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The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes*

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Factorially designed experiments have been used to study the growth and survival of *Listeria monocytogenes* in different combinations of pH and salt concentrations at ambient and chill temperatures. Survival at low pH and high salt concentration was strongly temperature dependent. The minimum pH values that allowed survival after 4 weeks from an initial 10^4 cells were 4·66 at 30°C, 4·36 at 10°C and 4·19 at 5°C. These limits were salt dependent, low (4–6%) salt concentrations improved and higher concentrations reduced survival at limiting pH values. The lowest pH that allowed a 100-fold increase in cell numbers within 60 d was 4·66 at 30°C but this was increased to 4·83 at 10°C. At 5°C growth occurred at pH 7·0 but not at pH 5·13. Simple predictive models describing the effect of hydrogen-ion and salt concentration on the time for at least a 100-fold increase in numbers at 10°C and 30°C were constructed after analysis of the results for a least squares fit to a quadratic model. The interactions between salt and hydrogen-ion concentration on growth were found to be purely additive.

There is now a great deal of information about the epidemiology, thermotolerance and ability to grow at low temperatures of *Listeria monocytogenes*. This Gram-positive, non-sporeforming aerobic bacterium is pathogenic to animals in which it can cause abortion (Smith *et al.* 1955) and mastitis (Gitter *et al.* 1980); and to man, where, in susceptible individuals, infection is often fatal. The organism can cause abortion in pregnant women as well as meningitis in new-born infants and immunocompromised adults (Seeliger 1961; Ralovich 1984).

Thermotolerance is perhaps one of the most studied aspects of this organism largely because dairy products have been implicated as a vehicle of transmission (Fleming *et al.* 1985; James *et al.* 1985; Ho *et al.* 1986) and *Listeria* has been isolated from pasteurized milk (Fleming *et al.* 1985; Fernandez-Garayzabal *et al.* 1986). The lower temperature limit for growth of *L. monocytogenes* is 0°C–0·3°C (Seeliger & Jones 1986; Walker & Stringer 1987), which makes this organism particularly relevant to chilled foods. Its ability to grow at low pH values is obviously greatly influenced by the nature of the acidulant. In media acidified by HCl the minimum pH permitting growth was 4·39 (George *et al.* 1988) whilst in unclarified cabbage juice acidified with lactic acid no growth occurred at pH 4·8 (Conner *et al.* 1986). The effectiveness of the weak acid preservatives against *L. monocytogenes* is also predictably influenced by pH. For example, at pH 5·0 and 13°C the bacteria could not grow at concentrations of 0·05% of sodium benzoate but did grow in the presence of 0·1% sodium benzoate when the pH was increased to 5·6 (El-Shenawy & Marth 1988a). The influence of pH on the effectiveness of sorbic acid has also been demonstrated by the same authors (El-Shenawy & Marth 1988b). Despite this, relatively little quantitative data on

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the effect of preservative factors on this organism are available, particularly where two more factors may be acting in combination. In the present work the effect of temperature, salt concentration and pH on both the survival and growth of *L. monocytogenes* is established by factorially designed experiments. The results have also been used to construct a simple predictive model relating the above factors to the time to visible growth.

Materials and Methods

BACTERIAL STRAIN AND MEDIA

Listeria monocytogenes ATCC 19115 (human strain) was obtained from Dr H. Seeliger*. It was maintained on Heart Infusion Agar (Difco) and grown in tryptic phosphate broth (TPB; Conner *et al.* 1986) buffered with a combination of citric acid (0·1 mol/l) and di-potassium hydrogen orthophosphate (0·2 mol/l) to achieve pH values between 7·0 and 4·19 (McIlvaine 1921). Solutions of different salt concentrations in citrate/phosphate buffer were autoclaved separately (121°C for 10 min) from the medium then combined aseptically to give final concentrations of 0·5–18·5% w/v.

SURVIVAL EXPERIMENTS

A microtitre-plate system was used to assess the effect of salt concentration and hydrogen-ion concentration on the survival of *L. monocytogenes*. In a factorially designed experiment 10 concentrations of salt (0–18%, equal increments) were combined with eight hydrogen-ion concentrations in equal increments from 14·8 μmol/l (pH 4·83) to 64·6 μmol/l (pH 4·19). Duplicate microtitre plates were inoculated with 10^4 cells/well taken from a stationary phase culture (24 h), sealed with non-toxic Titretek sealers (Flow Laboratories, Rickmansworth, Herts) and incubated at 30°C, 10°C and 5°C. After incubation for 1, 2, 3 and 4 weeks 50 μl was removed from each well and transferred to a corresponding well in recovery plates that contained TPB (250 μl) at pH 7 and no additional salt. Recovery plates were incubated at the same temperatures as the original plates. Wells were assessed visually for growth daily and time to visible growth within 60 d recorded; samples from selected wells that showed growth were plated on Tryptone Soya Agar (TSA, Oxoid) to check for purity. Plate counts from selected wells indicated that 'visual growth' represented at least a 100-fold increase in cell numbers.

