Probability of Growth of *Clostridium botulinum* at Various Temperatures in a Model Broth System

By

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Thesis

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DEDICATION

My congratulations and thanks to my family, Gretchen, Brian and Barbara for getting me through it all.

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INTRODUCTION

The probability of growth of bacterial pathogens in foods is dependent on the actions and interactions of numerous food parameters (pH, a, Eh, microbial flora, preservatives), enviromental parameters (temperature, atmosphere, relative humidity), and bacterial characteristics (species, type, strain, numbers, physiological state) (Genigeorgis, 1981). Parameters affecting the growth and toxigenesis of Clostridium botulinum in foods have been studied and reported extensively (Genigeorgis and Riemann, 1979, Hauschild, 1982, Sperber, 1982). With the exceptions of the effects of heat and radiation on C. botulinum survival (Stumbo, 1965, Genigeorgis and Riemann, 1979, Licciardello, 1983), quantitative methodologies and data to permit the prediction of the effect of one or more of the above mentioned parameters on C. botulinum growth or destruction are extremely limited (Riemann, 1966, Wilson, 1980, Roberts et al, a, b, 1981, 1982, Hauschild, 1982). Such information is needed because it will allow the evaluation of the effect of old and the development of new preservation approaches based on the use of less intensive conditions to accomplish an acceptable degree of same safety as now obtained using extreme conditions, and to assist in the establishment of realistic quality control standards for C. botulinum in foods.

The use of modified atmospheres (vacuum packaging and

storage under selective gas or hypobaric atmospheres) during the refrigerated storage of fresh fish has been shown to be the an efficient step to extend product shelf-life (Huss, 1981, Parkin and Brown, 1982, Wilhelm, 1982, Finne, 1982). However, concern for the potential of C. botulinum growth during fish storage has hindered the wider application of modified atmospheres by the fish industry (Eyles and Warth, 1981, Eklund, 1982). The problem arises from the fact that C. botulinum is sometimes highly prevalent in fish (Cann et al, 1966, Huss, 1974, Huss and Pedersen, 1979) and fish products (Pace et al, 1973, Hayes, 1983), that nonproteolytic types B, E and F can grow during extended storage at 3.3 °C (Ohye and Scott, 1957, Roberts and Hobbs, 1968, Eklund et al, 1967, Sugiyama, 1980, Lynt et al, 1982) and that the inhibition of the usual aerobic spoilage flora of fresh fish by modified atmospheres may make the environment more conducive to C. botulinum growth. Overall, the effect of modified atmospheres on the food flora may also change the patterns of spoilage as well as delay it to an extent that toxigenesis may take place before the spoilage signal alarms the consumer (Cann et al, 1980, Huss, 1981, Eyles and Warth, 1981, Stier et al, 1981, Eklund, 1982, Seaward, 1982, Lee and Solberg, 1983, Lindsay, 1983).

With all the recent progress on the subject we are still lacking a quantitative evaluation of the risk of C. botulinum growth and toxigenesis in fresh fish as it relates to parameters like type of fish, type and load of C. botuli-

num cells or spores, temperature and time of storage, and the nature of the modified atmosphere. This paper is the first of a series addressing the above subject. More specifically, it deals with the development of quantitative methodology which can predict the probability of growth of C. botulinum in a model broth system as it is affected by cell type (vegetative cell, spore), serological type (A, B, E and F), proteolytic activity (proteolytic, nonproteolytic), inoculum size and length and temperature of incubation.

METHODS

EXPERIMENTAL STRAINS

A total of 17 *Clostridium botulinum* strains were used. Strains A 69, A 62, A CDC-HALL, B OKRA, B KA-40, B 213, F Langeland, and E KA-2 were obtained from Dr. Hans Riemann, Department of Epidemiology and Preventive Medicine, U.C. Davis. Strains E 250 and F 187 were obtained from Dr. Crowther, Unilever Research, Bedford, England. Strains B 133-4803, B 706, E 211, E 4062 and F 3194 were obtained from Dr. Charles Hatheway, Centers for Disease Control, Atlanta, Georgia. Strains B 17 and E Beluga were obtained from Dr. M.W. Eklund, Northwest and Alaska Fisheries Center, Seattle, Washington. Toxicity of strains was confirmed by standard toxin neutralization assay (CDC, 1974). Proteolysis was confirmed by characteristic growth in piece meat media (Difco Laboratories, Detroit, MI), and in brain heart infusion (BHI) broth (Difco) with 5% gelatin.

STOCK CULTURES

The stock cultures were prepared by growing cells in BHI broth with 0.25% L-ascorbic acid and incubating under vaspar seal at 30 °C for two to five days. A 48 hour subculture of this was inoculated into a sporulation medium in a 16x125mm test tube with a vaspar seal. For proteolytic

strains the sporulation medium was made of BHI broth with 0.1% L-cysteine hydrochloride (Sigma), and 0.1% soluble starch (Difco), pH 7.4. For nonproteolytic strains the sporulation medium was made of 5.0% trypticase peptone (BBL Microbiology Systems, Cockeysville, MD), 0.5% Bacto-peptone (Difco), 0.1% Na-thioglycolate (Difco), and 0.8% glucose (Difco), pH 7.4. Sporulation media were incubated for 10 to 14 days at 30°C. An amount of 0.1 ml of spore culture was placed in a 4 ml glass vial containing 12 porcelain beads (Star Porcelain Co., Trenton, N.J.). After vortexing, the beads were dried at room temperature for 24 hours, and placed in a desicator to be stored at -20°C.

PREPARATION OF INOCULA

Vegetative cell inocula were prepared by taking one bead of stock culture and placing in 10 ml of BHI ascorbate broth (for strains to be incubated from 12° to 47°C), or in 10 ml of BHI with 0.1% cysteine hydrochloride (for strains to be incubated from 4° to 8°C), in 16x125mm size test tubes with vaspar seal. Depending on strain, the tubes were incubated at 30°C for 24 to 72 hours.A 10 ml subculture (one ml inoculum to nine ml broth) was incubated at 30°C for 12 to 20 hours (depending on strain). One ml of this exponentially growing culture was used as inoculum for vegetative cell experiments in the probability of growth study.

Spore inocula were prepared by placing a stock culture bead into 10 ml BHI-cysteine broth, subculturing once, and

then inoculating the exponentially growing cells into 300ml of sporulation media in an Ehrlenmeyer flask. The culture was incubated at 30°C for 10 to 14 days, and when sufficient numbers of spores were formed, the culture was centrifuged (5000 x g, for 10 minutes). The spores were resuspeneded in 50% ethanol, mixed, and recentrifuged. Spore pellets were washed twice in sterile distilled water, resuspended in sterile distilled water, and stored in 4 ml aliquots at 1°C. The number of spores were determined by microscopic counting in a Petroff-Hauser chamber.

EXPERIMENTAL BROTHS AND ENUMERATION OF ORGANISMS

A 9 tube, ten-fold serial dilution of test organisms was made in either BHI-ascorbate broth (for vegetative cells to be incubated at 12 to 47°C), or BHI-cysteine broth (for vegetative cells to be incubated at 4 to 8°C, and for spores to be incubated at 4 to 47°C). The contents, nine ml, of each dilution tube was aliquoted into three screw-cap vials (size 15x45mm, 5ml capacity). Vials were sealed with 0.8 ml vaspar and incubated at the appropriate temperature. The 27 vials prepared for each strain and temperature were checked for growth at predetermined intervals of time, for a maximum of 28 days. Growth was determined by turbidity and/or gas production and confirmed by odor and phase contrast microscopy. The most probable number (MPN) of spores or cells initiating growth was determined as described previously for other bacteria

(Genigeorgis et al, 1971, Raevuori and Genigeorgis, 1975). From the number of vials out of 27 showing growth, the MPN of cells or spores of the inoculum which had initiated growth was calculated from the Tables of Fisher and Yates (1957).

In each experiment, a control set of 27 vials was incubated at 30 °C and used as a reference point to which growth at higher or lower temperatures was compared. The $\log_{10}$ of the MPN of spores or cells initiating growth at a particular temperature was subtracted from the $\log_{10}$ of the MPN of spores or cells initiating growth at 30 °C, to determine the magnitude of decimal reduction (DR) or increase in the number of cells required to initiate growth relative to 30 °C. The per cent probability of one cell or spore initiating growth at a particular temperature was calculated from the reciprocal of the antilog of DR (P=1/antilog DR). The per cent probability of growth after a particular length of time of incubation was calculated from the cumulative number of vials showing growth, by that day, leading to the determination of the MPN and DR.

STATISTICAL METHODS

The experiments were arranged in a factorial fashion (Snedecor and Cochran, 1967), involving 17 strains, a maximum of 9 temperatures, two cell types (spores and vegetative cells), a maximum of eight observations of growth during a 28 day incubation, and one replication. The

biomedical computer programs (Dixon and Brown, 1979) for multiple regression analysis BMDP 2R and 9R were used to develop equations relating the effects of strain, temperature, cell type, and time (independent variables) on the probability of growth by one cell or spore (dependent variable). In the regression analysis, the log of the per cent probability for each situation was used.

