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The effect of sodium chloride and temperature on the rate and extent of growth of *Clostridium botulinum* type A in pasteurized pork slurry

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A selective medium was used to enumerate *Clostridium botulinum* growing in the presence of natural spoilage organisms in a model cured pork slurry. The growth responses of a mixed spore inoculum of six strains of *Cl. botulinum* type A were studied at 15°, 20° and 27°C with 1·5, 2·5, 3·5 or 4·5% (w/v) salt added (a_w range 0·961–0·990). Gompertz and logistic curves, which have a sigmoid shape, were fitted to the data and lag times, growth rates, generation times and time to maximum growth rates were derived. Variation in germination rates of the spores occasionally gave a falsely extended lag time resulting in an exceptionally high estimate for growth rate. Products containing 4·5% (w/v) NaCl would be capable of supporting growth of proteolytic strains of *Cl. botulinum*, even at 15°C, although the lag period would be extended. In products where absence of *Cl. botulinum* cannot be assured additional preservative measures are essential. The information obtained provides a framework to investigate the effects of a wider range of additives or variables on the growth responses of *Cl. botulinum*.

Spores of *Clostridium botulinum* are naturally present in soil and are an occasional, though unavoidable, contaminant of raw meats (Greenberg *et al.* 1966, 1967; Roberts & Smart 1977). They survive curing and pasteurization processes and have been demonstrated in low numbers in processed meats (Taclindo *et al.* 1967; Insalata *et al.* 1969; Abrahamsson & Riemann 1971; Roberts & Smart 1976, 1977; Hauschild & Hilsheimer 1980). Notwithstanding that spores may remain viable for long periods within a product the safety record of commercially prepared pasteurized cured meats is excellent (Tompkin 1980).

It has long been recognized that salt and nitrite contribute to the inhibition of outgrowth of *Cl. botulinum* spores in cured meats, but it is also well established that several other factors are important. These include the pH of the product, processing practices such as the severity of any heat treatment, the presence of other additives or preservatives (nitrate, polyphosphate, isoascorbate/ascorbate, sorbate) and the effect of the environment, e.g. storage temperature (Riemann 1963; Roberts *et al.* 1981a; Roberts & Gibson 1986a). In the late 1960s, improved methods of chemical analysis led to the detection, in a variety of foods, of nitrosamines, which are carcinogenic to laboratory animals. Concern over the possible presence of those substances in cured meats led to investigations into the minimum level of nitrite, added to cured meats, which would ensure that *Cl. botulinum* was inhibited with certainty. The wish to reduce levels of nitrite added to cured meats, or even prohibit its use, prompted extensive research, particularly in the USA and the UK. Reports on studies on *Cl. botulinum* in cured meats were often expressed as 'growth' or 'no growth' under a given treatment combination with a specified incubation period. The 'time to first swell' was also used as an indicator of

growth of *Cl. botulinum* in canned cured meats. It was generally not related to determining growth rates of *Cl. botulinum*, merely to the effects on growth of *Cl. botulinum* of varying the level of curing salts and initial spore load within the product. These results were thoroughly reviewed by Sofos *et al.* (1979), Tompkin (1983) and Roberts & Gibson (1986b).

One difficulty in comparing work from different laboratories is the absence of standardized experimental methods and designs. Hauschild (1982) attempted to analyse the published data by calculating from selected sets the probability of one spore germinating, thereby allowing comparison of results from different workers who had used different experimental systems.

In an extensive study of factors variously reported to be important in controlling growth of *Cl. botulinum* in pasteurized cured meats, the relative contributions of factors were identified for the first time (Roberts *et al.* 1981a, 1981b, 1981c, 1982; Robinson *et al.* 1982; Gibson *et al.* 1982, 1984). The probability of toxin production within the 6 months' storage period was calculated but there was no expression of the rate of toxin production.

Despite the extensive scientific literature on *Cl. botulinum* in relation to the safety of cured meats, little relevant work has been published on factors affecting its rate of growth. Most research has been on pure cultures in laboratory medium, often at near-optimal pH and therefore of questionable relevance to growth in mixed culture, at lower pH values in meat products. Bonventre & Kempe (1960) studied toxin production kinetics of strain 62A in a laboratory medium and measured growth turbidometrically. The effect of temperature on generation times of single strains of *Cl. botulinum* types A and B in laboratory medium, with growth estimated nephelometrically, has also been reported (Ohye & Scott 1953). That work was extended by Ohye & Christian (1967) and Ohye *et al.* (1967) to a range of temperatures and a_w for individual strains of *Cl. botulinum* types A, B and E. To the food microbiologist and producer the deficiency in these data is the failure to express the rate or extent of growth of representative strains of *Cl. botulinum* in conditions simulating those in foods.

