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Effect of Water Activity and pH on Growth and Toxin Production by *Clostridium botulinum* Type G

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The combined effect of water activity (a_w) and pH on growth and toxin production by *Clostridium botulinum* type G strain 89 was investigated. The minimum a_w at which growth and toxin formation occurred was 0.965, for media in which the pH was adjusted with either sodium chloride or sucrose. The minimum pH (at the optimum a_w) for growth and toxin production of *C. botulinum* type G was found to be 5.6. Optimum conditions for toxin activation were a trypsin concentration of 0.1%, a pH of the medium of 6.5, and an incubation for 45 min at 37°C. These data did not show evidence of heat-labile spores, since a heat shock of 75°C for 10 min did not significantly decrease the spore count of strain 89G in media at pH 7.0 or 5.6. It was frequently observed that cells grown at reduced a_w or pH experienced severe morphological changes.

Type G is the most recently recognized type of *Clostridium botulinum*. The first isolate, designated strain 89G, was obtained by Giménez and Ciccarelli (11) from a soil sample in the province of Mendoza, Argentina. Recently, Sonnabend et al. (17) identified *C. botulinum* type G during a study of human autopsy specimens of five sudden and unexpected deaths in Switzerland. Ciccarelli et al. (6) studied cultures of strain 89G to examine its physiological characteristics and the sensitivity of certain animals to its toxin. The organism is slowly proteolytic, it does not appear to ferment any sugars, and its toxin is potentiated 100- to 1,000-fold by trypsin, as are the toxins of type E and the nonproteolytic strains of types B and F (15). Solomon and Kautter (15) determined the optimum conditions for sporulation and toxin production by strain 89G. They found that spores grown on solid media yielded higher counts than did those grown in liquid media and that higher toxin titers were obtained at 26 and 30°C than at 35°C in medium with 0.4% glucose. Lynt et al. (12) studied the heat resistance of two strains of *C. botulinum* type G: the Argentine strain 89G and the Swiss strain 2739. They reported that both strains were a mixture of heat-labile and heat-resistant spores (most frequently heat labile). Solomon et al. (16) studied the effect of low temperatures on the growth of type G in crabmeat and in broth and reported that strain 89G grew at 12°C but not at any lower temperature in the assay conditions.

Although Sonnabend et al. (17) identified *C. botulinum* type G in the five patients who died suddenly in Switzerland, the investigators could not prove that botulism was the cause of death. As mentioned by Lynt et al. (12), type G "is unique in having been isolated and identified without ever having caused an outbreak of botulism." *C. botulinum* type G has never been found in foods; however, the possibility of its occurrence makes necessary a thorough study of the conditions under which it can grow and produce toxin in foods. It is well known that any change from optimum physical or chemical conditions introduces a stress; the organism must either resist or adapt to this stress (10). Water activity (a_w) and pH are recognized as important environmental factors affecting bacterial growth; thus, alterations in these parameters may provide adequate protection against the growth of pathogens in foods. The influence of a_w and pH on growth and toxin production by *C. botulinum* types A, B, and E are well documented in the literature (18); however, no data are available regarding such relationships for type G. This study, therefore, investigated the combined effect of a_w and pH on growth and toxin production by *C. botulinum* type G strain 89.

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MATERIALS AND METHODS

Culture and spore stock. *C. botulinum* type G strain 89 was obtained from D. F. Giménez, Universidad Nacional de San Luis, Argentina. Strain 89G was cultured for biochemical identification by the method of Beerens (2). It was grown in Tarozzi medium (cooked-meat broth with about 15% chopped-meat pieces plus 2% bacteriological peptone [Difco Laboratories, Detroit, Mich.] and 0.4% NaCl) at 32°C for 15 days. The culture sporulated heavily; after centrifugation the spores were washed six times with physiological saline solution (PS) (0.85% [wt/wt] sodium chloride), suspended in the PS solution, and kept refrigerated in darkness. Periodical counts demonstrated the stability of the spore suspension ($\approx 10^6$ spores per ml) maintained in this way.