RESULTS

Using factorial design, 612 experiments were performed to determine the effect of serological type (A, B, E, and F), strain variation, proteolytic activity (proteolytic and nonproteolytic), cell type (spore and vegetative cell), temperature, and length of incubation on the probability of growth initiation of C. botulinum in a model broth system.

GROWTH OF NONPROTEOLYTIC STRAINS AT VARIOUS TEMPERATURES

Figure 1 presents the findings on the growth of nonproteolytic spores and vegetative cells after 28 days incubation at various temperatures. Data points represent the per cent probability of growth calculated at the end of the 28 day period without taking into account the rate of growth with respect to time. The data indicate a difference in behavior with respect to strain. No strain variation is noted at 30°C, because this temperature has been used as a base line for the relative comparison of the per cent probability (% probability 30°C = 100) of growth with the other temperatures. Temperature points showing a higher per cent probability of growth compared to 30°C, at times even more than 100%, reflect an enviroment more conducive to growth at that temperature than at 30°C. In the paper, the reported probability is per cent relative probability with respect to 30°C. Comparison of microscopic count and

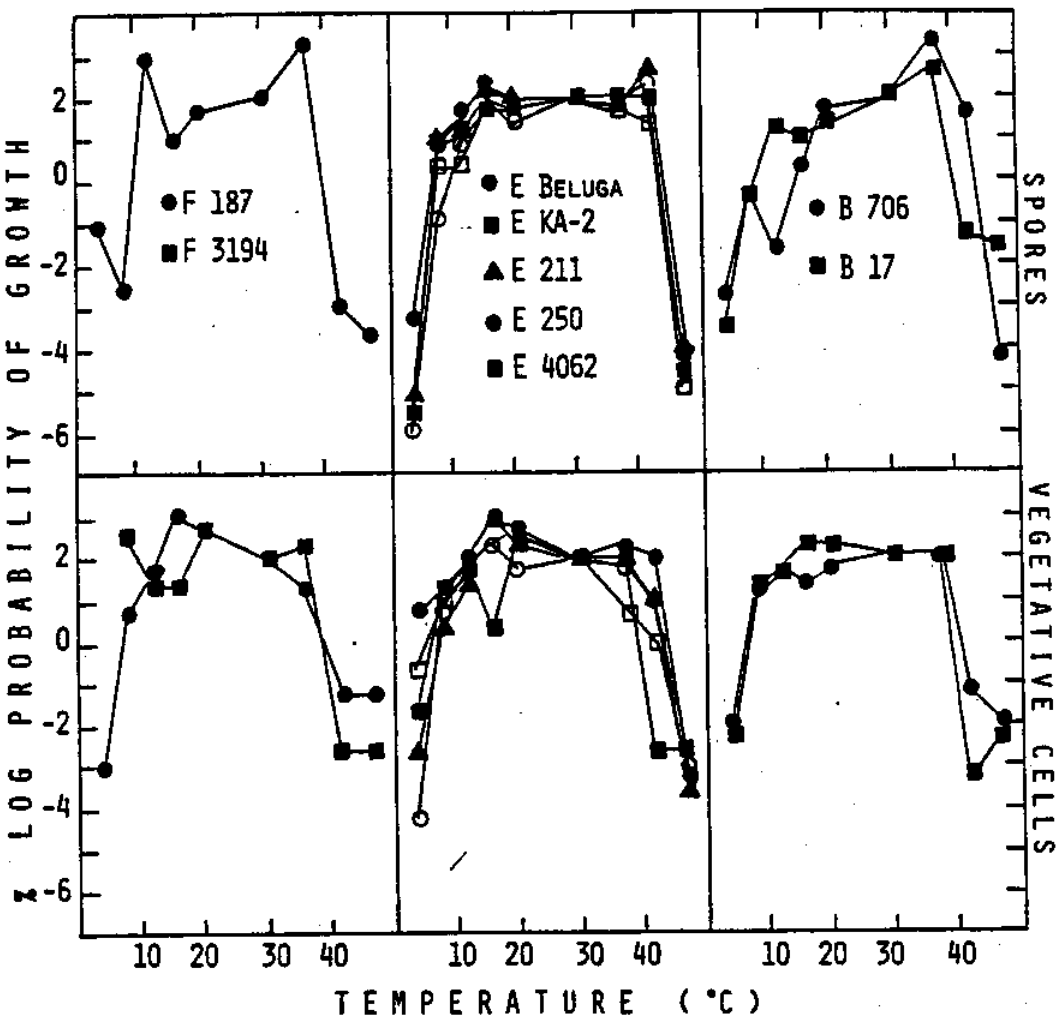

FIGURE 1. PROBABILITY OF GROWTH INITIATION OF NONPROTEOLYTIC *C. BOTULINUM* TYPES B, E, AND F SPORES AND VEGETATIVE CELLS IN A MODEL BROTH SYSTEM AFTER 28 DAYS INCUBATION AT VARIOUS TEMPERATURES.

MPN for spore preparations indicated a good correlation and the ability of even one spore to initiate growth at 30°C.

The general trend of probability of growth with respect to temperature is a bell shaped curve with maximum per cent probability of growth in the temperature range from approximately 15° to 40°C, and lower per cent probability at the extreme upper and lower temperatures. This means that as the temperature of incubation increases or decreases to either side of the midrange, higher numbers of spores or vegetative cells are needed to initiate growth.

As seen in Figure 1, nonproteolytic type B spore inocula displayed optimal growth probability at 37°C, with a steady decline in per cent probability of growth in the midrange area of 40° to 20°C. Vegetative cell inocula for type B strains had relatively even growth in the midrange, as did both spore and vegetative cell inocula for type E. Type F strains presented data too erratic and limited for noting general trends.

Type E vegetative cell inocula show a large variation in per cent probability of growth at 4°C. The log per cent probability ranged from 0.76 to -4.24 with a median of -1.67. These numbers mean that at 4°C, 17 to 1.7×10^6 cells/ml would be needed for growth initiation, with a median of 4.7×10^3 cells/ml. Spore inocula show a log per cent probability range from -1.00 to -5.92, with a median of -4.40. The difference of 2.73 between vegetative cell and spore inocula medians may be due to the added

Table 1a. Decimal reduction and per cent probability of growth of nonproteolytic vegetative cells at various temperatures.

		Incubation Temperature																							
		47 °C			42 °C			37 °C			30 °C			20 °C			16 °C			12 °C			8 °C			4 °C		
Type	Strain	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )
B	706	3.93	1.2×10^{-2}	(0)	3.23	5.8×10^{-2}	(0)	0	100	(12)	0	100	(12-21)	0.31	49	(17)	.66	21	(19)	0.31	49	(17)	0.69	28	(32)	5.58	2.6×10^{-4}	(0)
B	17	4.24	5.9×10^{-3}	(0)	5.24	5.0×10^{-4}	(0)	0	100	(18)	0	100	(19-24)	-.69	494	(26)	-1.0	1000	(22)	0	100	(24)	1.66	2	(19)	8.66	5.8×10^{-3}	(0)
E	Beluga	5.24	5.8×10^{-4}	(0)	0	100	(18)	-.34	218	(22)	0	100	(19-24)	-.69	494	(26)	-1.0	1000	(22)	0	100	(24)	1.66	2	(19)	3.24	6	(19)
E	HA-2	4.58	2.6×10^{-3}	(0)	4.58	2.6×10^{-3}	(0)	0	100	(16)	0	100	(13-23)	-.65	444	(22)	1.69	2	(18)	0.35	44	(19)	0.69	20	(21)	3.58	2.6×10^{-2}	(0)
E	211	5.58	2.6×10^{-4}	(0)	1.0	10	(16)	0	100	(19)	0	100	(16-22)	-.31	203	(21)	-1.0	1000	(22)	0.69	20	(10)	1.67	2	(16)	4.58	2.6×10^{-3}	(0)
E	250	4.93	1.2×10^{-3}	(0)	1.0	10	(14)	.35	44	(19)	0	100	(17-21)	.35	44	(19)	-.31	202	(21)	0.35	44	(19)	1.33	5	(17)	6.24	5.8×10^{-5}	(0)
E	4062	5.24	5.0×10^{-4}	(0)	2.0	1	(12)	1.31	5	(16)	0	100	(15-20)	-.69	494	(20)	-.31	202	(21)	0	100	(18)	1.00	10	(12)	2.69	2×10^{-1}	(12)
P	187	3.24	5.8×10^{-2}	(0)	3.24	5.8×10^{-2}	(0)	.69	20	(15)	0	100	(12-22)	-.69	494	(17)	-1.31	2032	(21)	0.31	49	(14)	1.34	5	(18)	4.93	1.2×10^{-3}	(0)
P	3194	4.58	2.6×10^{-3}	(0)	4.58	2.6×10^{-3}	(0)	-.34	219	(19)	0	100	(16-21)	-.78	208	(23)	.66	22	(19)	0.66	22	(19)	-.66	456	(18)	-------		

Table 1b. Decimal reduction and per cent probability of growth of nonproteolytic spores at various temperatures.