Studies on growth rates, lag times and extent of growth of micro-organisms in foods are important in assessing safety and shelf-life. Understanding the various factors which combine to control microbial growth (pH, a_w, temperature, preservatives, etc.) through data bases for specific organisms will lead to mathematical models that predict their growth responses from knowledge of the physical and chemical properties of the product. A manufacturer could use those models to predict shelf-life, or whether or not a product would be safe, when a product formulation is modified, or if it is subjected to temperature abuse. These principles apply equally to any pathogenic or food spoilage microbe in any food system.

In our previous work (Roberts & Gibson 1986b) we identified the main factors affecting the growth of *Cl. botulinum* in pasteurized cured meats. We have now determined the effect of a_w and temperature on growth rates and lag times of *Cl. botulinum* in the model pork slurry system. A method of quantifying growth of *Cl. botulinum* in the presence of competing organisms naturally present in the meat was first devised. An agar medium—selective botulinum medium (SBM)—was developed to allow quantitative estimation of *Cl. botulinum* in the presence of the natural spoilage organisms (Gibson 1986). This selective medium was used to follow the growth responses of a mixed *Cl. botulinum* type A spore inoculum in pasteurized pork slurry, after two mild heat treatments, at three storage temperatures and taking into account four different salt levels.

Materials and Methods

STRAINS USED

Clostridium botulinum type A strains 3806, 7272 and 9837 (National Collection of Type Cultures, London); ZK3 and BL22 (supplied by J.S. Crowther, Unilever Research Laboratories, Bedford) and 62A (from the National Food Processors Association, Berkeley, CA, USA) were used.

Spore crops

Spore crops of individual strains were prepared from actively growing cultures in Reinforced Clostridial Medium (RCM; Oxoid CM 149) after two successive subcultures incubated at 30°C for 4 h. Modified neopeptone sporulation agar supplemented with amino acids (Smelt *et*

al. 1982) was inoculated with 0·25 ml of cell suspension and incubated anaerobically at 30°C. Anaerobic conditions were achieved using *ca* 30 g 'Deoxo' pellets (Engelhard Products, Cinderford, Glos.), flushing the anaerobe jar twice with hydrogen, and then filling it. Plates were examined daily for spores by phase-contrast microscopy. Spores were produced over a period of 3–10 d, depending on the strain. Harvested spores were washed three times with sterile distilled water and stored at 4°C. Individual spore crops were counted on Reinforced Clostridial agar (Oxoid) supplemented with cysteine hydrochloride (0·05% w/v) and sodium bicarbonate (0·1% w/v) (see Gibson 1986) and diluted to *ca* 1×10^7 spores/ml. Two ml of each spore crop were added to 78 ml of sterile distilled water to give a final concentration of *ca* $1{\cdot}2 \times 10^6$ mixed spores/ml which was stored at 4°C during the experiment and used throughout as the spore inoculum.

PREPARATION OF SLURRIES

Pork leg muscle was defatted by hand, minced and mixed in a ratio of 1 : 1·5 pork : water and curing salts. The slurries contained salt (1·5, 2·5, 3·5 and 4·5% w/v) and polyphosphate (Curaphos 700, Fibrisol Services Ltd, London, 0·3% w/v). The salts and spore inoculum were mixed and then added to the minced meat and further mixed in a 1 l 'Atomix' jar to ensure even distribution of spores and curing salts in the final slurry. Slurries were prepared in 1 l volumes and dispensed into 32–35 sterile 28 ml wide-mouthed screw-capped bottles and subjected to one of two heat treatments designed to raise the centre temperature to 70°C and maintain it for 1 or 2 h (80°C/5 min + 70°C/1 h or 80°C/5 min + 70°C/2 h) and stored at 15°, 20° or 27°C. Immediately after heat treatment one slurry bottle was stored deep-frozen for subsequent fat, a_w and pH determinations.

Fat, pH and a_w measurement

Total fat levels were determined according to the British Standard method (Anon. 1970) for analysis of meat products. Water activity determinations were made using a Novasina Moisture Sensor (EEJ-3 measuring station, 4-Tebo temperature control box, Novasina, Zurich, Switzerland). A Kent EIL 7045/46 pH meter was used to determine pH values.