Culture media. Growth and toxin production by *C. botulinum* 89G were studied on TPPYGC broth (2% Trypticase [BBL Microbiology Systems, Cockeysville, Md.]–proteose–1% peptone [Oxoid Ltd., London, England]–0.5% yeast extract [Difco]–0.5% glucose [BDH, Poole, England]–0.5% L-cysteine chlorhydrate (Mallinckrodt, Inc., St. Louis, Mo.]) which was adjusted to different combinations of a_w and pH.

Adjustment of a_w and pH. The a_w of the TPPYGC medium was 0.996; this determination was derived by measuring the freezing point (9) with a milk cryoscope (model 4 DII; Advanced Instruments, Needham Heights, Mass.). Lower levels of a_w were obtained by adding sodium chloride or sucrose. The amount of each solute required to obtain a particular level of a_w was calculated from previously reported data (as described below) and was adjusted for the a_w of the TPPYGC medium (3). For sodium chloride, the values recommended by Chirife and Resnik (4) were used; for sucrose, the compilation of Teng and Lenzi (19) was

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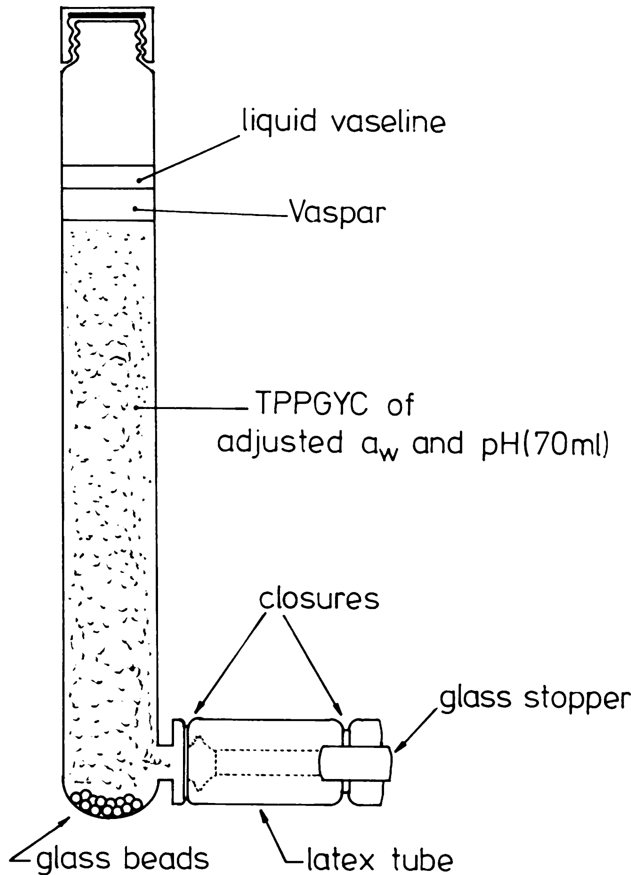

FIG. 1. Experimental device used for growth and toxin production experiments.

adopted. There is strong theoretical and experimental evidence that these previously reported a_w values are accurate to within 0.001 to 0.002. In several cases, a_w was experimentally determined for each different medium after sterilization, and excellent agreement was always found between calculated and measured a_w values. The values studied ranged between 0.996 and 0.950.

pH was measured by using an Orion Ioanalyzer 901 pH meter. Measurement of the freezing point of media adjusted to different pH values showed that the small additions of NaOH or HCl added to adjust the pH did not produce a significant alteration in a_w. The range of pH values in this study was 6.9 to 5.2.