		Incubation Temperature																							
		47 °C			42 °C			37 °C			30 °C			20 °C			16 °C			12 °C			8 °C			4 °C		
Type	Strain	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )
B	706	6.24	5.8×10^{-5}	(0)	.34	47	(18)	-1.35	2254	(23)	0	100	(16-23)	.33	47	(16)	1.65	2	(11)	3.65	2×10^{-2}	(5)	2.35	4×10^{-1}	(16)	4.69	2×10^{-3}	(9)
B	17	3.58	2.6×10^{-2}	(0)	3.34	4.6×10^{-2}	(3)	-.65	466	(15)	0	100	(11-19)	.66	22	(13)	1.0	10	(12)	0.66	22	(13)	2.34	5×10^{-1}	(12)	5.58	3×10^{-4}	(8)
E	Beluga	6.24	5.8×10^{-5}	(0)	-.31	202	(24)	.35	44	(22)	0	100	(2)-26)	0	100	(25)	-.35	225	(26)	0.34	46	(24)	1.35	4	(22)	5.24	5.8×10^{-4}	(10)
E	HA-2	6.58	2.6×10^{-5}	(0)	0	100	(22)	0	100	(22)	0	100	(22-25)	.31	49	(21)	.31	49	(23)	0.66	22	(22)	1.00	10	(22)	7.58	2.6×10^{-6}	(0)
E	211	6.24	5.8×10^{-5}	(0)	-.69	492	(23)	.31	49	(20)	0	100	(21-24)	0	100	(22)	-.35	225	(23)	1.00	10	(19)	1.00	10	(21)	7.24	5.8×10^{-6}	(0)
E	250	6.93	1.2×10^{-5}	(0)	0	100	(23)	0	100	(23)	0	100	(23-24)	.66	22	(22)	0	100	(24)	1.31	5	(20)	3.00	1×10^{-1}	(12)	7.93	1.2×10^{-6}	(0)
E	4062	6.93	1.2×10^{-5}	(0)	.66	22	(22)	.31	49	(23)	0	100	(24-25)	.34	46	(24)	0	100	(25)	1.65	2	(23)	1.69	2	(18)	4.00	1×10^{-2}	(11)
P	187	5.58	2.6×10^{-4}	(0)	5	1X10	(4)	-1.35	2254	(23)	0	100	(19-21)	.31	49	(20)	1.0	10	(18)	-1.00	995	(24)	4.65	2×10^{-3}	(5)	1.00	1×10^{-1}	(10)

Numbers in parentheses refers to number of positive tubes, cumulative by day, out of a maximum of 10.

factor of germination. Overall, for both spore and vegetative cell inocula of all nonproteolytic strains, the log per cent probability of growth at 4^{o} C ranged from 0.76 to -5.92, with a median of -2.90. No growth was observed at 47^{o} C with vegetative cell inoculum levels of $2X10^{3}$ to $4X10^{5}$ /ml and spore inoculum levels of $4X10^{3}$ to $8X10^{6}$ /ml.

A detailed list of data concerning decimal reduction or increase and per cent probability of growth of the nonproteolytic vegetative cell and spore preparations is presented in Table 1.

GROWTH OF PROTEOLYTIC STRAINS AT VARIOUS TEMPERATURES

Figure 2 presents the data on the log per cent probability of growth of proteolytic types and strains with respect to temperature. The general trends of strain variation and bell shaped response of log probability to temperature observed for the nonproteolytic strains are also true for the proteolytic strains.

Type A spore inocula show a higher per cent probability of growth at high temperatures than do type A vegetative cell inocula. At 47^{o} C, the log per cent probability of spore inocula for two strains (A 69 and A CDC-HALL) was 0.31 and 0.66 respectively. Strain A 62 was sensitive at 47^{0} C, with a log probability of growth of -5.59. The log per cent probability at 47^{0} C for vegetative cells for all three type A strains was -6.24. At 12^{o} C, the log per cent probability of growth by vegetative cells and spores of all proteolytic

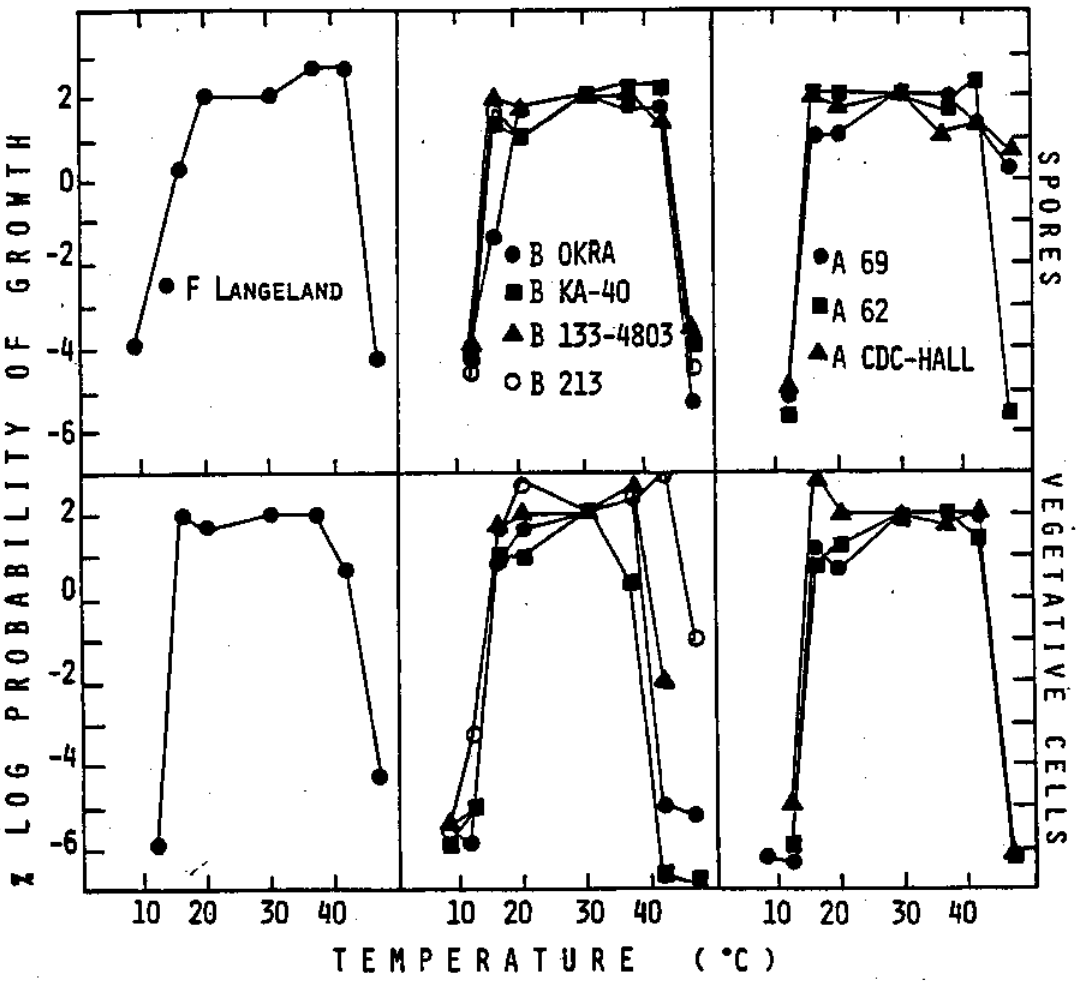

FIGURE 2. PROBABILITY OF GROWTH INITIATION OF PROTEOLYTIC C. BOTULINUM TYPES A, B, AND F SPORES AND VEGETATIVE CELLS IN A MODEL BROTH SYSTEM AFTER 28 DAYS INCUBATION AT VARIOUS TEMPERATURES.

Table 2a. Decimal reduction and per cent probability of growth of proteolytic vegetative cells at various temperatures.

Type	Strain	47 C			42 C			37 C			30 C			20 C			16 C			12 C		
		DR	Prob	( )	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )
A	69	8.24	5.8X10	-7 (0)	0	100	(26)	0	100	(26)	0	100	(26-28)	1.34	5	(24)	.66	22	(25)	8.34	4.6X10	-6 (1)
A	62	8.24	5.8X10	-7 (0)	.66	22	(25)	0	100	(27)	0	100	(24-27)	.70	20	(24)	1.31	5	(20)	7.93	1.2X10	-5 (0)
A	CDC-HALL	8.24	5.8X10	-6 (0)	0	100	(27)	.31	49	(26)	0	100	(21-27)	0	100	(26)	-1.0	995	(24)	7.00	1X10	-6 (0)
B	OKRA	7.24	5.8X10	-7 (0)	6.6	1X10	-5 (3)	-.34	219	(25)	0	100	(24-26)	.35	44	(25)	1.0	10	(23)	7.00	1X10	-5 (6)
B	KA-40	8.93	1.2X10	-6 (0)	7.0	1X10	-7 (3)	1.70	2	(24)	0	100	(26-29)	.35	44	(25)	.35	44	(25)	7.00	1X10	-4 (8)
B	133-4003				8.7	2X10	-7 (3)	-.70	494	(26)	0	100	(24-27)	0	100	(27)	.66	22	(22)	5.31	4.9X10	-6 (8)
B	213	3.0	1X10	-5 (11)	4.0	1X10	-7 (12)	-.70	494	(23)	0	100	(22-25)	-.69	494	(26)	0	100	(25)	7.93	1.2X10	-5 (0)
F	Langeland	6.2	5.8X10	-5 (0)	-1.0	1000	(25)	-.35	225	(23)	0	100	(25-27)	.35	44	(25)						
					1.31	5	(17)	0	100	(27)												

Table 2b. Decimal reduction and per cent probability of growth of proteolytic spores at various temperatures.