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GROWTH AND STATISTICAL ANALYSIS

The effect of salt and hydrogen-ion concentration on growth was also assessed by a microtitre-plate system. In a factorially designed experiment, seven concentrations of salt (0–18%, equal increments) were combined with seven hydrogen-ion concentrations in equal increments from 36·3 μmol/l (pH 4·44) to 0·1 μmol/l (pH 7·0) in broth. Microtitre plates were inoculated with 300 cells/well taken from a stationary phase culture (24 h), and sealed with non-toxic Titretek sealers. Plates were incubated at 30°C, 10°C and 5°C, and the experiment was repeated at least three times. Wells were assessed visually for growth every day for up to 60 d; samples from selected wells showing growth were plated on TSA and tested for purity. Results for the natural log time to visible growth at 30°C and 10°C were analysed for a least squares fit to a quadratic model using SAS statistical package (SAS Software Ltd) on a VAX mini-computer. The concentration of salt added to the medium and the measured pH values (converted to hydrogen-ion concentration) were used in the construction of the model. Insufficient information was obtained at 5°C for the construction of a three-dimensional model because growth occurred only at pH 7·0 and not at the next pH value studied (pH 5·13) within 60 d. Hence a simple quadratic model linking salt concentration to time to visible growth at 5°C and pH 7·0 was constructed using a least squares regression.

MEASUREMENT OF pH THROUGHOUT GROWTH

The pH of culture broth in control plates (uninoculated) was determined with an EA 940 pH meter (Orion Research Incorporated, Cambridge) and a micro electrode (3 mm diam). The pH remained constant to within 0·02 of a pH unit throughout the duration of the experiment.

Results

SURVIVAL

The conditions allowing survival of *L. monocytogenes* after 1, 2, 3 and 4 weeks at 30, 10 and 5°C are shown in Fig. 1a–c. Low pH values and high salt concentrations inactivated *L. monocytogenes* but in a temperature dependent manner. At 30°C after 4 weeks viable cells could be recovered only at pH values of 4·66 and above and at salt concentrations of 4% and below. After the same period at 10°C viable cells could be recovered at pH values of 4·36 and above and at salt concentrations of 10% and below. After the same period at 5°C viable cells could still be recovered at pH values as low as 4·19 and salt concentrations as high as 18%. The presence of low concentrations of salt in some instances allowed survival at lower pH values than in the absence of additional salt (Fig. 1a, 3 weeks; Fig 1b, 4 weeks). Near the limiting values for survival (low pH/high salt) the results obtained became increasingly erratic making the predictability of survival times less reliable.

GROWTH

The effect of pH and salt concentration on the time to visible growth within 60 days is presented in Table 1. The lowest pH values permitting growth within 60 d at 30°C, 10°C and 5°C were pH 4·66, pH 4·83 and pH 7·0 respectively. Salt increased the minimum pH values supporting growth at all temperatures.These results were also used to construct predictive models (Fig. 2). The statistical analysis methods applied are shown in Table 2.

Analysis 30°C

The following polynomial equation links the concentration of salt [S] (%) and hydrogen ions [H] (μmol/l) to the $\log_e$ time to visible growth (LTG) (days) of *L. monocytogenes* at 10°C and is shown in Fig. 2a.

$$\begin{aligned}LTG = {} & 5{\cdot}35 \times 10^{-2} + 1{\cdot}38 \times 10^{-2}[S] \\ & + 7{\cdot}45 \times 10^{-2}[H] + 2{\cdot}53 \times 10^{-2}[S][S] \\ & + 1{\cdot}85 \times 10^{-4}[H][H]^* \\ & - 1{\cdot}47 \times 10^{-3}[H][S]^* \qquad (1)\end{aligned}$$

* Terms insignificant at 95% level.

Analysis 10°C

The following polynomial equation links the concentration of salt [S] (%) and hydrogen ions [H] (μmol/l) to the $\log_e$ time to visible growth (LTG) (days) of *L. monocytogenes* at 10°C and is shown in Fig. 3b.

$$\begin{aligned}LTG = {} & 1{\cdot}44 - 1{\cdot}29 \times 10^{-1}[S] \\ & + 1{\cdot}45910^{-1}[H] + 3{\cdot}45 \times 10^{-2}[S][S] \\ & + 1{\cdot}70 \times 10^{-3}[H][H]^* \\ & - 3{\cdot}09 \times 10^{-3}[H][S]^* \qquad (2)\end{aligned}$$

* Terms insignificant at 95% level.