Spore inoculation. The experimental device used for the growth experiments in the different media is shown in Fig. 1; total volume was about 110 ml. Inoculation of the media was performed by injecting the spore suspension through the latex tube (Fig. 1) by using a 3-ml syringe (25 gauge, 5/8 in. [~ 1.59 cm]). Glass beads facilitated the homogenization of the inoculated media. The inoculum ranged between 1×10^3 and 5×10^3 spores per ml of medium. To avoid any modification of the media, the following procedure was used for inoculation. Spore suspension (1 ml) in PS solution was diluted 1/10 in TPPYGC (a_w, 0.996; pH 6.9) medium; after heat shock (10 min, 75°C), 1 ml was again diluted in 9 ml of TPPYGC medium (with the same a_w and pH as that of the medium under study). Samples for growth and toxin production analysis were taken at selected intervals through the latex tube (Fig. 1) by using a small syringe. This system allowed withdrawal of samples without the danger of modification of the atmosphere of the growth media.

In addition to the main growth system (Fig. 1), 20 screw-cap test tubes (16 by 160 mm) were also filled with the same growth media and inoculated with the spore suspension. All these tubes were sealed with Vaspar. Data obtained with these tubes were also analyzed periodically to monitor agreement with data obtained from the samples collected by using the system shown in Fig. 1.

Cells and spore counts. Cells were counted by using Brewer anaerobic agar with indicator (Difco) after 48 h of incubation at 32°C; longer incubation times did not change the counts. The diluent used was brain heart infusion (Difco) at one-fourth of its usual concentration plus 0.06% agar (Difco) and 0.05% L-cysteine. The solid medium was inoculated at 48 to 50°C and was immediately cooled in water. The counts were made in tubes by using a stereoscopic microscope (magnification, ×20).

Incubation. All media were incubated at 32°C in a constant-temperature incubator.

Toxin determination. The optimum conditions for the activation of toxin by trypsin were determined as follows. To produce toxin, strain 89G was cultured at 32°C in TPPYGC broth; after 6 days it was centrifuged, and the supernatant fluid containing the toxin was decanted and filtered through a membrane filter (0.45-μm pores; Millipore Corp.). The filtered fluid was divided into different portions which were adjusted to pH 5.5, 6.0, 6.5, and 7.0 and were trypsinized with trypsin solutions (Difco) of different concentrations (0.05 to 1.0%). They were then incubated for 45 min at 37°C. Another portion of supernatant fluid containing the toxin was adjusted to pH 6.5 and incubated at 37°C for various times. These experiments allowed for the selection of optimum conditions for toxin activation, as described in the Results and Discussion section.

Toxin titers in all experiments were determined by 0.5-ml intraperitoneal injection into white Swiss Webster mice and were expressed as minimal lethal dose per milliliter of culture; two mice per dilution were inoculated with filtered (Millipore filter; 0.45-μm pores) dilutions of the culture. Toxin type was confirmed by protecting mice with the appropriate antitoxin (Centers for Disease Control, Atlanta, Ga.; type G antitoxin). Grown cultures were tested for purity both aerobically and anaerobically in nutrient agar and in VL plus 10% horse blood (2). The trypsinized medium was diluted with gelatin buffer (pH 6.2).

Heat shock. A heat shock of 10 min at 75°C was always given to the spore suspension in TPPYGC (a_w = 0.996 [pH 6.9]) before inoculation into the medium under study. The effect of heat shock on spore count was studied in the following manner. Spore suspension (1 ml) was diluted either

TABLE 1. Effect of concentration of trypsin and pH on toxin activation in *C. botulinum* 89G[a]

pH	Toxin activation (MLD[b]/ml) at trypsin concn (%):		
	1.0	0.1	0.05
7.0	2,000	20,000	10,000
6.5	10,000	20,000	20,000
6.0	2,000	20,000	10,000
5.5	1,000	2,000	2,000

[a] Incubation was for 45 min at 37°C.
[b] MLD, Minimal lethal dose.