Type	Strain	47 C			42 C			37 C			30 C			20 C			16 C			12 C		
		DR	Prob	( )	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )	DR	Prob	( )
A	69	1.69	2	(18)	.69	20	(21)	0	100	(23)	0	100	(23-24)	1.0	10	(21)	1.0	10	(21)	7.14	7X10	-6 (1)
A	62	7.58	2.6X10	-6 (0)	-.35	224	(26)	1.0	10	(19)	0	100	(25)	0	100	(25)	0	100	(25)	7.58	2.6X10	-5 (0)
A	CDC-HALL	1.34	5	-6 (21)	.65	23	(23)	1.0	10	(24)	0	100	(26-21)	1.0	10	(18)	0	100	(23)	6.93	1.2X10	-5 (0)
A	OKRA	7.34	4.6X10	-4 (3)	.34	46	(24)	.34	46	(24)	0	100	(25)	0	100	(25)	0	100	(23)	6.34	4.6X10	-5 (6)
B	KA-40	5.93	1.2X10	-4 (0)	-.31	203	(21)	-.31	203	(21)	0	100	(23-25)	.35	44	(22)	.35	44	(22)	6.34	4.6X10	-5 (6)
B	133-4003	5.58	2.6X10	-5 (1)	.34	46	(21)	0	22	(22)	0	100	(19-20)	.35	44	(24)	3.34	4.6X10	(15)	6.24	5.8X10	-4 (0)
B	213	6.49	3.3X10	-5 (1)	-.69	492	(23)	-.69	492	(23)	0	100	(22)	1.0	10	(19)	.66	22	(19)	5.93	1.2X10	-5 (0)
F	Langeland	6.24	5.8X10	(0)	-.69	492	(23)	-.69	492	(23)	0	100	(21-26)	0	100	(19)	0	100	(20)	6.58	2.6X10	-6 (6)
																	.34	46	(21)	6.00	1X10	-6 (6)
																	1.69	2	(21)			

a Number in parenthesis refers to number of positive tubes, cumulative by day, out of a maximum of 30.

A, B and F strains ranged from -3.31 to -6.34, with a median of -4.95. At 47°C, the log per cent probability of growth by all vegetative cells and spores of all proteolytic A, B, and F strains ranged from 0.31 to -6.92, with a median of -4.15.

A detailed list of data concerning decimal reduction or increase and per cent probability of growth of proteolytic vegetative cell and spore preparations is presented in Table 2.

PROBABILITY OF GROWTH INITIATION WITH RESPECT TO LENGTH OF INCUBATION

Figures 3 and 4 present data on the per cent probability of growth initiation with respect to time by nonproteolytic spores and vegetative cells incubated at 8° to 20°C for 28 days. As mentioned before, a strain variation was also observed in these experiments. For one cell or spore in a hundred to initiate growth at 20°C (log per cent probability = 0), it required 3 to 4 days. At 16°C, it required 3 to 5 days. At 12°C, spores required 7 days, while vegetative cells required 3 days. At 8°C, spores required 15 days, while vegetative cells required 7 days.

Figures 5 and 6 present data on the per cent probability of growth initiation with respect to time by proteolytic spores and vegetative cells incubated at 12° to 20°C for 28 days. Strain variation is present as mentioned above. For one cell in a hundred to initiate growth at 20°C (log per cent probability = 0), it took 3 to 7 days for

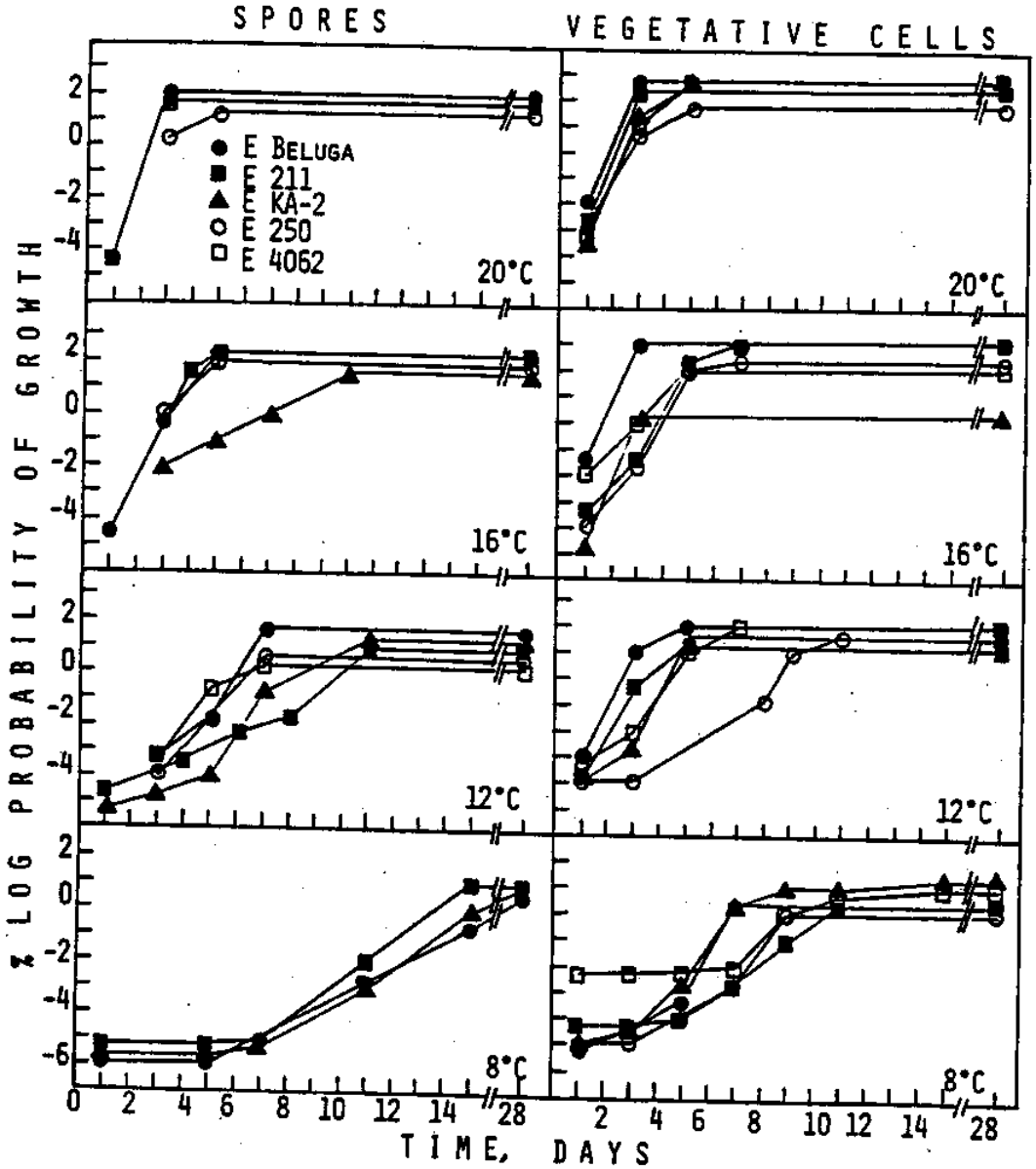

FIGURE 3. PROBABILITY OF GROWTH INITIATION OF NONPROTEOLYTIC *C. BOTULINUM* TYPE E SPORES AND VEGETATIVE CELLS IN A MODEL BROTH SYSTEM WITH RESPECT TO TIME OF INCUBATION (MAXIMUM OF 28 DAYS) AT 8 TO 20°C.