COUNTING METHODS

Two bottles were sampled immediately after heat treatment and the initial number of viable spores determined. At intervals during storage, individual bottles were removed and sampled by weighing the contents (*ca* 25 g) into a stomacher bag. Diluent (NaCl, 0·85% w/v and peptone, 0·1% w/v) was added to a total weight of *ca* 100 g (i.e. *ca* 1/4 dilution) and 'stomached' for 20 s (Colworth Stomacher Lab Blender, Seward Laboratories, Blackfriars Road, London). Decimal dilutions of the supernatant fluid were prepared and 0·1 ml volumes of each were distributed into 50 mm (deep form) petri dishes (Sterilin). Approximately 10 ml of molten SBM, previously tempered to 50°C, were added and allowed to set with lids angled to reduce condensation. Dishes were incubated anaerobically at 35°C for 48 h and presumptive botulinal counts made (Gibson 1986). When low counts were expected (i.e. <1000 colonies/g slurry) 10 ml of the initial $\times 4$ dilution in the stomacher bag were added to 90 ml of molten SBM, thoroughly mixed, and the total 100 ml poured into 10 50 mm petri dishes and incubated as above. The total number of colonies on the 10 plates was used to calculate the count.

CONFIRMATION OF *Clostridium botulinum*

Representative colonies from each presumptive botulinum count were subcultured and tested for their ability to produce *Cl. botulinum* type A toxin. Initially five colonies were subcultured from the greatest dilution giving growth into RCM and incubated at 35°C for 42 h. The cultures were then centrifuged and the five supernatant fluids were tested separately for *Cl. botulinum* type A toxin by mouse bioassay. Toxin type was confirmed using monovalent antitoxin. Over a period of several months all the colonies subcultured (>1100) were confirmed as *Cl. botulinum* type A (Gibson 1986). Subsequently the same procedure was followed but, to reduce animal testing, only one of the five supernatant fluids was injected. If it contained type A toxin the organisms were presumed to be *Cl. botulinum*. If it did not, the other four supernatant fluids were injected. In that way every colony count in the growth curves reported below was confirmed to represent *Cl. botulinum* type A.

EXPERIMENTAL PLAN

Factors studied in combination were: NaCl (% w/v), 1·5, 2·5, 3·5 and 4·5; heat treatment (centre temperature raised to 70°C, then maintained for 1 or 2 h), 80°C/5 min + 70°C/1 h and 80°C/5 min + 70°C/2 h; and storage temperature, 15°, 20° and 27°C.

Presumptive botulinal counts were made at appropriate intervals and growth curves plotted as $\log_{10}$ cfu/g slurry against time in days.

CURVE FITTING

Initially $\log_{10}$ counts of the number of *Cl. botulinum* were modelled as a function of time by both logistic (Einarssan & Eriksson 1986; Jason 1983) and Gompertz (Jeffries & Brain 1984) curves. These curves were chosen as both consist of four phases which may be compared with the four stages of microbial growth curves: an initial lag phase where no change occurs, followed by a period of accelerating change, a period of decelerating change and finally a stationary period.

The logistic curve is given by

$$L(t) = A + C/\{1 + \exp(-B(t - M))\}$$

and the Gompertz curve by

$$L(t) = A + C \exp\{-\exp(-B(t - M))\} .$$

For each curve $L(t)$ is the log count of the number of bacteria at time t (in days), A is the asymptotic log count as t decreases indefinitely, C is the asymptotic amount of growth that occurs as t increases indefinitely, and B is the relative growth rate at M, where M is the time at which the absolute growth rate is a maximum. The difference between the two models is that the logistic curve is symmetric about M, whereas the Gompertz is not. The following were derived from each curve: growth rate ($\log_{10}$ count/d), generation time (h) and lag time (d).

For the logistic curve:

$$\text{growth rate} = \frac{BC}{4}$$

$$\text{lag} = M - \frac{2}{B}$$

$$\text{generation time} = \frac{96 \times \log_{10}(2)}{B \times C} .$$

Table 1. The influence of NaCl on the water activity of pork slurries

Concentration of NaCl added (% w/v)	n	Mean	Range	Variance
1·5	5	0·9880	0·986–0·990	0·0016
2·5	4	0·9828	0·981–0·984	0·0015
3·5	4	0·9760	0·973–0·978	0·0022
4·5	4	0·9665	0·961–0·971	0·0048

For the Gompertz curve:

$$\text{growth rate} = \frac{BC}{e}$$

$$\text{lag} = M - \frac{1}{B}$$

$$\text{generation time} = \frac{24 \times \log_{10}(2)e}{B \times C} .$$

All curves were fitted and parameters derived using the Maximum Likelihood Program (MLP) package (Ross *et al.* 1980).