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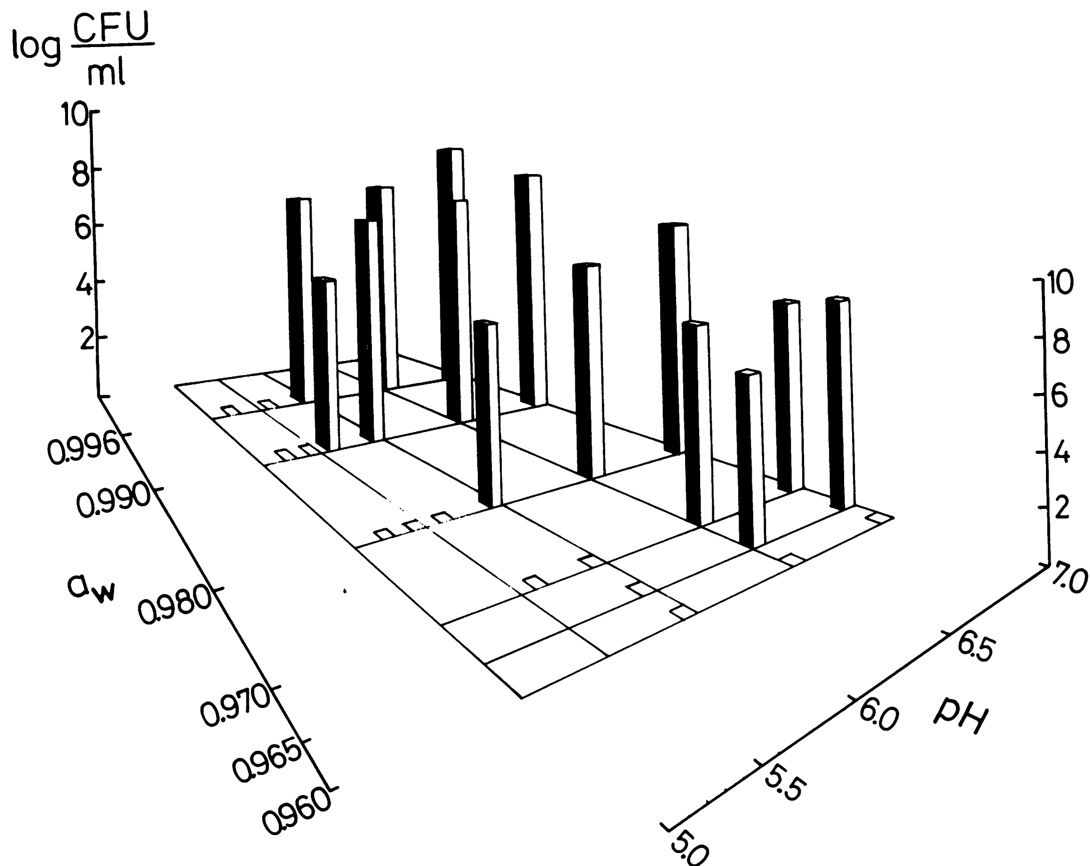

FIG. 2. Combined effect of a_w (adjusted with NaCl) and pH on the growth of *C. botulinum* type G strain 89 incubated at 32°C.

in 9 ml of TPPYGC which had been adjusted to pH 7.0 or 5.6 or in PS (pH 7.1) and subjected to a heat shock of 10 min at 75°C.

RESULTS AND DISCUSSION

Heat shock. It was found that a heat shock of 75°C for 10 min only slightly decreased the spore count of strain 89G, whether it had been suspended in TPPYGC medium (pH 7.0 or 5.6) or in PS solution (initial count, 8.5×10^5 CFU/ml; after heat shock, 5.8×10^5 CFU/ml). Lynt et al. (12) reported that *C. botulinum* G strain 89 produces two kinds of spores: one that is readily destroyed by the usual heat shock for spores of proteolytic strains (80°C, 10 min) and another that survives much higher temperatures. They found that a spore count of approximately 10^5 before heat shock was reduced to <100 after heat shock. These authors also reported quantitative data on the thermal resistance of this strain (D and Z values). Present data did not provide evidence of heat-labile spores, even after allowing for the difference in heat shock temperature (75°C instead of 80°C). Perhaps the difference with the results of Lynt et al. (12) may be attributed to the different media used for sporulation; Lynt et al. (12) used anaerobic agar without egg yolk, but in this study, Tarozzi medium was used.