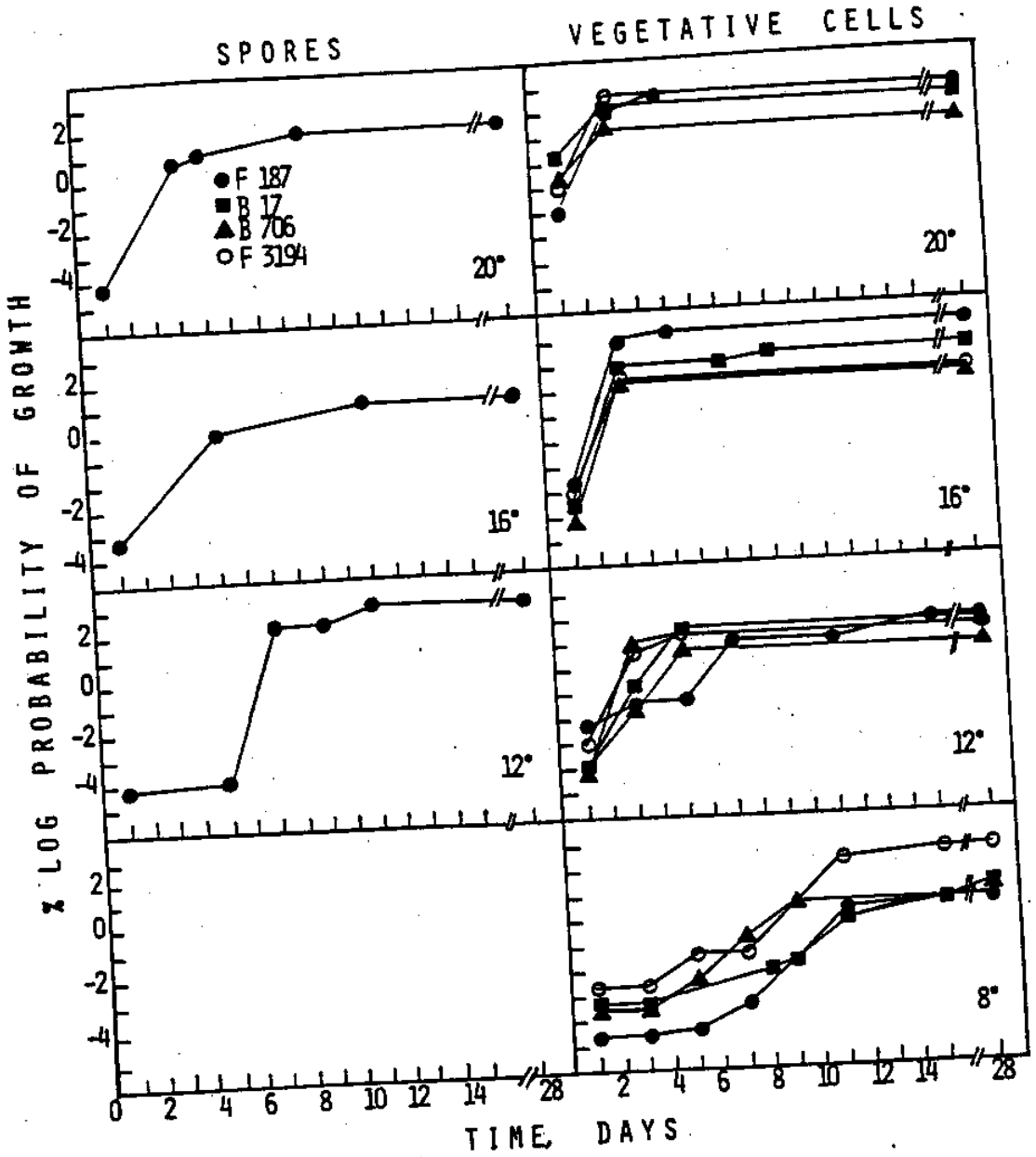

FIGURE 4. PROBABILITY OF GROWTH INITIATION OF NONPROTEOLYTIC C. BOTULINUM TYPES B AND F SPORES AND VEGETATIVE CELLS IN A MODEL BROTH SYSTEM WITH RESPECT TO TIME OF INCUBATION AT 8 TO 20°C.

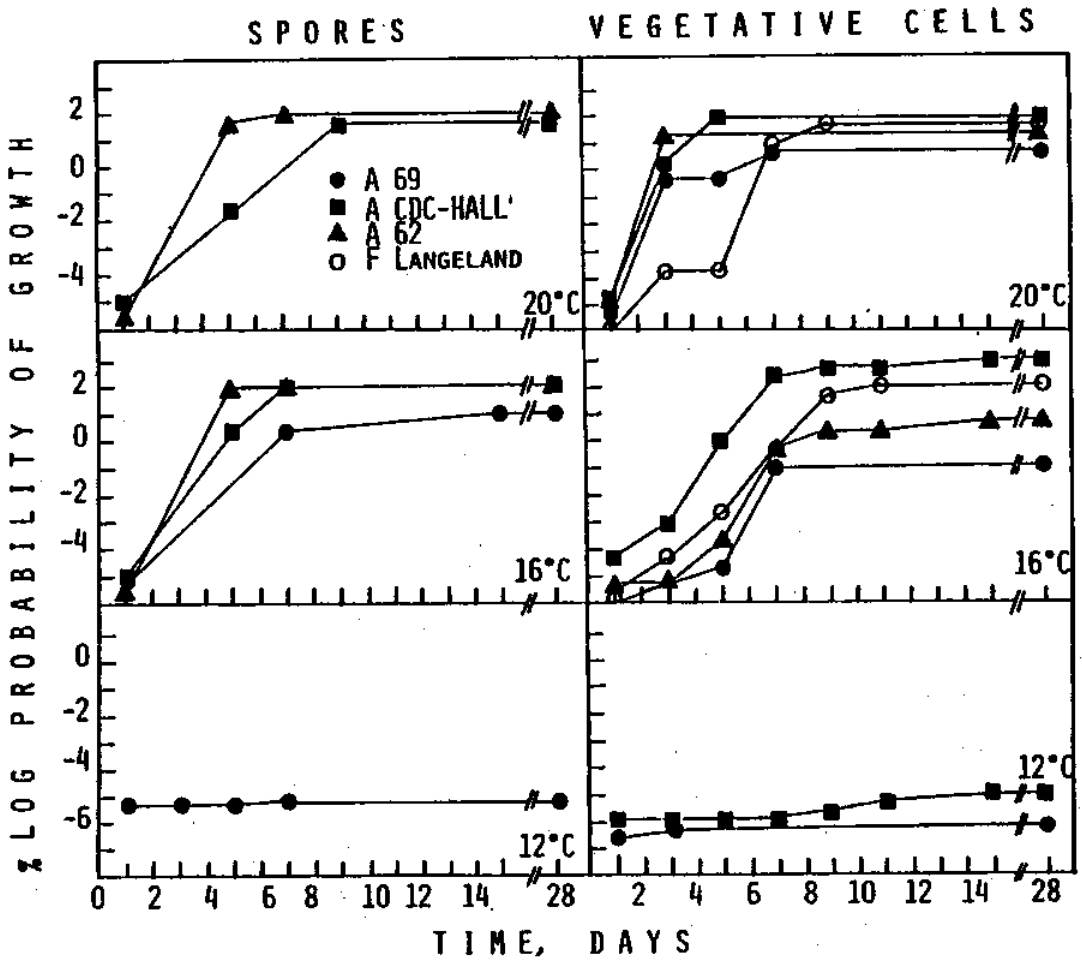

FIGURE 5. PROBABILITY OF GROWTH INITIATION OF PROTEOLYTIC C. BOTULINUM TYPES A AND F SPORES AND VEGETATIVE CELLS IN A MODEL BROTH SYSTEM WITH RESPECT TO TIME OF INCUBATION (MAXIMUM OF 28 DAYS) AT 12 TO 20°C.