Analysis 5°C

The following simple quadratic model links the concentration of salt [S] (%) to the $\log_e$ time to visible growth (LTG) (days) of *L. monocytogenes* at 5°C and at pH 7·0 (correlation coefficient $R^2 = 0{\cdot}97$).

$$\begin{aligned}LTG = {} & 2{\cdot}10 + 7{\cdot}56 \times 10^{-2}[S] \\ & + 1{\cdot}66 \times 10^{-2}[S][S] \qquad (3)\end{aligned}$$

The resulting predicted values from the polynomial equations 1 and 2 are also shown in Fig. 3 and compared with raw data values.

Discussion

The survival of *L. monocytogenes* under conditions of osmotic and low pH stress is greatly influenced by temperature (Fig. 1). Low temperatures (5°C, 10°C) at which metabolism and growth are reduced allow *L. monocytogenes* to survive low pH values and high salt concentrations for longer than at 30°C. Unexpectedly, low concentrations of salt provide a slight protective effect against inactivation of *L. monocytogenes* at low pH values. This is evident from the pattern of surival after 3 weeks at 30°C (Fig. 1a) and after 4 weeks at 10°C where viable cells could be recovered from lower pH values in the presence of 4–6% salt. The protective effect of low salt concentrations at low pH values has also been observed in the recovery of pH-injured cells. The time taken for cells to reach visible growth was more rapid after injury in the presence of low concentrations of salt (4–8%) than in the absence of salt (results not shown).

