

Sporostatic, Sporicidal, and Heat-Sensitizing Action of Maleic Acid against Spores of Proteolytic *Clostridium botulinum*

ARTHUR J. MILLER,* JEFFREY E. CALL, and BOBBY L. BOWLES

ABSTRACT

The antibotulinal potential of maleic acid was investigated in an uncured turkey product and model systems. There was a dose-related delay in neurotoxin detection, with no toxin developing after 40 days at 28°C with 500 or 1,500 *Clostridium botulinum* spores per g and 2% maleic acid. Anaerobic and aerobic microflora were suppressed by maleic acid, and pH remained constant during the incubation period. Sporicidal and sporostatic concentrations were 19.5 and 2.4 mM, respectively, in broth. The 8-day sporostatic levels were >5 and 2 mM for canned chicken and beef broths, respectively. Dipicolinic acid release was reduced by 50% with 0.25 mM maleic acid. Maleic acid also lowered spore thermal resistance at 80°C. These data indicate that maleic acid inactivates *C. botulinum* spores, delays spore outgrowth, and reduces thermal resistance.

Key words: Maleic acid, *Clostridium botulinum*, spores, thermal resistance, dipicolinic acid

Although rare, botulism produced the second highest death-to-case ratio (192/1,000) among foodborne illnesses occurring in the U.S. between 1973 and 1987 (3). Soil and sediments harboring *Clostridium botulinum* spores are the principal routes for entry into the food chain. While commercial sterilization destroys the thermotolerant spores, combinations of sublethal preservation techniques, such as refrigeration and acidification, are used frequently to inhibit spore germination. *C. botulinum* intoxication usually involves consumption of either improperly prepared home-processed foods or temperature-abused commercial products. Recently, however, there have been some outbreaks from consumption of ethnic foods or those that were manufactured using new technologies (11, 20, 28, 36, 38).

Uncured ready-to-eat turkey products, now common retail items, are moderately heat-processed, low-salt, nitrite-free, vacuum-packaged products with an excellent safety record. Since there is no additional growth barrier, product safety depends upon good manufacturing practices and proper refrigeration. Additional measures are necessary to

prevent pathogen growth or toxigenesis if such products are temperature abused.

Organic acids and their corresponding salts are promising antibacterial agents because they are generally inexpensive and enjoy consumer acceptance. We observed recently (23) that among a series of four-carbon dicarboxylic acids, including the naturally occurring compounds fumaric, aspartic, malic, succinic, tartaric, and maleic acids, only maleic acid had inhibitory activity toward proteolytic strains of *C. botulinum*. Earlier Huhtanen et al. (14) showed that maleic acid inhibited gas production by *C. botulinum* in cans of comminuted, nitrite-free temperature-abused bacon. Using structure-activity relationships, Dymicky et al. (9) demonstrated that mono-C₁₃₋₁₈ alkyl maleates had maximum antibotulinal activity.

Maleic acid (1,2-ethylenedicarboxylic acid, F.W. 116.07), the *cis* isomer of fumaric acid, is manufactured synthetically by the hydrolysis of maleic anhydride. Its reactivity stems from the conjugation to the ethylene backbone with the oxo functions of the two carboxyl groups, making maleic acid bifunctionally reactive. Maleic acid can undergo reactions including amidation of primary and secondary amines, decarboxylation, and electrophilic or nucleophilic addition (18). Maleic acid is found in fruits and fermented foods and was shown to be an effective preservative (1, 26). Limited toxicity data exists and was reported previously (23). Thus, the previously reported functional and preservative activity of this unique four-carbon unsaturated dicarboxylic acid in foods prompted us to investigate further the antibotulinal properties of maleic acid.

MATERIALS AND METHODS

Spore cultures

A spore mixture containing three type A (33, 62A, 69) and three type B (999, 169, ATCC 7949) proteolytic *C. botulinum* strains was used throughout the study. Strains were from the culture collection at the USDA, Eastern Regional Research Center. Individual strain spore suspensions were prepared by culturing in botulinal assay medium without thioglycollate (BAM) at pH 7.0 (13) for up to 21 days as described previously (5, 6, 24). Equal numbers of the six individually prepared and then heat-shocked (80°C for 10 min) strains were pooled to yield a final concentration

of 5.7 log number of spores per ml. Spores were characterized, as described previously, and stored at 4°C in sterile distilled water. Viability and germination rate were tested initially on individual strains. Before each experiment spore mixtures were enumerated after surface plating onto BAM agar plates and incubating anaerobically in a flexible chamber (Coy Industries, Ann Arbor, MI) for 48 h at 37°C. The atmosphere consisted of 90% nitrogen and 10% hydrogen. Residual oxygen was converted to water vapor by use of a palladium catalyst.