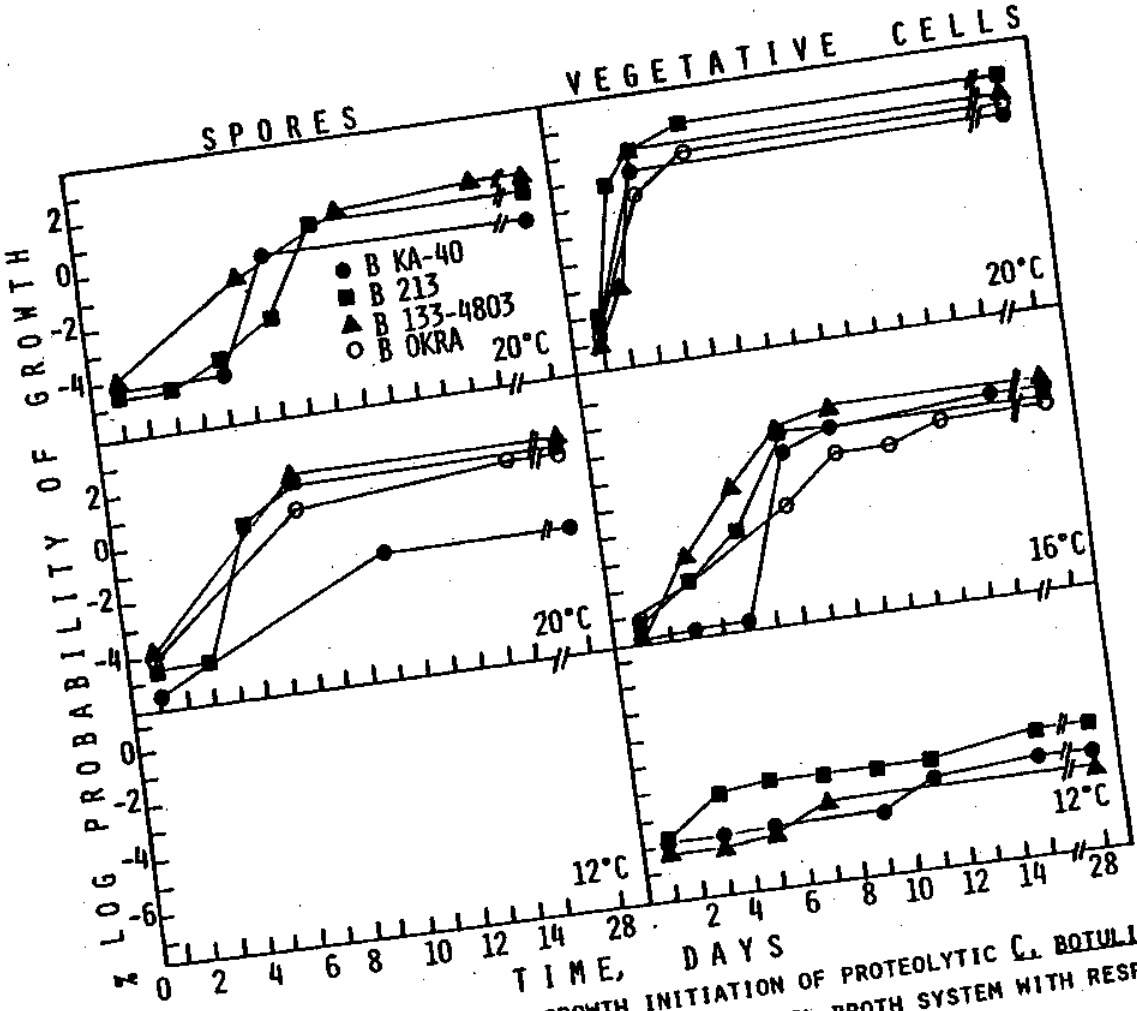

FIGURE 6. PROBABILITY OF GROWTH INITIATION OF PROTEOLYTIC C. BOTULINUM TYPE B SPORES AND VEGETATIVE CELLS IN A MODEL BROTH SYSTEM WITH RESPECT TO TIME OF INCUBATION (MAXIMUM OF 28 DAYS) AT 12 TO 20°C.

spore and vegetative cell inocula. At 16 C, 5 to 6 days were required. At 12 C, more than one cell or spore in a hundred (log per cent probabilities were all less than 0) was required for growth initiation. In comparing the log per cent probability means of the two populations, spore and vegetative cell, at each experimental temperature, the differences between the two were 1.89 (47 C), 2.45 (42 C), 0.01 (37 C), 0.21 (20 C), 0.35 (16 C), and 0.71 (12 C).

REGRESSION ANALYSIS

Using the biomedical computer program BMDP 2R and 9R (Dixon and Brown), the predictive value of known factors on the per centprobability of growth initiation of one cell or spore was evaluated. The factors evaluated were temperature (T), length of incubation (day), cell type (spore, vegetative cell), and proteolytic activity (proteolytic, nonproteolytic). These factors were used in the program as independent variables, with log per cent probability of growth as dependent variable. Regression equations generated were of the general form $Y = a + b_1 x_1 + b_2 x_2 + b_3 x_3 + \ldots + b_n x_n$. Where Y = estimated log probability of growth initation of one spore or cell, a = intercept, b_1, b_2, b_3, . . . b_n = regression coefficients, and x_1, x_2, x_3, . . . x_n = independent variables affecting the per cent probability of growth.

Table 3 presents the most important variables (T, T^2, T^3), intercepts, regression coefficients, squared multiple

Table 3. Summary of variables, intercepts, regression coefficients, squared multiple correlation coefficients (R^2), and standard errors (SE) of computer derived regression equations describing the log per cent probability of growth of spores and vegetative cells of *C. botulinum* incubated for 28 days at 4 to 47C in BHI broth.

Inoculum	Range[a]	Intercept (A)	Temperature (T)	T^2	T^3	R^2	SE
		Regression Coefficients					
All Cases							
Nonproteolytic vegetative cell	L	-5.76	1.23	-0.060	0.0009	0.69	0.85
	H	-3.26		0.016	-0.0003	0.67	1.25
Nonproteolytic spore	L	-0.79	1.60	-0.080	0.0013	0.78	1.07
	H	-13.49		0.048	-0.0008	0.85	1.13
Proteolytic vegetative cell	L	-29.60	2.76	-0.057		0.86	1.23
	H	-7.92		0.028	-0.0006	0.62	2.04
Proteolytic spore	L	-25.23	2.33	-0.048		0.86	1.07
	H	-12.16		0.036	-0.0007	0.73	1.38
Extreme Value							
Nonproteolytic vegetative cell	L	-1.84	0.59	-0.027	0.0004	0.73	0.72
	H	-10.54		0.032	-0.0006	0.99	0.27
Nonproteolytic spore	L	-6.09	1.56	-0.089	0.0015	0.94	0.52
	H	-20.90		0.056	-0.0010	0.98	0.87
Proteolytic vegetative cell	L	-25.81	2.59	-0.056		0.85	2.01
	H	-12.42		0.034	-0.0006	0.91	0.93
Proteolytic spore	L	-25.02	2.41	-0.050		0.88	1.80
	H	-6.59		0.020	-0.0004	0.96	0.34

[a] L refers to the low range of incubation temperature, 4 to 30 C. H refers to the high range of incubation temperature, 30 to 47 C.

Table 4. Summary of variables, intercepts, regression coefficients, squared multiple correlation coefficients (R^2), and standard errors (SE), of computer derived regression equations describing the log probability of growth of spores and vegetative cells of C. botulinum in BHI broth after an incubation of 1 to 28 days at 4 to 30C.

Inoculum	Intercept	Regression Coefficients				R^2	SE
		Temperature (T)	(1/T) e	1/day	T X e (1/day)		
All Cases	49.96	-0.21	-40.42	-8.02	0.12	0.74	1.07
Nonproteolytic vegetative cell	74.51	-0.10	-66.62	-5.91		0.72	1.35
Nonproteolytic spore	247.34	-0.28	-228.27	-4.38		0.79	1.46
Proteolytic vegetative cell	190.64	-0.26	-174.47	-6.12		0.67	1.65
Proteolytic spore							
Extreme Value	68.89	-0.29	-58.70	-5.65	0.09	0.73	0.90
Nonproteolytic vegetative cell	93.65	-0.30	-82.22	-8.64	0.10	0.75	1.53
Nonproteolytic spore	313.30	-0.53	-285.04	-4.17		0.79	1.48
Proteolytic vegetative cell	296.09	-0.43	-271.39	-3.53		0.75	1.74
Proteolytic spore							

correlation coefficients (R^2), and standard errors (SE) of regression equations for various types of C. botulinum inocula. The equations are based on per cent probabilities observed after a 28 day incubation, therefore, time of incubation has not been used as an independent variable. In order to develope the curve of best fit, the data was divided into two groups, one representing incubation temperatures of 4 to 30 °C, and another with temperatures of 30 °C to 47 °C. For each inoculum type two equations were generated, one in which all data points for strains were considered (all cases), and one in which only the data points corresponding to the growth of the strain showing the highest per cent probability of growth at a particular temperature was used (extreme value).

Table 4 presents intercepts, standardized regression coefficients, R^2, and SE for regression equations for various types of C. botulinum inocula, based on the use of temperature (T) and length of incubation (day) as independent variables. The regression coefficients are standardized so as to be independent of unit of measure, thus allowing a comparison of the absolute values of the coefficients in determining the effect of each independent variable on the dependent variable. As above, two equations, "all cases" and "extreme value", were generated for each type of inoculum. Only the low range incubation temperatures (below 30 °C) were examined over time of incubation.

DISCUSSION

GENERAL METHODOLOGY

Certain C. botulinum strains (B 17, B 706, F 187) did not consistently produce high yields of vegetative cells. As a result, the effect of a particular high or low temperature on the per cent probability of growth initiation could not be measured precisely with respect to the per cent probability at 30°C. In this case, the experiments were repeated until high yield inocula were obtained. This was not a problem for spore inocula, as spores were concentrated to acceptable levels for these strains. Computer derived regression equations are based on cell and spore inocula of at least 10^5/ml. In this study, the spores were not heat shocked in order to duplicate the natural conditions of growth.

Ascorbate was originally chosen as reductant for the media used, however, over long incubation times at 4°C, a color change in the media occurred which appeared to affect the per cent probability of growth. This was not observed at any other temperature. The problem was resolved by substituting cysteine hydrochloride for ascorbate in all subsequent experiments.

