which the calculated concentration of undissociated sorbic acid was 109 mg/liter (Table 1).

Effect of potassium sorbate on the probability of growth of

vegetative bacteria of nine proteolytic strains of *C. botulinum* at pH 5.1. The sensitivity of a further nine proteolytic strains of *C. botulinum* to sorbic acid in medium at pH 5.1 was tested. Potassium sorbate was added to give a total sorbic acid concentration of 1,000 mg/liter and a calculated undissociated sorbic acid concentration of 314 mg/liter. The additional strains of *C. botulinum* all grew well at pH 5.1, and the maximum MPN counts were recorded after 2 to 3 days at 30°C. In the presence of a total sorbic acid concentration of 1,000 mg/liter, the probability of growth within 14 days at 30°C was reduced by a factor between 10^5 and 10^7, and none of these strains was markedly more resistant to sorbic acid than was strain ZK3 (see Fig. 2).

Mathematical model of the effect of sorbic acid on strain ZK3. Graphical study of the experimental data showed that each series of measurements of probability of growth, over a range of incubation times, followed a curve whose shape varied randomly between series as well as deterministically in response to concentration of undissociated sorbic acid. This behavior suggested the fitting of a nonlinear, random-effects regression model (24) to the data to produce predictive equations relating the probability of growth to the factors investigated.

The model is of the form $-\log P = A + Be^{-C(t-2)}$, where t is the time in days and A, B, and C are random-effects parameters releated to the concentration of undissociated sorbic acid. All logarithms are to the base 10. Analysis of variance indicated no significant effect of pH in this range (pH $\geqslant$ 4.9) other than through its effect on the concentration of undissociated sorbic acid.

The relationship of A, B, and C to the concentration of

TABLE 2. Effect of sorbic acid and pH on the probability (P) of growth from a single vegetative bacterium of C. botulinum ZK3 in PYGS medium in 14 days at 30°C

Medium[b] parameter			−log P[c] of growth in 14 days in expt no.:		
pH	Total sorbic acid (mg/liter)	Undissociated sorbic acid[b] (mg/liter)	1	2	3
5.5	0	0	0	0.34	0.15
	1,015	156	0.38	1.25	0.28
	1,219	188			1.08
	1,421	219	3.23	1.67	3.34
	1,828	281	5.53	4.32	5.65
5.1	0	0	0.38		0.76
	500	156	0.20	2.20	1.53
	600	188			1.79
	700	219	1.50	3.56	1.58
	900	282	5.53	5.34	6.08
4.9	0	0	0.53	2.82	1.20
	374	157	0.28	3.41	1.49
	499	189			1.82
	524	220	4.53	3.48	6.00
	674	283	6.38	6.60	7.49

[a] See Table 1, footnote b.
[b] See Table 1, footnote c.
[c] See Table 1, footnote d.

undissociated sorbic acid was determined by the following two-step procedure. A separate exponential curve was fitted to each series of observations, estimating three parameters, A, B, and C of the curves. A regression model was used to relate the estimated values of the parameters to undissociated sorbic acid concentrations and pH. The parameters A, log B, and log C were each found to have a linear relationship to the concentration of undissociated sorbic acid in the range 150 to 300 mg/liter. The regression model was extended to the 0- to 150-mg/liter range by using the relationship derived in the 150- to 300-mg/liter range and the data obtained at zero concentration of sorbic acid; this gives a general guide to the effect of undissociated sorbic acid in this range.

The equations relating A, B, and C to the concentration of undissociated sorbic acid in grams per liter (x) are as follows:

$$A = 0.44 + 0.0338(x - 150) \qquad 150 < x < 300$$
$$A = 0.44 \qquad 0 < x < 150$$
$$\log B = 0.843 - 0.00309(x - 150) \qquad 150 < x < 300$$
$$\log B = 0.843 - 0.00761(150 - x) \qquad 0 < x < 150$$
$$\log C = 0.539 - 0.00522x \qquad 0 < x < 300$$

The predicted relationship between log P and time for six concentrations of undissociated sorbic acid is shown in Fig. 1.

On the basis of these results, and assuming that the pK$_a$ of sorbic acid in water is 4.76, the predicted effect of a total sorbic acid concentration of 1,000 mg/liter, at pH values between 7.0 and 4.8, on log P after incubation times of 3, 5, and 14 days is demonstrated in Fig. 2. The experimental results for the additional nine strains at pH 5.1 after incubation for 14 days are also included for comparison.