Activation of toxin with trypsin. Although it is known that the toxin of *C. botulinum* type G is fully potentiated by trypsin, the optimum conditions for its activation have not been investigated in detail. The combined effect of pH and trypsin concentration on toxin activation (expressed as toxin titers) is shown in Table 1. On the basis of these results, trypsin activation was always performed at pH 6.5 and at a final trypsin concentration of 0.1%. The effect of time of incubation at pH 6.5 with 0.1% trypsin was also investigated (data not shown). It was found that the highest toxin titer was obtained after 45 min of incubation at 37°C; longer times led to lower toxin titers.

Effects of a_w and pH. Growth and toxin production curves by *C. botulinum* type G strain 89 which had been incubated at 32°C and at various values of a_w (adjusted with NaCl or sucrose) and pH were obtained (data not shown). Reduction of a_w from its optimum value produced the following effects: (i) increase of the lag period, (ii) reduction of the specific growth or toxin production rate, (iii) reduction of the maximal number of cells or toxin titer, and (iv) inhibition of growth and toxin production below certain values of a_w. There are various comments which can be made concerning the results obtained regarding the effect of a_w. It appears that for almost all experiments, using either NaCl or sucrose to adjust a_w, the optimum growth and toxin production occur at an a_w of 0.990, rather than at an a_w of 0.996 (TPPYGC broth without added solute). This result agrees well with the findings of Christian and Waltho (5), who stated that "every nonhalophilic bacterium that has been examined with sufficient care exhibits an optimum a_w, which generally lies between 0.980 and 0.995." Only in one situation (pH 5.9; a_w adjusted with sucrose) was the toxin titer (a_w = 0.996) slightly higher than at a_w = 0.990.

It is interesting to note that at pH 6.9, a reduction of a_w to 0.965 with NaCl produced a lag time of 8 days for toxin production; at a slightly lower pH (6.5), the same a_w

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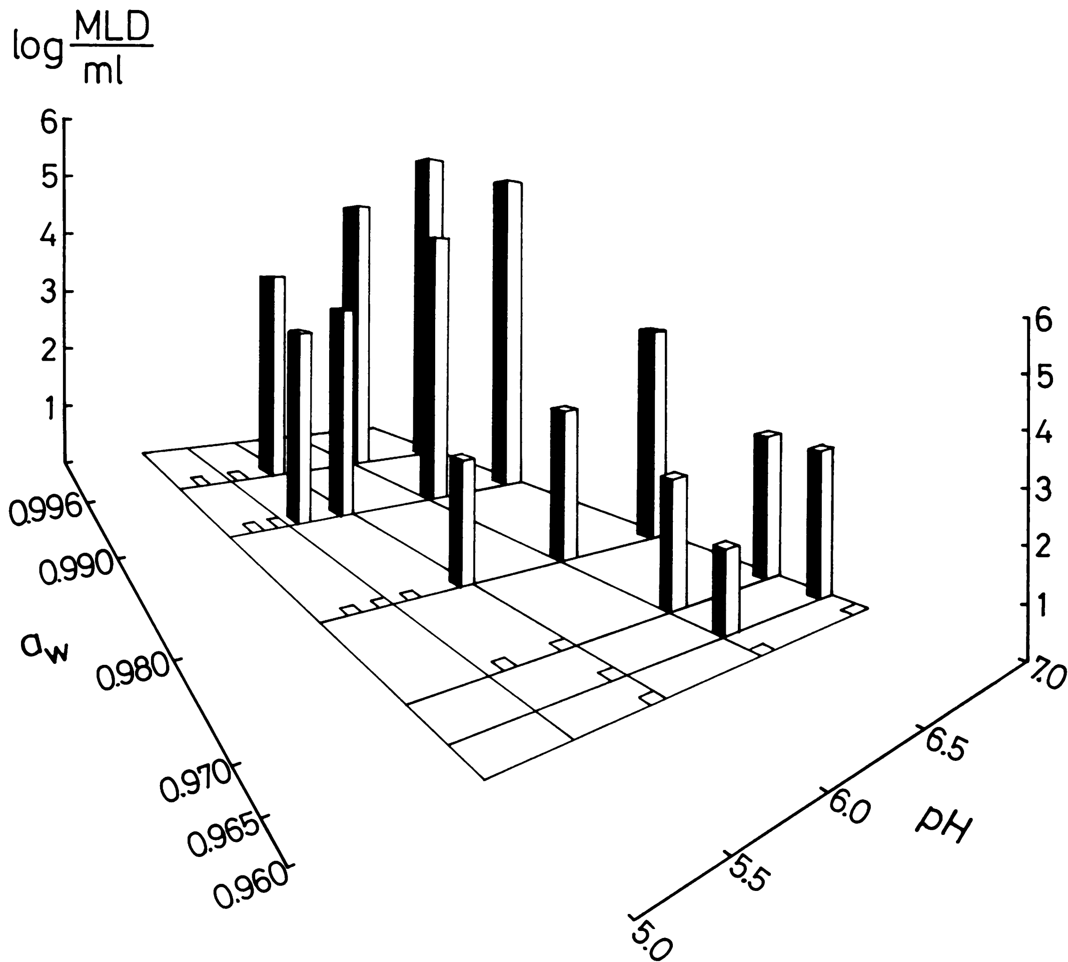