COMPARISON OF NONPROTEOLYTIC AND PROTEOLYTIC STRAINS

Reviews of C. botulinum have stressed the essential differences between the nonproteolytic and proteolytic types

(Sugiyama, 1980, Lynt et al, 1982, Eklund, 1982). Primary differences are proteolysis, heat resistance, somatic and spore antigens, salt tolerance and growth at low temperatures. Protelytic strains are unable to grow below 10° C, whereas nonproteolytic strains have exhibited growth at 3.3° C (Oosterom et al, 1981, Stier et al, 1982). This is demonstrated in Figures 1, 2 and 7. More specifically, Figure 7 shows this difference by comparing a nonproteolytic (E KA-2) and a proteolytic strain (A 69). Strain A 69 shows negligible growth at 12° C after 28 days. Only one spore in $1.4X10^{7}$ or one vegetative cell in $2.2X10^{8}$ would grow at 12° C. This means, it would take an inoculum of $1.4X10^{7}$ spores/ml or $2.2X10^{8}$ cells/ml to initiate growth at 12° C. For strain E KA-2, as few as 4.6 spores/ml or 2.3 vegetative cells/ml would initiate growth at 12° C after 28 days incubation. No growth occurred for strain A 69 at 8° C. For strain E KA-2 10 spores/ml or 5 cells/ml were needed for initiation of growth at 8° C. Greater inocula of $3.8X10^{7}$ spores/ml or $3.8X10^{3}$ cells/ml were required for growth initiation at 4° C.

Figures 1 and 2 illustrate growth initiation at 4° to 47° C after a total of 28 days incubation. Both A 69 and E KA-2 required one cell or one spore/ml at 37° C in order to initiate growth. At 42° C, E KA-2 required one spore/ml for growth initiation, but no growth occurred with vegetative cell inocula of $3.8X10^{4}$ /ml, while A 69 required either one spore or one cell/ml. With respect to strain E KA-2, no

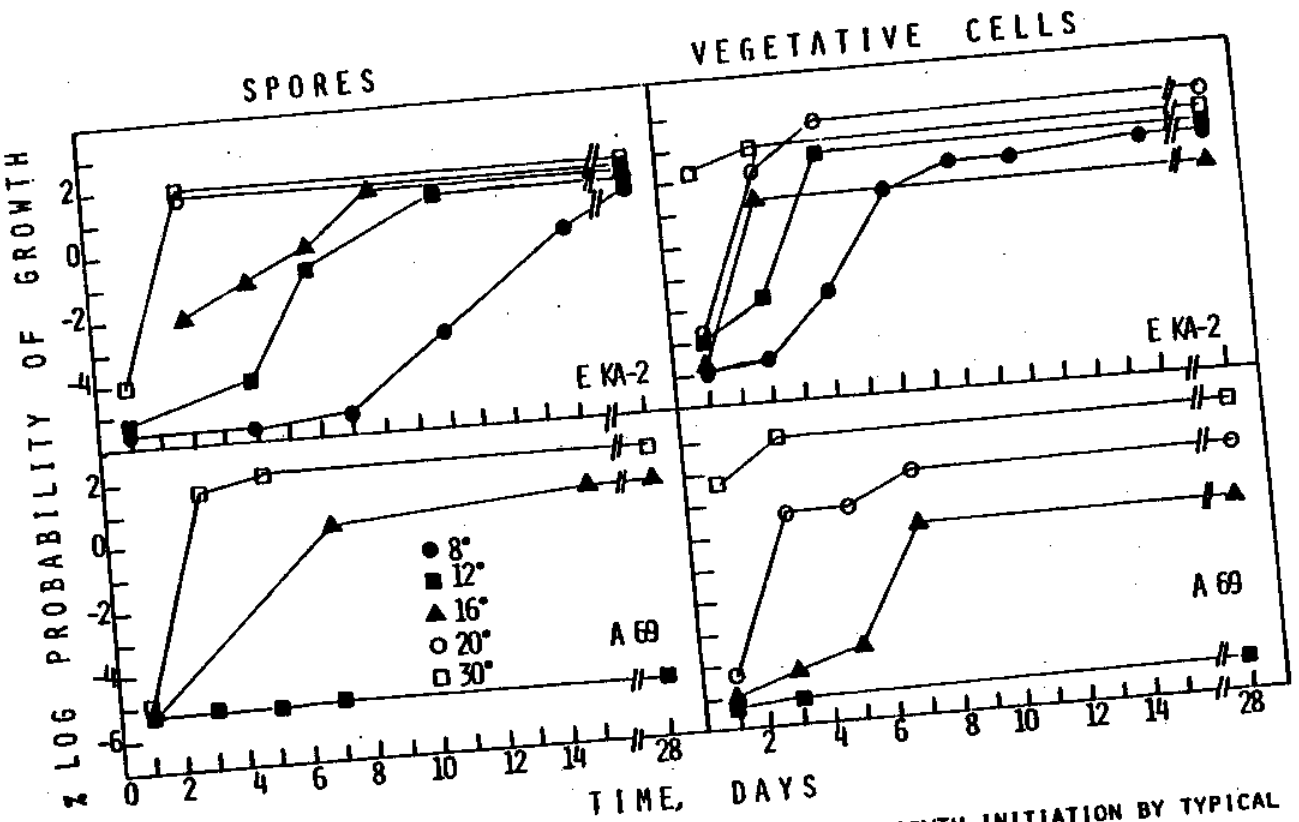

FIGURE 7. A COMPARISON OF THE LOG PROBABILITIES OF GROWTH INITIATION BY TYPICAL PROTEOLYTIC (A69) AND NONPROTEOLYTIC (E KA-2) C. BOTULINUM SPORES AND VEGETATIVE CELLS IN BHI BROTH WITH RESPECT TO TIME OF INCUBATION AT 8 TO 30°C.

growth was initiated at 47°C by inoculum of 3.8X106 spores/ml or $3.8X10^4$ cells/ml. For A 69, no growth occurred with inocula of $1.7X10^8$ cells/ml, but 50 spores/ml initiated growth. Overall, at the extreme upper level of temperatures tested, fewer proteolytic and nonproteolytic spores were required to initiate growth than vegetative cells. At temperatures below 20°C, fewer vegetative cells were required to initiate growth than spores.

EFFECT OF LOW TEMPERATURE ON GROWTH

The ability of nonproteolytic C. botulinum to grow at low temperatures has been mentioned above. Growth and toxin production by nonproteolytic types B and F took place at 3.3°C (Eklund et al, 1967), and for type E at 4° and 5.0°C (Ohye et al, 1957, Roberts et al, a, b, 1968, Solomon et al, 1977, Lindsay, 1983). In this study, after an incubation of 28 days, four nonproteolytic strains (one type B, one type F and two type E) initiated growth at 4°C with inoculum levels of $1X10^3$ to $1.7X10^5$ spores or 17 to $8.3X10^4$ vegetative cells/ml. Five nonproteolytic strains, one type B, one type F, and three type E did not show growth at 4°C, even at inoculum levels 3.8×10^5 to 1.7×10^7 spores or 3.8×10^3 to $1.7X10^6$ vegetative cells/ml. Eklund's demonstration of growth at 3.3°C was based on an incubation of greater than 109 days and inoculum levels of $5X10^5$ and $5X10^6$ /ml. It is possible that if an incubation time greater than 28 days was used in this study, more of the

nonproteolytic strains could have shown growth at 4°C.

The influence of low temperature on germination of C. botulinum has been investigated. Grecz and Arvay (1982) found that 9°C was optimum for germination of type E spores and 32.5°C for growth. At 2°C, at which no growth was observed, 50% of the spores germinated within 26 hours. A reduction in the per cent of spores germinating was seen above 14°C, with a maximum of 80% germinating at 9°C. Differences in the probability of growth were noted between cell and spore inocula used in the experiments being reported here. Table 5 illustrates the significantly higher numbers of nonproteolytic spores (1×10^3) needed to initiate growth at 4°C, as contrasted to the nonproteolytic vegetative cells (17) required at 4°C for growth initiation by the most capable strain. Although, according to Grecz, high rates of germination occur at 4°C, apparently other factors prevent spores from attaining growth probability rates comparable to those of vegetative cells.

Roberts et al, (1982), in constructing a model to describe the growth of C. botulinum types A and B as affected by temperature and salt concentration, noted that the data produced a non-linear curve with decreasing temperature. Temperatures tested were down to 15°C. In Figures 1 and 2 above, a non-linear, bell-shaped curve is also described, with decreasing per cent probability of growth to 4°C for nonproteolytic for 8°C for proteolytic. Thus, a general trend of non-linear growth initiation with

Table 5. Calculated minimum number of C. botulinum cells or spores needed to initiate growth in BHI broth at 4 to 47 C after 28 days incubation.[a]

Inoculum Type		Incubation Temperature								
		4 C	8 C	12 C	16 C	20 C	30 C	37 C	42 C	47 C
Nonproteolytic	spores	$1X10^3$	10	0.1	0.4	2.0	1.0[b]	0.5	10	$3.8X10^5$
	cells	17	0.2	1.0	0.04	0.5	1.0	0.5	10	$1.7X10^5$
Proteolytic	spores	no growth	no growth	$8.3X10^5$	1.0	1.0	1.0	0.2	0.2	22
	cells	no growth	no growth	$2.0X10^5$	0.1	0.2	1.0	0.2	0.2	$1X10^3$

[a] Based on formulas derived from data of the most capable strain with respect to temperature of incubation.