The results predicted by the model are only strictly valid for undissociated sorbic acid concentrations up to ca. 300 mg/liter. If these results can be extrapolated to higher concentrations of undissociated sorbic acid, the probability of growth in the presence of a total sorbic acid concentration of 1,000 mg/liter will be decreased by factors of the order of 10^{10} to 10^{12} at pH 4.9 to 4.8; at pH < 4.9, the probability of growth would be further reduced because of the effect of hydrogen ion concentration per se (B. M. Lund, A. F. Graham, and J. G. Franklin, Int. J. Food Microbiol., in press).

Breakdown of sorbic acid by C. botulinum. In medium at pH 5.1 that contained a total sorbic acid concentration of 600 mg/liter, an inoculum of 10^6 bacteria of strain ZK3 resulted in growth within 1 day and a decrease in sorbic acid concentration of 5 to 20% (wt/vol) (two vials). In vials that were incubated for a further 5 days after growth was first observed, the sorbic acid concentration had decreased by more than 95%. When 2.4 × 10^7 bacteria, consisting of approximately equal numbers of the mixture of strains shown in Fig. 2, was inoculated into medium at pH 5.1 containing sorbic acid at 1,000 mg/liter, growth was observed in 2 days with a decrease in concentration of sorbic acid of 16% (wt/vol). In
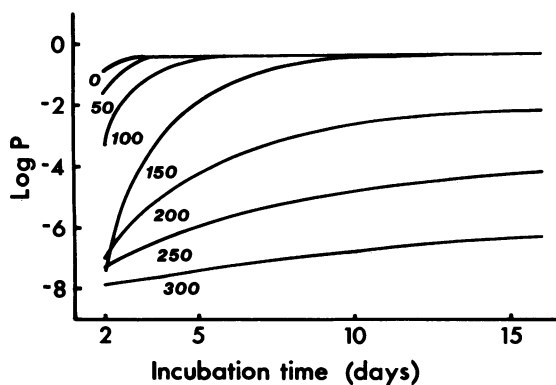

FIG. 1. Predicted effect of undissociated sorbic acid on the probability (P) of growth of C. botulinum as a function of incubation time. Data are given as the log probability of growth of a single vegetative bacterium at 30°C calculated from the model based on results for strain ZK3. Numbers in italics are concentrations of undissociated sorbic acid (in milligrams per liter).
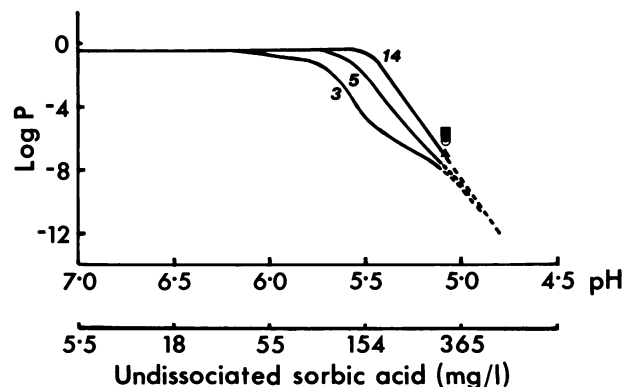

FIG. 2. Effect of pH on the probability (P) of growth of a single vegetative bacterium of C. botulinum within 3, 5, and 14 days at 30°C in the presence of a total sorbic acid concentration of 1,000 mg/liter. Continuous lines predicted from the model are based on results for strain ZK3 and a pK$_a$ for sorbic acid of 4.76. Numbers in italics are incubation times (days). Symbols represent experimental results after incubation for 14 days: ●, strain 62A; ○, strain VL1; ▲, strain 16037; ■, mixture of strains NCTC 3805, 2345, B6, NCIB 10657, 3262, and 3266.

vials incubated for 13 days after growth was first observed, the sorbic acid concentration decreased by more than 90%.

DISCUSSION

In assessing the effect of inhibitory conditions on the growth of *C. botulinum*, it is important to control, and usually to minimize, any inhibitory effect due to traces of oxygen and high redox potential. The degree to which anaerobiosis and low redox potential are achieved is seldom reported in studies of the effect of inhibitory factors on the growth of *C. botulinum*, although inhibition of a strain of type A by low pH, sodium chloride, or a high concentration of sucrose was shown to be greater at an E_h7 of -60 mV than at an E_h7 of -145 mV (26). In the present work, the experiments were done under strictly anaerobic conditions at redox potentials equivalent to lower than E_h7 of -350 mV, redox potentials which were probably influenced strongly by the hydrogen present in the gas phase.

