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Effect of Carbon Dioxide on Toxin Production by *Clostridium botulinum*

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Summary. Ten strains of *Clostridium botulinum* (5 type A and 5 type B) were combined and tested for their ability to produce toxin in modified reinforced clostridial medium, pH 5.7, under different amounts of pressure of carbon dioxide at 30° C. At atmospheric pressure (101 kPa), 100% CO_2 delayed toxin production when compared with an atmosphere of 100% N_2. Increasing the pressure level of CO_2 further delayed the onset of toxin production; however, CO_2 at 891 kPa absolute did not totally inhibit production of toxin, as two of 10 samples became toxic between 4 and 8 weeks of incubation. Pressurized CO_2 was lethal to *C. botulinum,* with the rate of decrease of recoverable colony-forming units being dependent on the amount of pressure of CO_2 and the length of exposure. However, CO_2 at 816 kPa absolute did not serve as a fully protective antibotulinal agent as some of the surviving spores were capable of germination and producing toxin within 8 weeks at 30° C.

Introduction

Depending on conditions, carbon dioxide may repress or stimulate microbial activity. For example, atmospheres enriched with carbon dioxide selectively retard the growth of gram-negative bacteria that spoil meats. Hence a modified atmosphere containing carbon dioxide is often used commercially to control microbial spoilage of fresh meats (Christopher et al. 1979; Silliker and Wolfe 1980; Silliker et al. 1977; Wolfe 1980). Carbon dioxide may also repress germination of spores of fungi and bacteria. Spores of *Rhizopus stolonifer, Botrytis cinerea* and *Cladosporium herbarum* will not germinate in the presence of 32% CO_2 (Wells and Uota 1970). At atmospheric pressure, 100% CO_2 inhibits germination of spores of *Bacillus cereus;* however, the same conditions stimulate germination of *Clostridium sporogenes* and *Clostridium perfringens* (Enfors and Molin 1978). Increased pressures of carbon dioxide are necessary to inhibit germination of *Clostridium* spores. Germination of *C. sporogenes* spores is inhibited slightly at 4 and almost completely at 10 atm, whereas germination of *C. perfringens* is slightly stimulated at 4, unaffected at 10, and stopped at 25 atm of carbon dioxide (Enfors and Molin 1978). These results prompted Enfors and Molin to speculate that storing food in carbon dioxide at atmospheric pressure can be hazardous with regard to the germination of *Clostridium botulinum,* but that high pressures of carbon dioxide can be used as an effective means of prolonging the storage life of perishable foodstuffs.

Besides decreasing the rate of germination, others have shown carbon dioxide at 719 kPa absolute to be lethal to clostridia (Hays et al. 1959). They found that the number of spores of *Clostridium butyricum, C. botulinum* and P.A. No. 3679 decreased after 42 days in the presence of 719 kPa absolute carbon dioxide. Hence under high pressure, carbon dioxide may not only inhibit germination but also may promote the death of *C. botulinum.* It was our interest to determine what effect carbon dioxide at different pressures has on the ability of *C. botulinum* to produce toxin.

Materials and Methods

Sample Containers and Medium. Aerosol cans (5.1 cm in diameter by 10.6 cm in height; 185-ml capacity) with double epoxy linings and stainless steel valves (without dip tubes) served as sample containers. The cans were filled to 25 or 70% of volume, 46 or 130 ml, respectively with reinforced clostridial medium without sodium chloride (RCM; Hirsch and Grinsted 1954) composed of (grams per liter): yeast extract (Difco), 3.0; beef extract (Difco), 10; peptone (Difco), 10; glucose, 5.0; soluble starch, 1.0; sodium acetate, 3.0; and l-cysteine HCl, 0.5, and adjusted with the

Table 1. Production of botulinal toxin in 130 ml of RCM, pH 5.7, in 5.1 by 10.6 cm aerosol cans and held at 30° C in the presence of nitrogen or carbon dioxide at different pressures

Treatment		No. of toxic samples/No. of samples tested at:								
Gas	Pressure (kPa)[a]	1 day		2 days		3 days		4 days		5 days
		Trial		Trial		Trial		Trial		Trial
		1	2	1	2	1	2	1	2	1
N_2	101	0/5	0/5	5/5	4/5	–	5/5	–	–	–
CO_2	101	0/5	0/5	0/5	0/5	2/5	5/5	5/5	–	–
CO_2	149	0/5	0/5	0/5	0/5	3/5	3/5	2/5	5/5	5/5
CO_2	204	0/5	0/5	0/5	0/5	1/5	0/5	4/5	5/5	5/5