Results

The mean total fat level of 17 slurry samples was 1·28% (range 0·89–1·51%, variance 0·19) and the mean pH level was 5·98 (range 5·83–6·09, variance 0·15). The water activity levels of 17 slurries were measured and mean values for each salt level are listed in Table 1.

DISTRIBUTION OF SPORES IN THE SLURRY

To test the efficiency of distribution of spores in slurries, six bottles were sampled at various times during distribution of one slurry. The calculated mean number of spores/g of slurry was 18, with a range of 13–24 (variance 3·6), showing that the spores were well distributed.

The target inoculum level was 10^3 spores/g slurry. Numbers detected throughout these experiments ranged from $10^{0·82}$ to $10^{2·42}$/g (mean $10^{1·8}$ with a variance of $10^{0·31}$), an apparent 10-fold loss in numbers detected.

GROWTH CURVES

A total of 43 growth curves was generated from 24 combinations of salt, heat treatment and storage temperature. The labour-intensive

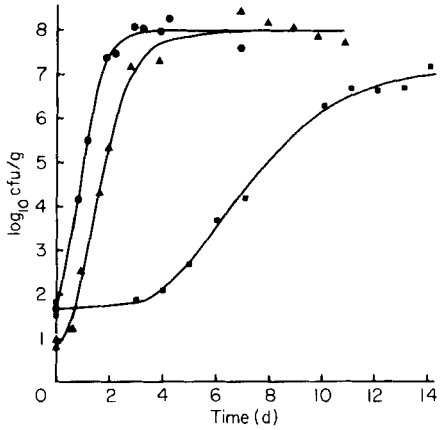

Fig. 1. Effect of temperature on growth of *Clostridium botulinum* type A in pork slurry containing 1·5% salt following heating for 2 h at 70°C. ●, Curve 77, 27°C storage; ▲, curve 96, 20°C storage; ■, curve 78, 15°C storage.

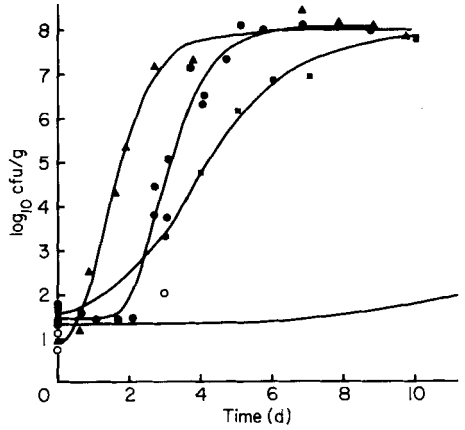

Fig. 2. Effect of salt concentration on growth of *Clostridium botulinum* type A in pork slurry stored at 20°C following heating for 2 h at 70°C. ▲, Curve 96, 1·5% salt; ●, curve 74, 2·5% salt; ■, curve 79, 3·5% salt; ○, curve 88, 4·5% salt.

counting methods, which included confirmatory tests of the presumptive *Cl. botulinum* colonies, precluded replication of all growth curves in the experimental plan. However, growth curves were usually repeated when insufficient data points prevented fitting a curve to the data, or when the fit appeared unreasonable.

As examples of the types of growth curves obtained, Fig. 1 illustrates the effect of decreasing storage temperature (with 1·5% salt added) on rate and extent of *Cl. botulinum* growth type A and Fig. 2 the effect of increasing salt concentration on growth during storage at 20°C.

REPRODUCIBILITY OF GROWTH CURVES

As a test of reproducibility of the system, data from replicate growth curves were plotted on common axes. There was good agreement between replicated data sets. For example, Fig. 3 shows four replicate growth curves (33, 77, 93 and 94 in Tables 2 and 3) prepared several months apart. Curve 33 was one of the trial data sets and contained only six data points, and hence was repeated with more frequent sampling and at different times as curves 77, 93 and 94. Differences in lag time between replicate curves were generally *ca* two-fold.

CALCULATED PARAMETERS

Tables 2 and 3 list values for time to maximum growth rate (M), maximum growth rate, lag time and generation time calculated from the parameters B, M, C and A obtained by fitting the Gompertz and logistic curves to the data.