In the absence of potassium sorbate, *C. botulinum* ZK3 grew rapidly at pH 5.5, 5.1, and 4.9. The probability of growth at pH 5.5 and 5.1 in 3 days at 30°C was not significantly different from that at pH 6.8 to 7.0, while at pH 4.9, the probability was 30-fold lower.

The addition of sufficient potassium sorbate to give a total sorbic acid concentration of 2,000 mg/liter caused no inhibition of *C. botulinum* at pH 7 and a relatively slight delay in growth at pH 6. Lower concentrations of total sorbic acid at pH 5.5, 5.1, and 4.9 caused a delay before growth and a decrease in the probability of growth of a single bacterium; the effect was dependent on the concentration of undissociated sorbic acid.

Eklund (7) reported that at pH 5.9 for *Bacillus cereus* and at pH 6.6 for two strains of *B. subtilis*, dissociated and undissociated sorbic acid contributed equally to inhibition. In his experiments, however, the MICs of total sorbic acid against *Bacillus* spp. at pH 6.6 were between 5,500 and 8,500 mg/liter, i.e., much higher than those used in our experiments or permitted as preservatives in foods in the United Kingdom.

The results of our work show that the inhibitory effect of potassium sorbate on the growth of *C. botulinum* increases markedly with decrease in pH between pH 7.0 and 4.9 and that the effect is dependent on the concentration of undissociated sorbic acid.

A nonlinear, random-effects model was found to be appropriate to describe the deterministic and random behavior observed in our experiments. Other workers have used a logistic regression model either for P (25) or for $\log P$ (16). These methods were inadequate for our results, first because of the form of the relationship of $\log P$, time, and sorbic acid concentration, particularly the marked differences in the level of $\log P$ towards the end of each time series, and second because of the random variation in the parameters of this relationship. A two-step procedure was used to fit the model; an improved technique would involve a more complex, single-step procedure involving the Expectation Maximization (EM) algorithm (32).

For most foods in which sorbic acid is permitted as a preservative in the United Kingdom the maximum concentration allowed is 1,000 mg/kg or lower (12). The model developed from the results with strain ZK3 was used to predict the influence of pH on the inhibitory effect of a total sorbic acid concentration of 1,000 mg/liter after incubation for 3, 5, and 14 days at 30°C (Fig. 2), and the experimental results for nine additional strains at pH 5.1 agreed reason-

ably with the prediction. At undissociated sorbic acid concentrations higher than 200 mg/liter, the rate of increase of $\log P$ had become very low after 14 days (Fig. 1). Foods with a pH higher than 4.5 are subject to the risk of growth of *C. botulinum*. Figure 2 shows the marked increase of the inhibitory effect of sorbic acid with a decrease in pH from 5.5 to 4.5. While the results cannot strictly be extrapolated to undissociated sorbic acid concentrations higher than about 300 mg/liter, the data indicate that the presence of a total sorbic acid concentration of 1000 mg/liter at pH values of 5.0 to 4.8 would decrease the probability of growth of vegetative *C. botulinum* cells by factors of the order of 10^8 to 10^{12}, and at pH < 4.9, the probability of growth would be further reduced by the effect of the concentration of hydrogen ions.

Growth of *C. botulinum* resulted in a decrease in the total concentration of sorbic acid, presumably as a result of utilization by the organism. Significant growth occurred before depletion of the major part of the sorbic acid. At pH 5.1 in the presence of total sorbic acid concentrations of 600 to 1,000 mg/liter, only 20% or less of the sorbic acid had been utilized when growth was observed, most of the remainder being used during longer incubations.

York and Vaughn (35) reported that proteolytic strains of *C. botulinum* were relatively resistant to sorbic acid; these workers routinely used liver infusion broth or agar containing 0.12% sorbic acid for isolation of *Clostridium* spp. According to our results, this concentration of total sorbic acid, at pH 5.5 or higher, would have little effect on the growth of strain ZK3 from an inoculum of 10^4 or more bacteria, although growth could be slightly delayed, and it is probable that the inoculum used by York and Vaughn was greater than this. York and Vaughn (35, 36) also reported that although the ability of spores of *C. botulinum* strains to result in growth decreased as the pH of the medium was lowered from 5.4 to 4.8, the minimum pH that permitted growth of each of 18 strains of type A and 16 strains of type B was the same in the presence and in the absence of a sorbic acid concentration of 1.2 g/liter.