[a] kPa, kilopascals absolute

Table 2. Production of botulinal toxin in 130 ml of RCM[a], pH 5.7, held at different pressures of carbon dioxide at 30° C

Pressure (kPa)[b]		No. of toxic samples/ No. of samples tested at:			
At fill	After 24 h	1 week	2 weeks	4 weeks	8 weeks
101	101	5/5	–	–	–
307	211	5/5	–	–	–
410	252	5/5	–	–	–
513	300	6/10	8/10	10/10	–
616	341	2/10	9/10	8/10	10/10
719	417	2/10	4/10	4/10	6/10
822	465	2/5	2/5	3/5	4/5
925	513	2/5	2/5	3/5	3/5
1029	575	0/5	0/5	0/5	3/5

[a] The amount of RCM comprised 70% of the volume of the can

[b] kPa, kilopascals absolute

appropriate amounts of HCl or NaOH to obtain pH 5.7 following treatment with CO_2 or N_2. Measurement of pH was determined after equilibration with the appropriate gas.

Spore Inoculum. A 10-strain mixture of *C. botulinum* spores, types A (56A, 62A, 69A, 77A and 90A) and B (53B, 113B, 213B, 13983B and Lamanna-okra B), was heat-shocked at 80° C for 15 min and added to RCM (27° C) at 1.0×10^3 spores/ml. Spores were prepared by growing each strain in a manner conducive to sporulation (Christiansen et al. 1974). Spores were harvested by centrifugation and washed with sterile water. The washed spores were sonicated briefly (30 s at a time; a total sonication time of 5 min), washed with sterile water using centrifugation, and stored in sterile, distilled water at −20° C.

Gas Treatment of Media. Media were treated with carbon dioxide or nitrogen (99.99% purity) by sparging the appropriate gas (Filter-sterilized; CO_2 preheated at 30° C) through the media for 1 min, crimping sterile valves onto the sample containers, and charging to the desired pressure. Samples not pressurized were sparged with the appropriate gas for an additional 4 min before being capped.

Analyses of Samples. Cans were incubated at 30° C and 5 or 10 samples of each treatment were assayed at each sampling interval for pressure, pH, growth (turbidity), botulinal toxin, and, in one study, *C. botulinum* colony counts. Growth, as measured by turbidity, was present in only, but all, media that contained botulinal toxin. For detection of toxin, each of two mice was injected i.p. with 0.5 ml of medium from the test sample. The mice were held up to 4 days and examined for symptoms and death characteristic of *C. botulinum* poisoning. When death occurred, two additional mice were challenged with a sample-antitoxin mixture which was preincubated at 37° C for 30 min (Food and Drug Administration, 1978). Unneutralized extract was again injected into two more mice as a control. To determine counts of *C. botulinum* colony-forming units, media of all samples showing no evidence of microbial growth were serially diluted in 0.1% peptone and subcultured in Prickett tubes containing Andersen's pork pea agar (Speck 1976). The tubes were overlaid with sterile petrolatum and incubated at 30° C until colony counts were stable (3–4 weeks). The average number of colonies counted for each treatment at each sampling interval was reported.

Results and Discussion

Effect of Low Pressure Carbon Dioxide on Botulinal Toxin Production

The effect of CO_2 at atmospheric pressure (101 kPa absolute) and at 149 and 204 kPa absolute on botulinal toxin production was compared to a control treatment of N_2 at atmospheric pressure. Relative to the N_2 control, the presence of CO_2 at all three pressures delayed the production of botulinal toxin (Table 1). By day 2, five of five, and four of five samples treated with N_2 were toxic for trials 1 and 2, respectively, whereas none of the CO_2-treated samples became toxic until day 3. In most instances, all of the CO_2-treated samples within a sampling interval did not become toxic until day 4 or 5, whereas all of the N_2-treated samples were toxic by day 3. Although others have shown that germination of clostridia spores is stimulated in the presence of, in some instances, atmospheres of up to 100% CO_2 (Enfors and Molin 1978; Hambleton and Rigby 1970; Wynne and Foster 1948), it appears that slightly or non-pres-

Table 3. Survival of *C. botulinum*[a] and production of botulinum toxin in 46 ml of RCM[b], pH 5.7, held at different pressures of carbon dioxide at 30° C