Maleic acid

Maleic acid purchased from Sigma Chemical Company (St. Louis, MO) was used without further purification. For food challenge studies maleic acid was freshly diluted in sterile deionized and distilled water. For other experiments a 5.0 M stock solution was prepared in 95% ethanol and stored at 5°C. Final ethanol concentrations in testing treatments were below those reported to be inhibitory (15, 19).

Product formulation and treatment

Turkey breast meat was obtained, formulated, inoculated, and incubated as described previously (23, 24). Briefly, 500-g batches of ground turkey were prepared with 1.4% (7 g, wt/wt) sodium chloride, 0.3% (1.5 g, wt/wt) sodium pyrophosphate, and 0 to 3% maleic acid (0 to 0.26 M, 0 to 15 g, wt/wt). The pH levels of the turkey formulations were adjusted with hydrochloric acid or sodium hydroxide to 6.0 and blended in a food processor. A positive control containing botulin spores and no maleic acid, and a negative control, containing neither spores nor maleic acid, was included in each experimental trial.

Two inoculum levels were tested. Formulations were placed into heat-sealable plastic bags and weighed, and either 500 or 1,500 spores per g were inoculated into all but the negative control samples by adding approximately 5 ml of an appropriately diluted suspension of the spore mixture. Spores were mixed into the turkey formulations by systematic hand kneading. After mixing, 10 ± 0.1-g samples were weighed into filter Stomacher bags (Tekmar, Cincinnati, OH). Stomacher bags were folded and placed into high-oxygen-barrier bags, with an O₂ transmission rate of 3.5 cm³/100 in²/24 h, at 24°C and 75% RH, as defined by the manufacturer (Koch Supplies, Inc., Kansas City, MO). Bags were vacuum sealed at -950 mbar in a Multivac A300/16 packaging machine (Sepp Haggemüller, West Germany), heated immersed for 20 min in 75°C water, cooled in crushed ice, and incubated at 28°C for 0 to 40 days. Preliminary heating trials on uninoculated turkey formulations indicated that the samples reached final temperature within 5 min after immersion. At the end of each incubation period, samples were removed, frozen, and stored at -18°C until tested. Three replicate samples per treatment were tested at each sampling time. Duplicate trials were performed.

Product evaluation

Products were evaluated periodically for pH, aerobic and anaerobic/facultative bacterial load, neurotoxin, and sensory characteristics. Neurotoxin mouse bioassays (8), pH determinations, and sensory evaluations were described in detail previously (23, 24). Bioassays were conducted on duplicate Swiss-Weber laboratory mice that were observed for typical botulism symptoms for 72 h.

For bacteriological evaluation, treated or control samples were removed from opened vacuum pouches, diluted using 20 ml of 0.1% peptone (Difco Laboratories, Detroit, MI) to yield a 1:3 dilution, and blended for 2 min using a Model 400 Stomacher (Tekmar). Each filtrate was serially diluted in 0.1% peptone water and plated onto nutrient agar and *Lactobacillus* MRS plates (Difco)

using a spiral plating instrument (Model D, Spiral Systems, Cincinnati, OH). Nutrient agar plates were incubated in a forced-air incubator, while the MRS dishes were incubated in a Coy anaerobic chamber. Both sets of Petri dishes were incubated at 37° for 48 h. Quantification was performed by manual counting. Bacterial assays were performed through the 40-day sampling, until a toxigenic response was obtained by the neurotoxin assay, or until the cells reached the stationary phase of the growth cycle.

Spore inhibitory concentrations

Maleic acid was serially diluted from 2,500 mM (23%) to extinction in BAM broth, without pH adjustment, and then inoculated with 0.1 ml of culture containing 4.7 log heat-shocked (80°C for 10 min) spores. The pH range of the final solutions ranged from 3.0 to 7.0. After anaerobic exposure for 6 h at 32°C, 0.1 ml from the broth tubes was subcultured to each of 5 replicate thioglycollate (TG) broth tubes, before and after a 10-min, 80°C heat treatment, to destroy germinated spores or outgrown vegetative cells. Tubes were incubated for 48 h at 32°C and then examined visually for turbidity. Sporicidal activity was defined as no visible growth in ≥4 tubes containing non-heat-shocked maleic acid-exposed cultures. Sporostatic activity was defined as growth in <4 tubes containing heat-shocked maleic acid-exposed cultures.