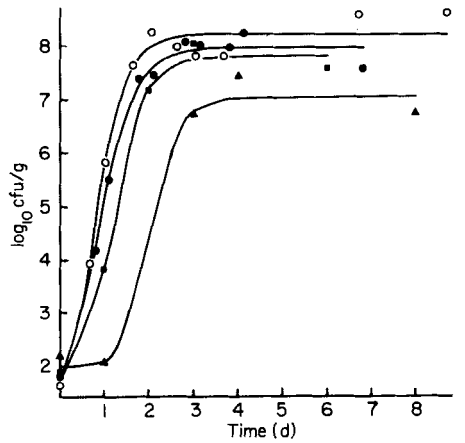

Fig. 3. Replicate growth curves of *Clostridium botulinum* type A in pasteurized pork slurry containing 1·5% salt and stored at 27°C following heating for 2 h at 70°C. ○, Curve 94; ●, curve 77; ■, curve 93; ▲, curve 33.

Table 2. Effect of salt and incubation temperature on rate of growth of *Clostridium botulinum* type A in pork slurry: Gompertz analysis

Temperature (°C)	NaCl (% w/v)	Heat	Code	Calculated values			
				M†	Growth rate†	Lag†	Generation time†
27	1·5	1	34	1·33	*	*	*
		1	95	0·62	5·16	0·12	1·4
		2	33	1·65	*	*	*
		2	77	0·78	4·81	0·29	1·50
		2	93	1·03	5·18	0·60	1·39
		2	94	0·70	5·81	0·29	1·24
	2·5	1	27	4·5**	*	*	*
		1	36	4·3	1·14	2·37	6·34
		2	28	3·64	3·93	3·15	1·84
		2	35	4·0	2·21	3·08	3·26
	3·5	1	38	4·56	2·31	3·70	3·13
		1	97	2·58	3·04	1·90	2·38
		2	37	2·63	2·20	1·76	3·28
	4·5	1	40	*	*	*	*
		1	98	4·61	1·41	3·34	5·10
		2	39	4·32	1·60	3·08	4·63
20	1·5	1	42	3·23	2·30	2·34	3·14
		2	41	*	*	*	*
		2	73	2·46	6·02	2·09	1·20
		2	96	1·27	3·46	0·52	2·09
	2·5	1	44	4·28	1·22	2·60	5·93
		2	43	2·35	1·98	1·34	3·64
		2	74	2·77	3·07	1·98	2·35
	3·5	1	46	7·54	1·55	6·17	4·67
		2	45	5·59	1·09	3·72	6·60
		2	79	3·4	1·43	1·77	5·06
	4·5	1	48	8·51	0·51	6·30	14·12
		1	90	9·28	0·43	3·83	16·79
		2	47	9·74	0·45	6·79	16·06
		2	88	8·28	0·39	2·86	18·49
15	1·5	1	50	9·80	0·75	7·27	9·59
		1	91	6·93	0·81	4·34	8·97
		2	49	*	*	*	*
		2	78	6·25	0·86	3·88	8·42
	2·5	1	52	11·82**	0·58	8·67	12·49
		1	92	11·93	0·58	8·48	12·43
		2	51	12·24**	*	*	*
		2	87	13·03	0·49	8·16	14·76
	3·5	1	54	27·54	0·17	17·67	41·68
		1	89	28·58	0·18	19·48	41·24
		2	53	*	*	*	*
		2	86	35·46	0·20	28·46	36·11
	4·5	1	56	*	*	>60·00	*
		1	85	*	*	<211·00	*
		2	55	*	*	>60·00	*
		2	82	*	*	<211·00	*

* No fit.

** No standard errors given.

† Values for time (d) to maximum growth rate (*M*), maximum growth rate ($\log_{10}$ counts/d), lag time (d) and generation time (h) calculated from Gompertz formula. Standard errors were calculated, but are not tabulated for simplicity. Data are available on request.

<, Growth detected on day 211, lag period *not* accurately determined; >, insufficient samples to make counts after day 60.

Heat 1, Centre temperature of slurry bottle raised to 70°C and maintained for 1 h; heat 2, centre temperature of slurry bottle raised to 70°C and maintained for 2 h.