Pressure (kPa)[c]		Sampled at:							
At fill	After 24 h	1 week		2 weeks		4 weeks		8 weeks	
		No. of toxic samples/ No. of samples tested	CFU/ml[d]	No. of toxic samples/ No. of samples tested	CFU/ml	No. of toxic samples/ No. of samples tested	CFU/ml	No. of toxic samples/ No. of samples tested	CFU/ml
101	101	5/5	–	–	–	–	–	–	–
513	438	0/5	280	2/5	190	4/5	72	5/5	–
616	548	0/5	250	0/5	120	0/5	62	5/5	–
719	616	0/5	180	0/5	150	0/5	33	3/5	28
822	719	0/5	180	0/5	140	0/5	22	5/5	–
925	816	0/5	160	0/5	140	0/5	20	5/5	–
1029	891	0/5	140	0/5	93	0/5	31	2/10	12

[a] Initial number of spores was 1.0×10^3/ml; spores were heat-shocked before inoculation
[b] The amount of RCM comprised 25% of the volume of the can
[c] kPa, kilopascals absolute
[d] CFU/ml, colony-forming units/ml. Counts were determined on nontoxic samples having no evidence of microbial growth

surized atmospheres containing 100% CO_2 do not increase, but rather reduce, the hazard of botulism. Perhaps the presence of carbon dioxide stimulates germination of *C. botulinum* spores but slows enzymatic reactions critical for toxin production. It has been suggested that carbon dioxide inhibits enzymatic reactions, such as carboxylation/decarboxylation reactions, critical for growth of *Pseudomonas* and *Sclerotium rolfsii* (King and Nagel 1975; Kritzman et al. 1977).

Effect of Higher Pressures of Carbon Dioxide on Botulinal Toxin Production

Two additional studies were done to determine if carbon dioxide at higher pressures may serve as an effective long-term antibotulinal agent.

In one study, 130 ml of RCM (70% of the volume of the can) was pressurized with up to 1,029 kPa absolute of CO_2. However, after 24 h, the pressure in the cans was approximately one-half of the pressure present when the cans were filled (Table 2). Once equlibrated (within 24 h), the pressures in the cans did not change during the 8 weeks of storage. Within 1 week, some samples from all treatments, except cans containing 575 kPa absolute of CO_2 (initially filled at 1,029 kPa absolute), were toxic. Three of five samples pressurized at 575 kPa absolute became toxic between 4 and 8 weeks of storage.

Because of the substantial difference in pressures within cans at the time of fill and after equilibration, another study was done using less medium which would result in greater pressure levels within cans after equilibration. In this study, 46 ml of RCM (25% of the volume of the can) was pressurized with up to 1,029 kPa absolute of CO_2. After equlibration, the pressure in the cans was over 80% of the pressure at the time of fill (Table 3). As occurred before, once equilibrated, the pressures in the cans changed little during the 8 weeks of storage. Only one group of pressurized samples, those initially filled at 513 kPa absolute (438 kPa absolute after equilibration) became toxic within 2 weeks of incubation. The other pressurized samples, ranging from 548 to 891 kPa absolute after equilibration, became toxic between 4 and 8 weeks of storage.

Additionally, counts of *Clostridium botulinum* colony-forming units were determined on all samples showing no evidence of microbial growth. As was observed by Hays et al. (1959), pressurized carbon dioxide was lethal to *C. botulinum*. For example, after 8 weeks of exposure to 891 kPa absolute of CO_2, the recoverable population was reduced from 1.0×10^3 CFU/ml to 1.2×10^1 CFU/ml. The rate of decrease of recoverable colony-forming units was dependent on the amount of pressurized carbon dioxide and the length of exposure. Although pressurized carbon dioxide was lethal to *C. botulinum*, some of the surviving colony-forming units were capable of producing toxin in the presence of pressures of over 788 kPa absolute of CO_2. Hence it appears that CO_2 pressurized at levels that are commonly used in aerosol cans (719–788 kPa absolute) will not function as a fully protective

antibotulinal agent in a medium supportive of the production of botulinal toxin and held at an abusive temperature. However, since highly pressurized carbon dioxide did delay toxin production for at least 4 weeks, it is possible that pressurized carbon dioxide in combination with other antibotulinal agents can be used effectively to prevent production of botulinal toxin under otherwise hazardous conditions.

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