In contrast, in our experiments, strain ZK3 grew rapidly at pH 5.1 and 4.9 in the absence of sorbic acid from a very small inoculum, but the presence of a total sorbic acid concentration of 0.9 g/liter at pH 5.1 or 0.67 g/liter at pH 4.9 prevented growth from an inoculum of 10^5 to 10^6 bacteria. Similar results were obtained with nine additional strains at pH 5.1. The greater resistance to sorbic acid reported by York and Vaughn may have been due in part to their use of a relatively high inoculum, but it is unlikely that this is a sufficient explanation for the difference between their results and ours.

Potassium sorbate has been shown to inhibit the germination and outgrowth of *C. botulinum* spores in addition to inhibiting the growth of vegetative bacteria (6, 31). There is evidence, however, that the concentrations necessary to inhibit germination are, at least in some cases, higher than those needed to inhibit the growth of vegetative bacteria. At pH 5.7, a potassium sorbate concentration of 5,200 mg/liter, equivalent to an undissociated sorbic acid concentration of 400 mg/liter, was required to inhibit the germination of 95% of the spores of *C. botulinum* 62A during incubation for 2 h at 35°C (27). Potassium sorbate caused a similar delay in growth from spore inocula and vegetative inocula of the same species (1, 3). Germination of spores of strain 53B at pH 5.5 was not significantly inhibited by a potassium sorbate concentration of 2,600 mg/liter, equivalent to an undissociated sorbic acid concentration of 293 mg/liter (2). The use of sorbic acid to reduce the risk of growth of *C. botulinum* in

foods is likely, therefore, to depend on its ability to inhibit the growth of vegetative bacteria.

Sofos and Busta (29) concluded that 0.20% sorbic acid retards the growth of *C. botulinum* in uncured meat products and may be used to replace nitrite as an inhibitor of *C. botulinum* in cured meat products. Both uncured and cured meat products usually have a pH between 5.5 and 6.5. Assuming that the dissociation of sorbic acid is not affected by components of the meat products and that the fat content is not sufficient to take up significant sorbic acid, then at pH 5.5, 2 g of sorbic acid per kg would be equivalent to approximately 308 mg of undissociated sorbic acid per kg; on the basis of our results, this should reduce the probability of growth of *C. botulinum* by a factor of at least 10^5. At pH 6.5, this total concentration of sorbic acid would be equivalent to approximately 35 mg of undissociated sorbic acid per kg, which, on the basis of our results, would not significantly inhibit the growth of vegetative cells of *C. botulinum*.

The highest concentrations of sorbic acid permitted in foods in the United Kingdom are 2,000 mg/kg in low-fat products consisting principally of a water-in-oil emulsion and 1,000 mg/kg in several other products including cheese, certain sauces, and salad cream (12). The results reported here show that in a nutrient anaerobic medium at pH 5.5, a total sorbic acid concentration of 1,000 mg/liter delayed the growth of proteolytic strains of *C. botulinum* but did not significantly reduce the probability of growth from a single bacterium after 14 days at 30°C. At pH 5.1, this concentration reduced the probability of growth in 14 days by a factor of approximately 10^6, while at pH 4.9, a total sorbic acid concentration of 670 mg/liter reduced the probability of growth by a similar factor.

The conditions necessary to inhibit the growth of bacteria depend on the size of the inoculum. In designing preservative systems to prevent the growth of *C. botulinum* in foods, it is important to show that these systems will effect a significant reduction in the probability that a single spore or bacterium will survive, grow, and form toxin. The heat processing used in general in the production of low-acid canned foods reduces this probability by a factor of at least 10^{12}. Previous workers have reported that potassium sorbate can delay growth of and toxin formation by *C. botulinum* in foods and in culture media at pH 5.5 and above. The work reported here provides a systematic study of the effect of potassium sorbate on the probability of growth of a single bacterium in a culture medium. It demonstrates that the inhibition is due mainly to undissociated sorbic acid, provides a mathematical model describing the effect, and shows that at the maximum concentration permitted in foods in the United Kingdom, 1,000 mg/kg, sorbic acid significantly reduced the probability of growth of *C. botulinum* in media at pH lower than 5.5. The combination of sorbic acid with other inhibitory factors is likely to provide very effective preservation systems against the growth of *C. botulinum* in foods at pH 4.5 to 5.5.

ACKNOWLEDGMENTS

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