Anti-outgrowth potential of maleic acid was also assessed using commercial canned chicken and beef broths, as described previously (7). Briefly, 2 to 5 mM maleic acid, without pH adjustment, was added to the commercial products along with 4.7 log heat-shocked *C. botulinum* spores per 0.1 ml. The pH values of the final mixtures ranged from 6.0 to 7.0. The broths were incubated anaerobically at 25°C and visually examined for turbidity. Controls included both inoculated and uninoculated commercial broths.

Dipicolinic acid (DPA) release

DPA analysis was modified from the colorimetric assay of Janssen et al. as described by Bowles and Miller (5, 6). Maleic acid levels of 0.5 to 5 mM were added to 4.7 log heat-shocked spores in 0.1 ml and incubated for 9 h anaerobically at 32°C. The chromogenic reagent was added, optical density was measured at 440 nm, and DPA content was calculated from a standard curve that was determined for each experiment.

Effect on spore thermal resistance

C. botulinum spores (7 log spores per ml) were aerobically exposed to 0 or 100 mM (1.2%) maleic acid for 30 min at 25°C, then transferred to an 80°C water bath. The samples, in 5.0 ml glass vials, reached equilibrium temperature in 95 s. Spore thermal resistance was evaluated by comparing the population densities of maleic acid-treated and untreated samples by anaerobic plating on BAM agar and incubation for 48 h at 37°C. To separate heating from maleic acid effects, a control was performed for each experiment consisting of spores incubated 50 min at 25°C in 100 mM maleic acid.

RESULTS

Turkey product evaluation

Changes in the turkey product pH levels were slight during the 40-day trial (data not shown). Control samples, containing neither spores nor maleic acid, exhibited increased pH levels, from 6.4 to 7 during the 28°C incubation. Control samples inoculated with spores had slightly higher initial pH levels (6.5), then increased further to 6.7 within 5

days, while the pH values of maleic acid-containing samples dropped only 0.1 pH unit during the incubation period, regardless of ingoing acid concentration and spore challenge load.

The results from the total aerobic plate counts from the 500 spores per g of turkey samples are presented in Fig. 1a. For Fig. 1a and b data are presented that show only the lag, growth, and early stationary phases of the growth cycle. Control samples that received no maleic acid, or spores that were incubated at 28°C, reached a maximum population density of 6.2 log CFU/g by 10 days. Similarly, positive (spore-only) controls reached a maximum population density of 5.4 log CFU/g by 10 days. There was an inverse relationship between increased maleic acid levels and the growth rate of aerobic bacteria. At 10 days, for example, aerobic bacteria levels in the experimental samples were 4.5, 3.5, and 1.8 log CFU/g for 1 (86 mM), 2 (115 mM), and 3% (258 mM) maleic acid, respectively. Maximum population densities were observed at 10 days for the 1 and 2% maleic acid-treated samples, while 3% maleic acid samples reached a maximum population density of 4.1 log CFU/g at 20 days, demonstrating further the growth-suppressing effect of maleic acid on total aerobic bacterial load. Increasing spore concentrations to 1,500/g resulted in shorter lag duration periods (data not shown).

Similar results were obtained for facultative/anaerobic bacteria in the 500-spores per g turkey samples that were observed on lactobacillus MRS agar, permitting growth of organisms including lactic acid bacteria (Fig. 1b). Negative and positive control samples reached a maximum population density of 5.7 log CFU/g in 10 days. There was a dose-related suppressing effect on the growth of facultative/anaerobic bacteria when maleic acid was added to the turkey product. The 1,500-spores-per-g products (data not shown) had shorter lag duration periods at each maleic acid level, compared to the lower inoculum size samples.

The mouse bioassay was employed to qualitatively assess botulinum neurotoxin detection in uncured turkey product that was challenged with two levels of *C. botulinum* spores and incubated at 28°C. Results are summarized in

TABLE 1. Time to detection of neurotoxin as measured by mouse bioassay in vacuum-packaged uncured turkey inoculated with spores of proteolytic *C. botulinum* and treated with maleic acid; incubation at 28°C

Inoculum (spores/g of turkey)	Days to neurotoxin detection				
	Maleic acid, % (mM)				
	0 (0)	0.5 (43)	1.0 (86)	1.5 (115)	2.0 (172)
500	3	4	5	20	>40
1,500	2	2	4	8	>40

Table 1. At the 500 spores per g challenge level, turkey samples contained neurotoxin at 4, 5, and 20 days in the presence of 0.5, 1, and 1.5% maleic acid, respectively. The time to neurotoxin detection decreased when the spore inoculum was elevated threefold, to 1,500 spores per g. No neurotoxin was detected at 40 days in samples containing 2% maleic acid, regardless of spore inoculation level.