Table 3. Effect of salt and incubation temperature on rate of growth of *Clostridium botulinum* type A in pork slurry: logistic analysis

Temperature (°C)	NaCl (% w/v)	Heat	Code	Calculated values			
				M†	Growth rate†	Lag†	Generation time†
27	1·5	1	34	1·32	*	*	*
		1	95	0·67	4·7	0	1·54
		2	33	1·32	*	*	*
		2	77	0·86	4·24	0·04	1·71
		2	93	1·21	4·34	0·48	1·66
		2	94	0·81	5·25	0·45	1·37
	2·5	1	27	3·66	*	*	*
		1	36	5·09	1·05	2·09	6·90
		2	28	*	*	*	*
		2	35	4·28	2·59	3·21	2·79
	3·5	1	38	5·16	2·05	3·84	3·53
		1	97	2·86	2·96	1·90	2·44
		2	37	3·11	2·23	1·93	3·24
	4·5	1	40	7·10	3·05	6·39	2·37
		1	98	5·41	1·43	3·71	5·06
		2	39	4·94	1·36	2·98	5·31
20	1·5	1	42	3·59	2·23	2·34	3·24
		2	41	3·88	5·85	1·95	1·24
		2	73	2·60	6·07	2·11	1·19
		2	96	1·51	3·41	0·43	2·12
	2·5	1	44	4·09	*	*	*
		2	43	3·09	2·32	1·91	3·11
		2	74	3·06	2·95	1·96	2·45
	3·5	1	46	7·93	1·24	5·62	5·84
		2	45	6·20	*	*	*
		2	79	3·90	1·28	1·31	5·66
	4·5	1	48	9·3	0·42	5·65	17·12
		1	90	11·18	0·54	6·05	13·35
		2	47	13·52	0·37	5·84	19·77
		2	88	13·64	0·49	5·34	14·78
15	1·5	1	50	11·12	0·78	7·89	9·28
		1	91	7·82	0·80	4·29	9·03
		2	49	12·46	7·85	12·12	0·92
		2	78	6·98	0·89	3·94	8·14
	2·5	1	52	12·78	0·51	8·06	14·17
		1	92	13·15	0·60	8·62	12·14
		2	51	12·57	1·50	11·14	4·80
		2	87	14·52	0·48	7·87	15·02
	3·5	1	54	32·60	0·18	18·57	40·42
		1	89	31·64	0·18	19·46	40·92
		2	53	107·41	*	*	*
		2	86	35·46	0·20	28·16	36·84
	4·5	1	56	*	*	>60·00	*
		1	85	*	*	<211·00	*
		2	55	*	*	>60·00	*
		2	82	*	*	<211·00	*

* No fit.

** No standard errors given.

† Values for time (d) to maximum growth rate (*M*), growth rate ($\log_{10}$ counts/d), lag time (d) and generation time (h) calculated from logistic formula. Standard errors were calculated but are not tabulated for simplicity. Data are available on request.

<, Growth detected on day 211, lag period *not* accurately determined; >, insufficient samples to make counts after day 60.

Heat 1, Centre temperature of slurry bottle raised to 70°C and maintained for 1 h; heat 2, centre temperature of slurry bottle raised to 70°C and maintained for 2 h.

Discussion

DISTRIBUTION OF SPORES IN SLURRY

Spores were added to the slurry during mixing to ensure even distribution throughout the slurry, attempting to simulate the natural dispersion of relatively low numbers of spores in the product. Inoculation of spores directly into the bottle after distribution of slurry could have resulted in a concentrated 'pocket' of spores which might not have responded as individual spores present in foods.

The inoculum was chosen as a compromise between the very low level of spores naturally present in meat products (approx. 1/kg or less) and the minimum that could be counted in the initial samples of slurry. Inoculated spores were well distributed throughout the slurry. Since the heat treatment was relatively mild, loss of spore viability was not expected, but during evaluation of SBM the presence of antimicrobials reduced the apparent numbers of spores recovered by *ca* 0·5 $\log_{10}$ (Gibson 1986).

Having ensured that the slurry was evenly mixed, each bottle became a separate sample unit once it was distributed. When a count was made, that sample was necessarily destroyed. Individual spores separated from an apparently homogeneous spore crop germinated at widely varying rates (Roberts & Thomas 1982), consequently relatively low numbers of spores in different bottles will not necessarily germinate at the same time, or at the same rate.

REPRODUCIBILITY OF GROWTH CURVES

Considering the cumbersome techniques and errors involved in the counting method, the similarity of the replicate curves gives considerable confidence in both the experimental system and the counting method (see Fig. 3) especially since the results were obtained over more than 6 months. There was also close similarity between corresponding curves for the two heat treatments.