[b] Numbers reported for other than 30 C, are relative numbers with respect to 30 C.

decreasing temperature, as affected by the variables of strain, cell type, proteolytic activity, inoculum level, and incubation length can be shown.

EFFECT OF INOCULUM LEVEL AND LENGTH OF INCUBATION ON GROWTH

Length of incubation and inoculum level is also a factor in the probability of growth at low temperatures (Hauschild, 1982). Eklund et al (1967) noted that increasing lengths of time were required for C. botulinum nonproteolytic type B production of visible gas and toxin (used as growth indicators) when temperature was decreased. Seventeen days were required at 5.6°C 24 days at 4.4°C, and over 109 days at 3.3°C. Decreasing the inoculum level by one log, from 5×10^6 to 5×10^5 increased the length of incubation required for toxin and gas production. Thus, 21 days was needed for growth at 5.6°C, 33 days at 4.4°C, and over 109 days at 3.3°C. In a separate study Eklund et al (1967), reported similar data for nonproteolytic type F, with both lower temperature and lower inoculum levels requiring increasing length of incubation for appearance of growth indicators.

Data from experiments reported here confirm and expand these findings. Figures 3 and 4 present data on the per cent log probability of growth of nonproteolytic strains of E, B and F as affected by temperatures and time of incubation. Arithmetic conversion (100/probability of growth) gives the number of cells (level of inocula) needed

for initiation of growth under the test conditions. Thus, the present findings can be directly compared with those of Eklund. Figures 3 and 4 show that lengthened incubation time increases the per cent probability of growth, and that it requires lower inoculum levels for growth initiation. Also, lower temperatures require longer incubation to reach the same per cent probability of growth as at a higher temperature.

PREDICTION OF PROBABILITY OF GROWTH

A computerized multiple regression analysis was used to derive a set of equations in order to predict the per cent probability of growth initiation at various temperatures. Cell type (vegetative, spore) and proteolytic ability (proteolytic, nonproteolytic) were used as defining characteristics in dividing data into subsets. Table 6 presents the experimentally observed log per cent probabilities for growth with the ones calculated from the regression equations for the same temperature of incubation. It should be noted that calculated log per cent probability values at the temperatures where no experimental growth was observed, (eg. proteolytic spore and vegetative cell inocula below 12 ^{o}C) required theoretical inoculum levels of 4 X 1010 to 2 X 10^{18} cells or spores/ml (per cent log probabilities of -8.6 to -16.3) for growth initiation. Such levels are unattainable under natural conditions, and in essence, these conditions represent no growth.

Table 6. Examples of calculated and observed log per cent probabilities of growth of proteolytic and nonproteolytic C. botulinum vegetative cells and spores in BHI broth after 28 days incubation at various temperatures.

Inoculum Type	Probability	Incubation Temperature								
		4 °C	8 °C	12 °C	16 °C	20 °C	30 °C	37 °C	42 °C	47 °C
Nonproteolytic spore	observed[a]	-1.0	1.0	3.0	2.4	1.7	2.0[b]	3.4	2.7	-3.6
	calculated	-1.2	1.5	2.5	2.4	1.9	2.1 L[c] 1.9 H	4.0	2.1	-3.3
Nonproteolytic cell	observed	0.8	2.7	2.0	3.3	2.3	2.0	2.3	1.0	-3.2
	calculated	0.9	2.1	2.7	2.9	2.8	2.0 L 2.0 H	2.5	0.9	-3.1
Proteolytic spore	observed	no growth	no growth	-3.9	2.0	2.0	2.0	2.7	2.7	0.7
	calculated	-16.2	-9.0	-3.4	0.6	3.0	1.9 L 1.9 H	2.9	2.4	0.7
Proteolytic cells	observed	no growth	no growth	-3.3	3.0	2.7	2.0	2.7	3.0	-1.0
	calculated	-16.3	-8.6	-2.7	1.4	3.8	1.9 L 1.8 H	3.3	2.4	-0.8

[a] Log per cent probabilities for most capable strain to initiate growth at a particular temperature.

[b] Log per cent probabilities reported for temperatures other than 30 °C are relative to the probability of growth at 30 °C.

[c] Caluculated per cent probabilities are based on two regression equations, one for temperatures below 30 °C (L) and one for temperatures above 30 °C (H).

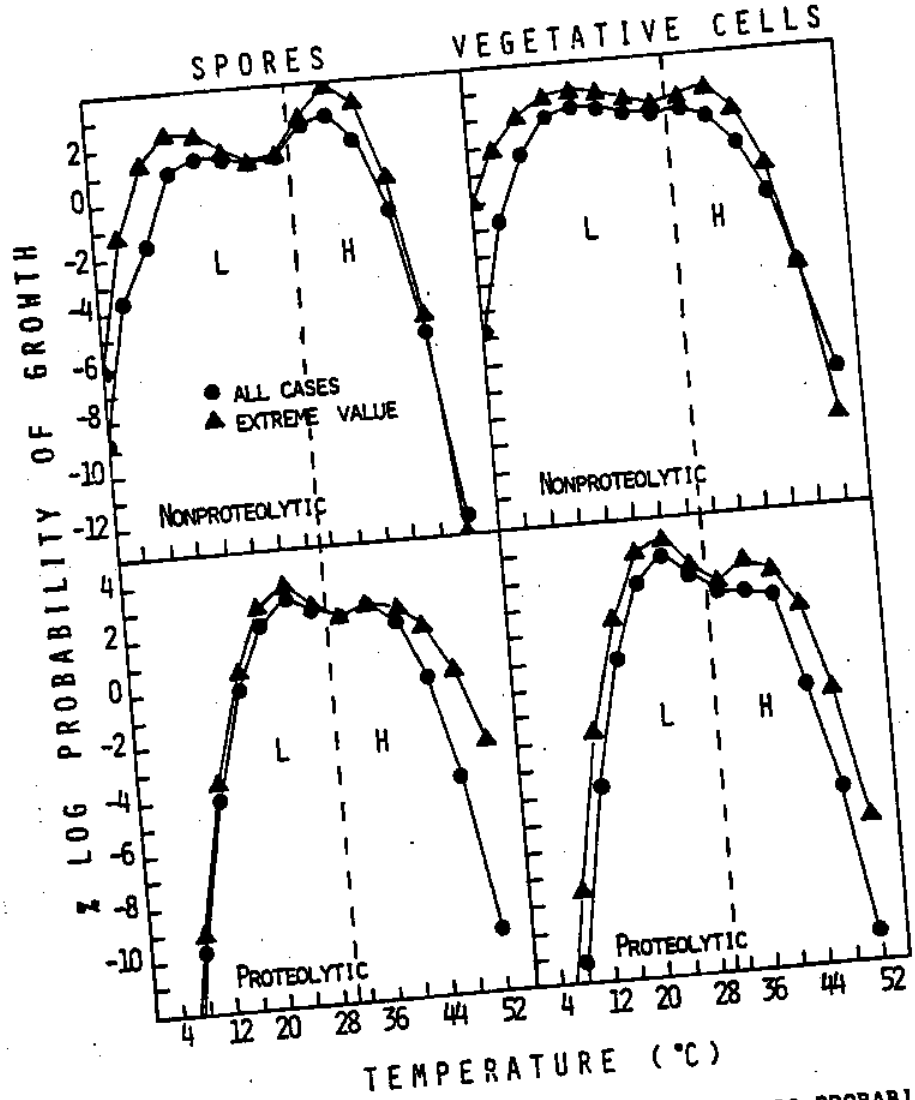

FIGURE 8. COMPARISON OF COMPUTER DERIVED LOG PROBABILITIES OF GROWTH INITIATION BY SPORES AND VEGETATIVE CELLS OF NONPROTEOLYTIC AND PROTEOLYTIC C. BOTULINUM INOCULATED INTO BHI BROTH AND INCUBATED FOR 28 DAYS AT 4 TO 47°C. CALCULATED PROBABILITIES ARE BASED ON TWO REGRESSION EQUATIONS, ONE FOR TEMPERATURES BELOW 30°C (L) AND ONE FOR TEMPERATURES ABOVE 30°C (H). THE REGRESSION EQUATIONS WERE DERIVED FROM DATA REFLECTING THE GROWTH OF ALL STRAINS (●) AND FROM DATA REFLECTING THE GROWTH OF THE MOST CAPABLE STRAINS AT EACH INDIVIDUAL TEMPERATURE (▲).

who in classical experiments used in developing minimum heat processing standards for the canning industry, also chose the most resistant strain for inclusion in their model.

In summary, this study has demonstrated the feasibility of utilizing multiple regression approaches to develop equations capable of predicting the probability of C. botulinum growth as it is affected by cell type (spore, vegetative cell), serological type (A, B, E and F), proteolytic activity (proteolytic, nonproteolytic), size of inoculum, length of incubation and temperature.

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