Off odors or abnormal texture or color were inadequate warnings for neurotoxin presence in the product (data not shown). Only the positive control (no maleic acid) and 0.5% maleic acid-treated turkey samples, at the 500-spores-per-g level, exhibited obvious evidence of spoilage. Conversely, the 1.5% maleic acid samples exhibited neurotoxin at least 1 day prior to a noticeable product change. The poor correlation between neurotoxin development and obvious product changes was also evident in the 1,500-spores-per-g samples. Although the positive control samples exhibited off odors at 2 days, coinciding with neurotoxin detection, there were no textural, color, or olfactory clues from the observation of the product that would suggest neurotoxin presence in any maleic acid-treated sample.

Spore inhibitory concentration

The spore static concentration was 2.4 mM maleic acid for *C. botulinum* spores in BAM broth at 32°C (data not shown). Nonviable spores were produced with 19.5 mM maleic acid (data not shown). *C. botulinum* spores were

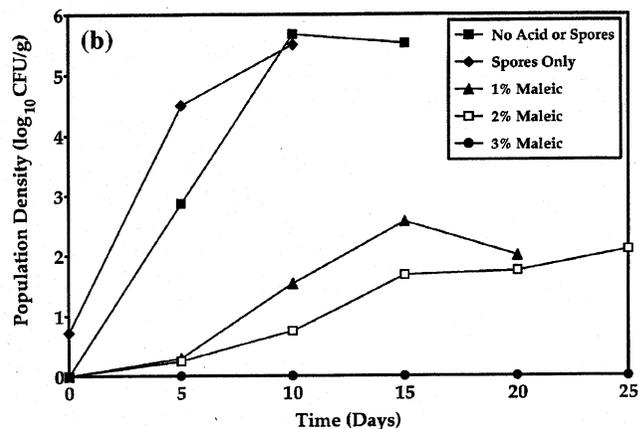
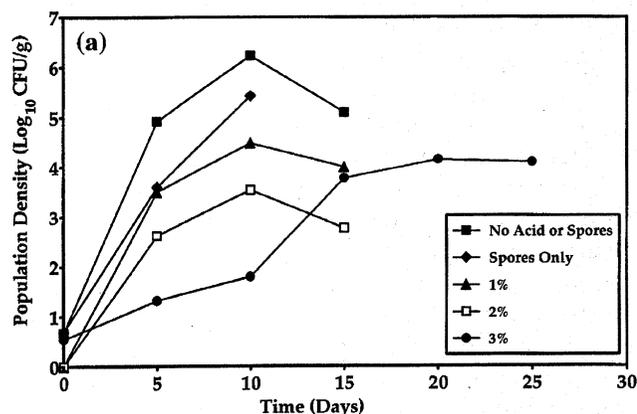


FIGURE 1. (a) Aerobic bacterial growth on nutrient agar of uncured turkey product containing 0 to 3% maleic acid and inoculated with spores of proteolytic *C. botulinum*; incubation at 28°C. (b) Anaerobic/facultative bacterial growth on MRS lactobacillus agar of uncured turkey product containing 0 to 3% maleic acid and inoculated with spores of proteolytic *C. botulinum*; incubation at 28°C.

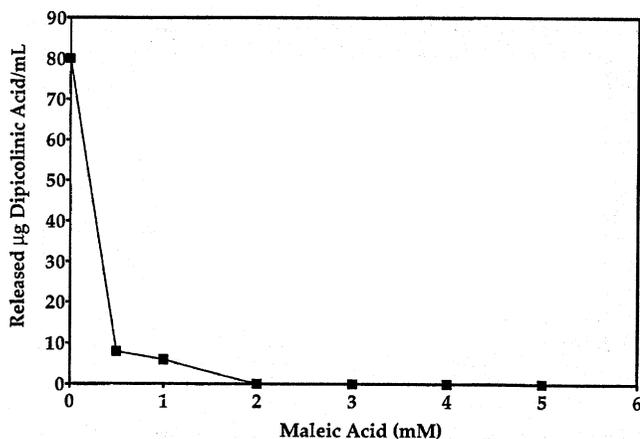


FIGURE 2. Level of DPA released into surrounding medium after exposure of spores of proteolytic *C. botulinum* to 0 to 5 mM maleic acid for 9 h at 32°C.

inhibited from outgrowth in beef broth containing 2 mM maleic acid, as measured by comparing visual turbidity in the experimental tubes to the controls, for up to 8 days. Outgrowth was inhibited in chicken broth, but the concentration required varied with end-point time. For example, 2, 4, >5, and >5 mM maleic acid was required to prevent outgrowth for 2, 4, 6, and 8 days, respectively.