Differences between the earlier and the later data sets (see Fig. 3, and compare curve 33 with 77, 93 and 94) were due in part to the fewer observations in the earlier trial data sets, and probably greater expertise in the methods with time.

CALCULATED GROWTH RATES

Generally at any given storage temperature maximum growth rate decreased and lag time, generation time and time to maximum growth rate (M) increased as salt concentration increased. Similarly at any given salt level growth rate decreased and lag time, generation time and M increased as storage temperature decreased. There was generally good agreement between replicate growth curves, although there were a few notable exceptions. These differences were often a result of too few data points in one of the replicate curves, or apparently extended lag periods, which together with a lack of data points at the lower asymptote resulted in the calculation of an exceptionally fast growth rate (see Table 2, curves 73 and 96). Apparently extended lag periods may have been the result of variability in germination rates of spores in the separate bottles.

Failure to fit a curve occasionally occurred. A very short lag time and rapid growth rate with no observations during those phases resulted in a step function being fitted with B infinite and M indefinite. Failure to fit a curve also occurred when lag time was long and growth rate slow, with no observations during the stationary phase, in which case either excessive estimates for M and C were obtained, or there was incomplete convergence of the curve-fitting method in the MLP package. In such cases the information gained from those observations was used to obtain a further, more complete, set of data to which a curve could be fitted. When growth was rapid (high temperature, low salt concentration) there were often fewer observations during the lag and acceleration phases than in the deceleration and stationary phases. Consequently, a single observation in the earlier phase of growth had a greater influence on the shape of curve fitted than a single observation during the later growth phases. This was a greater problem with the Gompertz fit, which is asymmetrical about M, than the logistic fit, which is symmetrical about M and whose overall shape can be determined more easily from only one half of the curve. Such problems illustrate the importance of an adequate number of data points, evenly spaced throughout the whole period of growth, for this type of curve fitting. Those problems will remain unsolved whilst manual counting methods have to be used, because of the

difficulties in obtaining counts during the night, and anticipating when a count is warranted.

Many of the growth curves were obviously asymmetric. Hence attempting to fit the logistic, which is symmetrical about M, seemed inappropriate. Since the logistic curve has been fitted previously to microbial growth curves (Jason 1983; Einarsson & Eriksson 1986) both logistic and Gompertz curves were fitted, and the parameters derived are listed in Tables 2 (Gompertz) and 3 (logistic). All curve parameters discussed herein relate to the Gompertz fits.

Mean growth rates from 10 strains of *Cl. botulinum* type A, grown in laboratory medium and expressed as generations per hour (Ohye & Scott 1953), were converted to generation times and compared with those calculated from the Gompertz curve for mixed strains of *Cl. botulinum* type A grown in pasteurized pork slurries containing 1·5% added salt (the lowest level tested). At 30°C the calculated generation time in laboratory medium was 1·09 h and at 25°C, 1·87 h (Ohye & Scott 1953), while in pork slurry at 27°C the calculated generation time was 1·4 h following the 1 h heat treatment and 1·24–1·5 h following the 2 h heat treatment. At 20°C the calculated generation times were 3·85 h in laboratory medium (Ohye & Scott 1953) and 3·14 h in our pork slurry following the 1 h heat treatment and 1·2–2·13 h following the 2 h heat treatment. Considering the inherent differences in the two experimental systems, on the one hand, pure cultures grown in laboratory medium with growth measured nephelometrically and, on the other, mixed spores of six strains inoculated into pork slurry naturally contaminated with spoilage organisms and growth estimated by selective isolation on solid medium, these generation times are remarkably similar. At 15°C, however, calculated generation times were 27·86 h from Ohye & Scott's (1953) data but 8·42–9·59 h in the pork slurry system. Hence, at temperatures approaching those limiting growth, *Cl. botulinum* appears to grow better in meat product than their laboratory medium (comprising neopeptone, yeast extract and glucose at pH 7·0 with no salt added). Rapid growth of proteolytic *Cl. botulinum* type A in inoculated, autoclaved chicken has also been reported: toxin production occurred within 1 d at 35°C and numbers increased from the 1×10^3 spores inoculated to millions/g within the same period (Hobbs & Spooner 1966).