FIG. 3. Combined effect of a_w (adjusted with NaCl) and pH on toxin production by *C. botulinum* type G strain 89 incubated at 32°C. MLD, Minimal lethal dose.

(0.965) increased the lag time to about 27 days (data not shown).

In all experiments it was observed that when the toxin was trypsinized, toxin titers were dramatically higher (130- to 600-fold).

It was observed that at the optimum values of a_w and pH (0.990 and 6.9) studied here, the highest cell concentration of the strain 89G of *C. botulinum* reached about 10^8 CFU/ml, and the toxin titer reached approximately 2×10^6 minimal lethal doses per ml.

The minimum a_w at which growth and toxin production of strain 89 occurred (at 32°C) was 0.965, in media in which the a_w was adjusted either with NaCl or with sucrose. Growth of *C. botulinum* was also observed at an a_w of 0.960 in media adjusted with sucrose, but toxin was not detected after 75 days of incubation. This behavior was the subject of the present investigation. Reduction of a_w with sucrose instead of NaCl appeared to be somewhat less inhibitory for toxin production, since lag times were shorter and higher toxin titers at each a_w were obtained.

It is useful to compare the minimum a_w for growth of type G with that of the other types of *C. botulinum*, as reported previously. The minimum a_w for growth of *C. botulinum* types A and B has been reported to be in the range 0.94 to 0.96 (7, 11, 13, 14). The minimum a_w for the growth of type E is reported to be 0.97 (1, 8, 13). Therefore, the a_w limit for growth and toxin production of type G (0.965) is closer to the limit reported for type E than it is to that reported for types A and B.

It was found in the present work that the minimum pH (at the optimum a_w) for the growth of *C. botulinum* type G strain 89 was 5.6; no growth (or toxin production) was observed at pH 5.4, 5.3, or 5.2 after 75 days of incubation at 32°C. A substantial body of research reports no growth of the common types of *C. botulinum* at pH 4.8 or lower (18). Thus, with regard to the pH limit for growth, type G is more sensitive to pH than are the other types of *C. botulinum*.

The combined effect of a_w and pH on the growth and toxin production by *C. botulinum* 89G was summarized in a three-dimensional plot (Fig. 2 and 3).

Morphological changes. It was frequently observed that cells grown at reduced a_w or pH experienced severe morphological changes; chain formation of bacilli was frequently observed when the a_w was decreased to 0.965 (by adding sucrose), and lysis was noted in this case after 3 days of growth. Long bacilli (about 50 to 70 μm) were observed at pH 5.6 (at the optimum a_w).

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