DPA release

DPA levels were estimated colorimetrically after spores were exposed to 0 to 5 mM maleic acid for 9 h at 32°C. Results are presented in Fig. 2. Control samples, without any maleic acid, released 80 µg of DPA/ml. When maleic acid was added, however, an exponential decay relationship was observed between inhibitor concentration and DPA release. Approximately 0.25 mM maleic acid was calculated as the inhibitory concentration required to lower DPA release by 50%, while 2.0 mM maleic acid was experimentally determined to completely inhibit DPA release. This later value coincides well with the experimentally derived sporostatic levels.

TABLE 2. The effect of maleic acid on thermal resistance of spores of proteolytic *C. botulinum* in BAM broth

Time ^a at 80°C (min)	log ₁₀ CFU/ml	
	Maleic acid (mM)	
	0	100
0	7.0	6.3
5	7.0	5.2
10	7.0	4.5
15	6.9	3.8
20	6.8	3.3

^a Initial exposure was 30 min at 25°C. Experimental samples were then transferred to an 80°C water bath. Controls were maintained at 25°C and varied only between 6.8 and 6.0 log CFU/ml throughout the heating trial.

Effects on spore thermal resistance

Maleic acid effects on thermal resistance of proteolytic *C. botulinum* spores are shown in Table 2. Maleic acid (100 mM) controls were incubated for 50 min at 25°C, in parallel with the experimental samples, which were exposed to 0 or 100 mM maleic acid for 30 min at 25°C, then heated for 0 to 20 min at 80°C. Results indicated that the maleic acid-treated controls remained viable, as indicated by growth on BAM agar, throughout the 50-min 25°C incubation. Likewise, spores that were unexposed to maleic acid (0 mM) but received the 25°C incubation and 80°C thermal treatment lost no viability. Spores levels exposed to 100 mM maleic acid, however, decreased 1,000-fold after 20 min at 80°C.

DISCUSSION

Earlier reports demonstrating the antibotulinal efficacy of maleic acid and its derivatives provided little information about the mechanism of inhibition (9, 14, 23). The current study further characterized the inhibitory potential of maleic acid by demonstrating decreased total bacterial growth and a dose-related delay in neurotoxin detection in a food product at two spore inoculum levels. Moreover, the study determined sporocidal and sporostatic potential in a bacteriological medium and in commercial soups, showed that maleic acid inhibited DPA release in a concentration-dependent manner, and provided evidence that maleic acid lowered spore thermal resistance.

The lower pH values observed in the maleic acid-treated turkey samples, compared to controls, indicated an ability to overcome the tendency of the control samples to increase pH levels, with respect to time. The use of maleic acid as a food acidulant was demonstrated previously (26). It is unlikely that the antibotulinal action of maleic acid, however, was due to a direct acidifying effect, since *C. botulinum* growth could occur at the pH values observed in all of the experimental products. More likely, maleic acid retarded spoilage organisms and pathogen growth, which could form alkaline breakdown products, like ammonia, by proteolysis of food components. This hypothesis is supported by the decreased bacterial population growth that paralleled increased maleic acid concentrations. Food preservation by maleic acid was reported previously (39).

The observation that increased maleic acid levels lowered levels of aerobic and anaerobic bacteria in the turkey product shows the general dose-related antimicrobial activity of this compound. Furthermore, the retarding of neurotoxin development in the turkey product was also concentration-dependent. The additional observation that time to neurotoxin detection varied inversely with initial spore levels is consistent with the report by Baker and Genigeorgis (2). The lack of off odors or texture in the inoculated turkey product underscores the potential consumer hazard associated with foods containing botulinal neurotoxin, since contaminated products often lack either spoilage signs or gas and swelling.

The present study also confirms the earlier observation that higher levels of *C. botulinum* inhibitors are required to impart a sporocidal effect than for sporostasis (5, 6, 7). The

DPA data further show inhibitory potential above 2 mM maleic acid, a level consistent with the experimentally determined inhibitory levels in BAM broth and the two commercial soups.