CALCULATED TIMES TO MAXIMUM GROWTH RATE (M)

The specific growth rate of an organism is not constant over the growth period but increases to a maximum and then decreases (Jason 1983). This is borne out by the form of our results which show a continual change in the rate of growth. Consequently all growth rates quoted here refer to the maximum growth rate achieved. The M value (time to reach maximum growth rate) is an appropriate parameter for modelling because it takes into account both lag time and growth rate. Time to reach a particular increase in numbers (e.g. 1×10^7) (Daud *et al.* 1978) or increase in optical density (Ratkowsky *et al.* 1982, 1983) has also been used as a means of comparing growth of organisms at different temperatures. Additionally the time at which maximum growth rate occurs has been directly related to the log of the initial numbers of cells inoculated (Jason 1983). In these experiments inoculum level remained constant throughout, thus M can be used to estimate the effect of varying the level of salt added, or the storage temperature.

A three-dimensional representation of the combined effect of storage temperature and salt concentration on time to maximum growth rate is shown in Fig. 4 where the higher the block the longer the time, in days, to reach the maximum growth rate. Estimates for M at 15°C storage with 4·5% salt added could not be determined as no growth had occurred at day 148, but growth had occurred by day 211. Consequently the M value in Fig. 4 for 4·5% salt at 15°C is represented as a line of indeterminate height.

Such a representation gives a good indication of the general relationship which ultimately could be used to describe the growth responses of *Cl. botulinum* under the conditions tested. A surface has been drawn over the diagram by linking the corners of each block, which slopes gradually from the front along the two horizontal axes, but slopes steeply towards the back along the vertical axis. A wider range of storage temperatures and salt levels, with more replication at each combination, would be required to describe the surface more precisely.

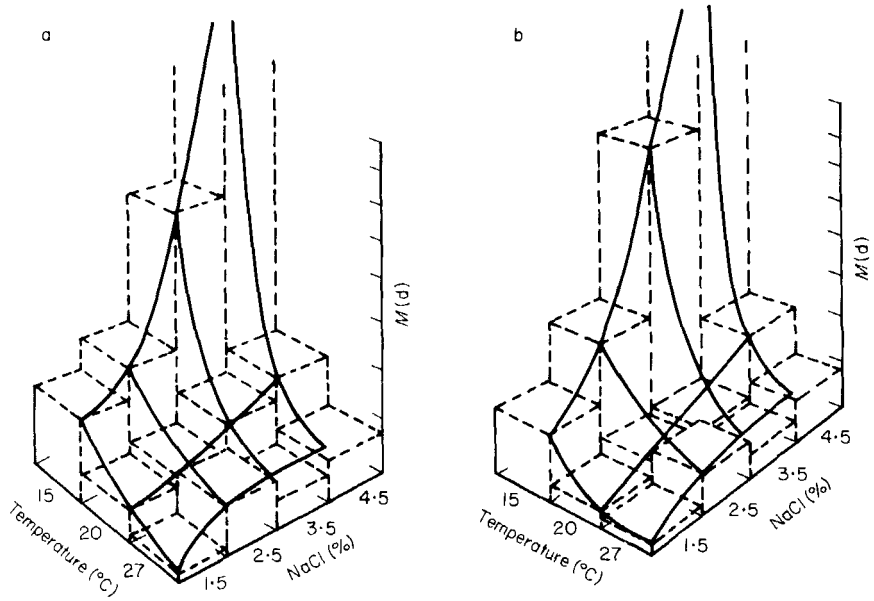

Fig. 4. Effect of storage temperature and salt concentration on time to reach maximum growth rate (M) of *Clostridium botulinum* type A, in pork slurry following two heat treatments. The taller the block the longer the time to reach maximum growth rate. A mean value is indicated when several values for M were obtained. a, Heated for 1 h; b, heated for 2 h.

There are many cured meat products on the market at a_w levels within the range studied here which have enjoyed an excellent safety record. A proportion of those products are stored below *ca* 10°C, below which temperature *Cl. botulinum* type A does not grow. The fact that salt and temperature alone are not adequate to prevent outgrowth and toxin production of *Cl. botulinum* indicates that other factors are also important in maintaining the bacteriological safety of those products. From previous work (see Tompkin 1983; Roberts & Gibson 1986a, 1986b) nitrite and ascorbate/isoascorbate are both very important and nitrate and polyphosphate may play a role in particular circumstances.

Studies such as this provide a greater understanding of the growth responses of *Cl. botulinum* at varying temperatures and a_w, but also indicate the potential dangers should the factors which prevent growth be modified without due regard to their combined effects.

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