A comparison can be made between the sporostatic efficacy of maleic acid and that of various other preservatives, using the experimentally derived value of 2 mM (233 µg/ml) as a benchmark level. Reddy et al. (31) inhibited *C. botulinum* spore outgrowth in broth with 200 to 400 µg/ml BHA, BHT, and TBHQ. Sodium hypophosphite was inhibitory to *C. botulinum* at 3,000 µg/g of bacon (30). Winarno et al. (41) showed that EDTA inhibited germination of *C. botulinum* 62A spores in a food product at 2.5 mM. The level of nitrite approved for use in cure-pumped bacon, and taken here as an antibotulinal level, is 120 µg/g of bacon. From these studies it can be concluded that maleic acid inhibits *C. botulinum* at concentrations comparable to other antimicrobial agents.

The thermal challenge experiment showed that neither mild heat treatment nor short maleic acid exposure, alone, affected spore viability. Yet, their combined effects decreased viability, thus indicating a synergistic relationship. Maleic acid appears to reduce the thermal resistance of *C. botulinum* spores to heat, most likely in the dormant spores. Pretreatment of *C. botulinum* spores by a variety of agents can lower thermal resistance. Examples of previously reported sensitizing agents include: ionizing irradiation, hypochlorite, hydrogen peroxide, caffeic acid, and certain aliphatic and aromatic ketones and aldehydes (5, 6, 7, 17).

Our understanding of maleic acid metabolism in *C. botulinum* is poor, compared to information available on other bacteria. For example, despite studies demonstrating four-carbon dicarboxylate permeases that allow maleic acid to move into vegetative bacteria (35), there is no information regarding such transport systems in *C. botulinum*. Many microorganisms can use maleate as a carbon and energy source (29). Maleate also is an intermediate in the microbial degradation of gentisate (12) and pyridine rings (4). Furthermore, a variety of microorganisms produce fumarate (29) or D-malate (40) from maleate. Fumarate also can be incorporated into the citric acid cycle in aerobic microorganisms. D-malate can be degraded to either pyruvate and CO₂ (10) or to acetyl-coA and glyoxylate (37).

Knowledge about the antimicrobial potential and the mechanism of action of maleic acid and its close derivatives in *C. botulinum* is, likewise, limited. Maleic acid blocked nicotinic acid metabolism in *C. barkeri* by the competitive inhibition of methyleneglutarate mutase (21). Maleic anhydride, the synthetic precursor of maleic acid, was shown to reduce the lethal hemolytic and platelet-aggregating activities and phospholipase C activity of *C. perfringens* α-toxin (32). Similarly, Sakurai and Nagahama (33) demonstrated that the lethal activity of *C. perfringens* ε-prototoxin and e-toxin was lost after treatment with 2,3-dimethylmaleic anhydride. These reports indicate that maleic acid and its derivatives can inhibit some clostridial species by disturbing intermediary metabolism or by modifying the biological activity of clostridial toxins. The present study supports the notion that metabolism, as assessed by viability and growth

studies, is adversely affected by maleic acid. The potential interference of maleic acid with the biological activity of neurotoxin remains unclear.

In the present study maleic acid suppressed proteolytic *C. botulinum* prior to the outgrowth stage in its life cycle. More specifically, the sporicidal and sporostatic activity of the compound, and the temperature-sensitizing effect, indicate that it affects the spore in its dormant state. It is uncertain if maleic acid inhibits *C. botulinum* by affecting the spore coat or if its mode of action is on an internal receptor. It is unlikely, however, that the inhibitory action resulted from interference with an active transport system, since dormant spores were affected.

Maleic acid occurs naturally in plants, and is produced as a fermentation product in some foods (16, 22, 25). While the compound is unapproved as a direct food additive, maleic acid has been awarded food application patents, was tested in foods, and was efficacious for acidulation (26), color enhancement (27, 42), shelf-life extension (39), and odor reduction (1). Schroeder and Hosenev (34), moreover, demonstrated that maleic acid reacted with free radicals in gluten proteins. In view of these properties, as well as the antibotulinal activity shown in the present study, further studies need to be conducted to gain a fuller understanding of the mechanism of action of this multifunctional compound.

REFERENCES

1. Ajinomoto Co., assignee. Week 17, 1970. Garlic smell reduction. British patent 1,190,156.
2. Baker, D. A., and C. Genigorgis. 1990. Predicting the safe storage of fresh fish under modified atmospheres, with respect to *Clostridium botulinum* toxigenesis by modeling length of the lag phase of growth. *J. Food Prot.* 53:131-140.
3. Bean, N. H., and P. M. Griffin. 1990. Foodborne disease outbreaks in the United States, 1973-1987: pathogens, vehicles, and trends. *J. Food Prot.* 53:804-817.
4. Behrman, E. J., and R. Y. Stanier. 1957. The bacterial oxidation of nicotinic acid. *J. Biol. Chem.* 228:923-945.
5. Bowles, B. L., and A. J. Miller. 1993. Antibotulinal properties of selected aromatic and aliphatic aldehydes. *J. Food Prot.* 56:788-794.
6. Bowles, B. L., and A. J. Miller. 1993. Antibotulinal properties of selected aromatic and aliphatic ketones. *J. Food Prot.* 56:795-800.
7. Bowles, B. L., and A. J. Miller. 1994. Caffeic acid activity against *Clostridium botulinum* spores. *J. Food Sci.* 59:1-4.
8. Center for Disease Control. 1974. Detection of *Clostridium botulinum* and botulinal toxin. p. 41-44. *In* Laboratory Methods in Anaerobic Bacteriology, CDC laboratory manual. Center for Disease Control, Atlanta, Georgia.
9. Dymicky, M., M. Bencivengo, R. L. Buchanan, and J. L. Smith. 1987. Inhibition of *Clostridium botulinum* 62A by fumarates and maleates and relationship of activity to some physicochemical constants. *Appl. Environ. Microbiol.* 53:110-113.
10. Giffhorn, F., and A. Kuhn. 1983. Purification and characterization of a bifunctional L-(+)-tartrate dehydrogenase-D-(+)-malate dehydrogenase (decarboxylating) from *Rhodospseudomonas sphaeroides* Y. *J. Bacteriol.* 155:281-290.
11. Hayashi, K., S. Sakaguchi, and G. Sakaguchi. 1986. Primary multiplication of *Clostridium botulinum* type A in mustard-miso stuffing of 'karashi-renkon' (deep-fried mustard-stuffed lotus root). *Int. J. Food Microbiol.* 3:311-320.
12. Hopper, D. J., P. J. Chapman, and S. Dagley. 1968. Enzymatic formation of D-malate. *Biochem. J.* 110:798-800.
13. Huhtanen, C. N. 1975. Some observations on Perigo-type inhibition of *Clostridium botulinum* in a simplified medium. *J. Milk Food Technol.* 38:762.

14. Huhtanen, C. N., H. Trenchard, and L. Milnes-McCaffrey. 1985. Inhibition of *Clostridium botulinum* in comminuted bacon by short-chain alkenoic and alkenoic acids and esters. *J. Food Prot.* 48:570-573.
15. Johnston, R. S., S. Haramon, and D. Kautter. 1964. Method to facilitate the isolation of *Clostridium botulinum* type E. *J. Bacteriol.* 88:1521-1522.
16. Kim, H. O., and H. S. Rhee. 1975. Studies on the nonvolatile organic acids in kimchis fermented at different temperatures. *Korean J. Food Sci. Technol.* 7:74-81.
17. Kim, J., and P. M. Foegeding. 1993. Principles of control, p. 121-176. In, A. H. W. Hauschild and K. L. Dodds ed. *Clostridium botulinum: ecology and control in foods*. Marcel Dekker, Inc., New York.
18. Kirk, R. E., and D. F. Othermer (ed.). 1981. *Encyclopedia of Chemical Technology*, 3rd ed., vol. 14, p. 770. John Wiley & Sons, New York.
19. Koransky, J. R., S. D. Allen, and V. R. Dowell, Jr. 1978. Use of ethanol for selective isolation of sporeforming microorganisms. *Appl. Environ. Microbiol.* 35:762-765.
20. Kotev, S., A. Leventhal, A. Bashary, H. Zahavi, A. Cohen, P. Slater, A. Ruston, E. Baron, B. Farber, J. Greenspan, M. Tenenbaum, R. Amerongon, V. Tulumello, J. Lynch, S. Schultz, C. Reisberg, S. Shahidi, S. Joseph, L. Crowell, J. Ferrara, J. Guzewich, M. Shayegani, G. Hannett, and D. L. Morse. 1987. International outbreak of type E botulism associated with ungutted, salted whitefish. *Morbidity and Mortality Weekly Rep.* 36:812-813.
21. Kung, H.-F., and T. C. Stadtman. 1971. Nicotinic acid metabolism. VI. Purification and properties of α -methylglutamate mutase (B_{12} -dependent) and methylglutamate isomerase. *J. Biol. Chem.* 246:3378-3388.
22. Lee, D. S., S. K. Woo, and C. B. Yang. 1972. Studies on the chemical composition of major fruits in Korea. On non-volatile organic acid and sugar contents of apricot, peach, grape, apple and pear and its seasonal variation. *Korean J. Food Sci. Technol.* 4:134-139.
23. Miller, A. J., and J. E. Call. 1994. Inhibitory potential of four-carbon dicarboxylic acids on *Clostridium botulinum* spores in an uncured turkey product. *J. Food Prot.* 57:679-683.
24. Miller, A. J., J. E. Call, and R. C. Whiting. 1993. Comparison of organic acid salts for *Clostridium botulinum* control in an uncured turkey product. *J. Food Prot.* 56:958-962.
25. Nangniot, P. 1985. Applications of polarographic and voltametric analysis in the fields of agriculture and alimentation. *Trends Anal. Chem.* 4:155-161.
26. Nesty, G. A. August 1970. Food acidulent. U.S. patent 3,523,024.
27. Ohta, H., and Y. Osajima. 1978. Quality of grape juice. III. Effects of organic acids on anthocyanin pigment from juice of Campbell Early grapes. *J. Jpn. Soc. Food Sci. Technol.* 25:78-82.
28. O'Mahony, M., E. Mitchell, R. J. Gilbert, D. N. Hutchinson, N. T. Begg, J. C. Rodhouse, and J. E. Morris. 1990. An outbreak of foodborne botulism associated with contaminated hazelnut yoghurt. *Epidemiol. Infect.* 104:389-395.
29. Otsuka, K. 1961. *cis-trans* isomerase. Isomerisation from maleic acid to fumaric acid. *Agric. Biol. Chem.* 25:726-730.
30. Pierson, M. D., K. M. Rice, and J. F. Jadlocki. 1981. Sodium hypophosphite inhibition of *Clostridium botulinum*. In *Proc. 27th European Meat Res. Workers Congr., Vienna*, vol. 2, p. 651.
31. Reddy, N. R., M. D. Rierison, and R. V. Lechowich. 1982. Inhibition of *Clostridium botulinum* by antioxidants, phenols, and related compounds. *Appl. Environ. Microbiol.* 43:835-839.
32. Sakurai, J., Y. Fujii, K. Torii, and K. Kobayashi. 1989. Dissociation of various biological activities of *Clostridium perfringens* alpha toxin by chemical modification. *Toxicon* 27:317-324.
33. Sakurai, J., and M. Nagahama. 1986. Amino groups in *Clostridium perfringens* epsilon prototoxin and epsilon toxin. *Microbiol. Pathol.* 1:417-424.
34. Schroeder, L. F., and R. C. Hosenev. 1978. Mixograph studies. II. Effect of activated double-bond compounds on dough-mixing properties. *Cereal Chem.* 55:348-360.
35. Shaw, J. G., and D. J. Kelly. 1990. Binding protein dependent transport of C4-dicarboxylates in *Rhodobacter capsulatus*. *Arch. Microbiol.* 155:466-472.
36. Solomon, H. M., D. A. Kautter, T. Lilly, and E. J. Rhodehamel. 1990. Outgrowth of *Clostridium botulinum* in shredded cabbage at room temperature under a modified atmosphere. *J. Food Prot.* 53:831-833.
37. Stern, J. R. 1963. Enzymic activation and cleavage of D- and L-malate. *Biochim. Biophys. Acta* 69:435-437.
38. St. Louis, M. E., S. H. S. Peck, D. Bowering, G. B. Morgan, J. Blatherwick, S. Banerjee, G. D. M. Kettyls, W. A. Black, M. E. Milling, A. H. W. Hauschild, R. U. Tauxe, and P. A. Blake. 1988. Botulism from chopped garlic: delayed recognition of a major outbreak. *Ann. Intern. Med.* 108:363-368.
39. Ueno Seiyaku, assignee. Week 26, 1972. Fish preservation. Japanese patent 22,264/72.
40. van der Were, M., W. J. J. Van den Tweel, and S. Hartmans. 1992. Screening for microorganisms producing D-malate from maleate. *Appl. Environ. Microbiol.* 58:2854-2860.
41. Winarno, F. G., C. R. Stumbo, and K. M. Hays. 1971. Effect of EDTA on the germination of and outgrowth from spores of *Clostridium botulinum* 62A. *J. Food Sci.* 36:781-785.
42. Zenkoku Kamaboko Suisan, assignee. Week 45, 1971. Fish paste product. Japanese patent 39,059/71.