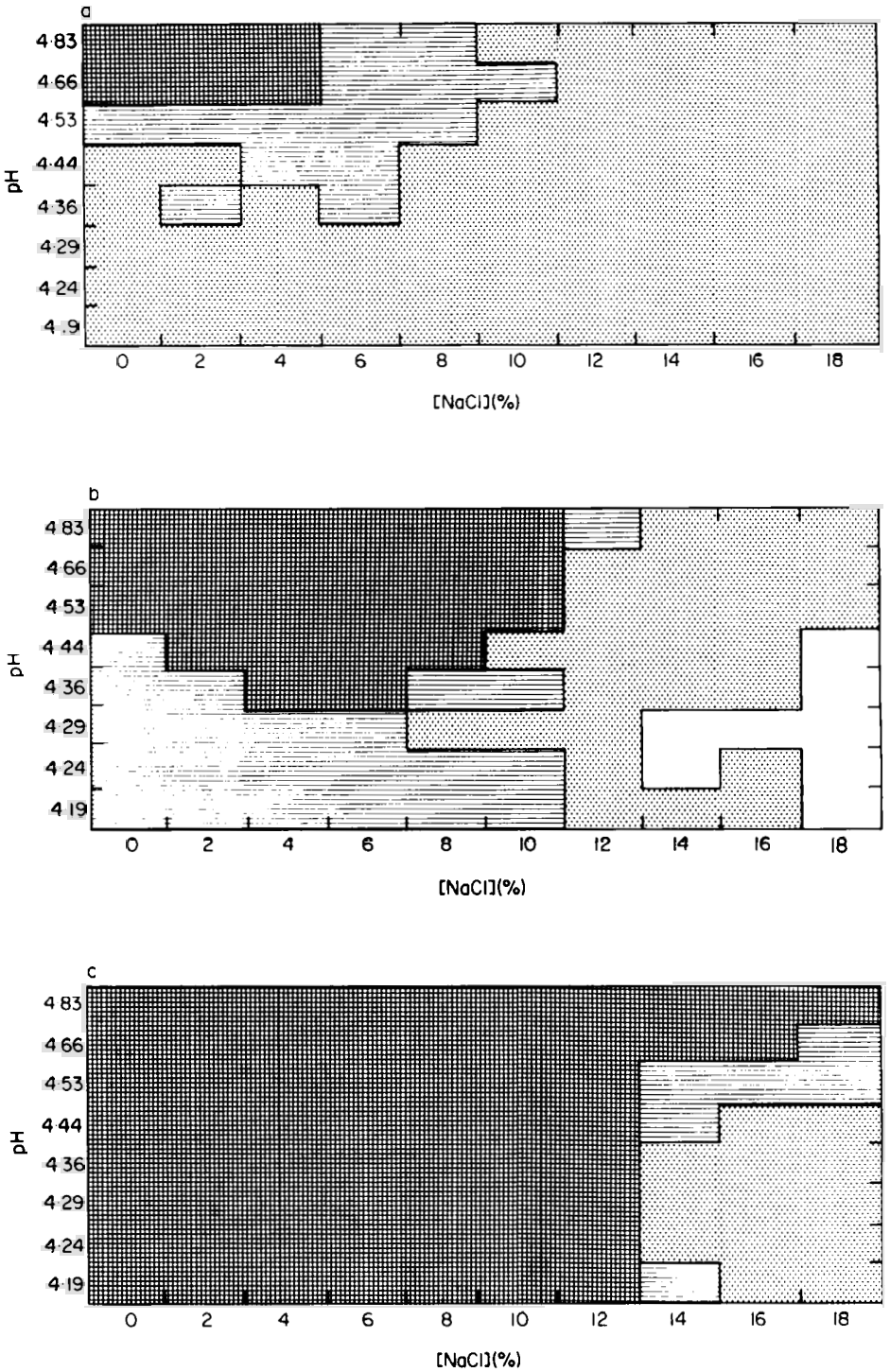

Fig. 1. Effect of salt concentration, pH and temperature on the survival of *Listeria monocytogenes*. Shaded areas represent growth of subcultured samples, incubated under optimum conditions (pH 7·0, 0% NaCl) within 60 days after incubation under the indicated conditions for 1, 2, 3 and 4 weeks at (a) 30°C, (b) 10°C and (c) 5°C. Survival: ▦, after 4 weeks; ▤, after 3, but not 4 weeks; ▨, after 2, but not 3 weeks; □, after 1, but not 2 weeks.

Table 1. Effect of pH and salt concentration on the growth of *L. monocytogenes*

		Mean time to visible growth (days) (n = 3)					
	[Salt]	30°C		10°C		5°C	
pH	(% Added)	Actual	Predicted	Actual	Predicted	Actual	Predicted
7·0	0	1·0	1·06	4·0	4·26	9·0	8·2
	2	1·0	1·20	4·0	3·78	8·6	10·1
	4	2·6	1·68	5·0	4·43	15·0	14·4
	6	3·0	2·85	7·0	6·82	28·0	23·3
	8	4·0	6·00	11·0	13·87	43·0	43·4
	10	25·0	15·34	45·0	37·33	NG	—
	12	38·3	48·08	NG	—	NG	—
	14	NG	—	NG	—	NG	—
	16	NG	—	NG	—	NG	—
	18	NG	—	NG	—	NG	—
5·13	0	2·0	1·85	11·3	11·24	NG	—
	2	2·0	2·06	11·0	10·48	NG	—
	4	2·0	2·80	12·0	12·80	NG	—
	6	4·3	4·66	21·6	20·70	NG	—
	8	12·0	9·58	NG	—	NG	—
	10	NG	—	NG	—	NG	—
	12	NG	—	NG	—	NG	—
	14	NG	—	NG	—	NG	—
	16	NG	—	NG	—	NG	—
	18	NG	—	NG	—	NG	—
4·83	0	4·0	3·31	25·0	25·03	NG	—
	2	4·0	3·59	24·3	24·29	NG	—
	4	4·0	4·81	NG	—	NG	—
	6	7·6	7·84	NG	—	NG	—
	8	19·5	15·73	NG	—	NG	—
	10	NG	—	NG	—	NG	—
	12	NG	—	NG	—	NG	—
	14	NG	—	NG	—	NG	—
	16	NG	—	NG	—	NG	—
	18	NG	—	NG	—	NG	—
4·66	0	5·0	5·87	NG	—	NG	—
	2	7·6	6·29	NG	—	NG	—
	4	8·0	8·17	NG	—	NG	—
	6	13·0	13·15	NG	—	NG	—
	8	NG	—	NG	—	NG	—
	10	NG	—	NG	—	NG	—
	12	NG	—	NG	—	NG	—
	14	NG	—	NG	—	NG	—
	16	NG	—	NG	—	NG	—
	18	NG	—	NG	—	NG	—

NG, no growth within 60 days.
No growth occurred at the two lowest pH values tested (pH 4·53 and 4·44).

The physiological basis of this protective effect of salt is unknown.

The extreme tolerance to salt of the strain of *L. monocytogenes* used in the present work is comparable to data available on the salt tolerance of other strains. For example, two strains of *L. monocytogenes* (Scott A and LCDC 81–861) grew in TSB (pH 7·3) at 30°C in the presence of 10% NaCl but not at 12% within two weeks (Conner *et al.* 1986). Growth studies in the present work were followed for 60 d and turbidity was detected in TSB (pH 7·0) at 30°C in the presence of 12% salt after 38 d incubation. The lower pH limit for growth of *Listeria* (four strains tested) in media acidified with HCl (George *et al.* 1988) was 4·39, whereas the lowest pH permitting growth of *L. monocytogenes* in this work was 4·66. Care must be taken, however, when such results are compared because of the use of different acidulants.

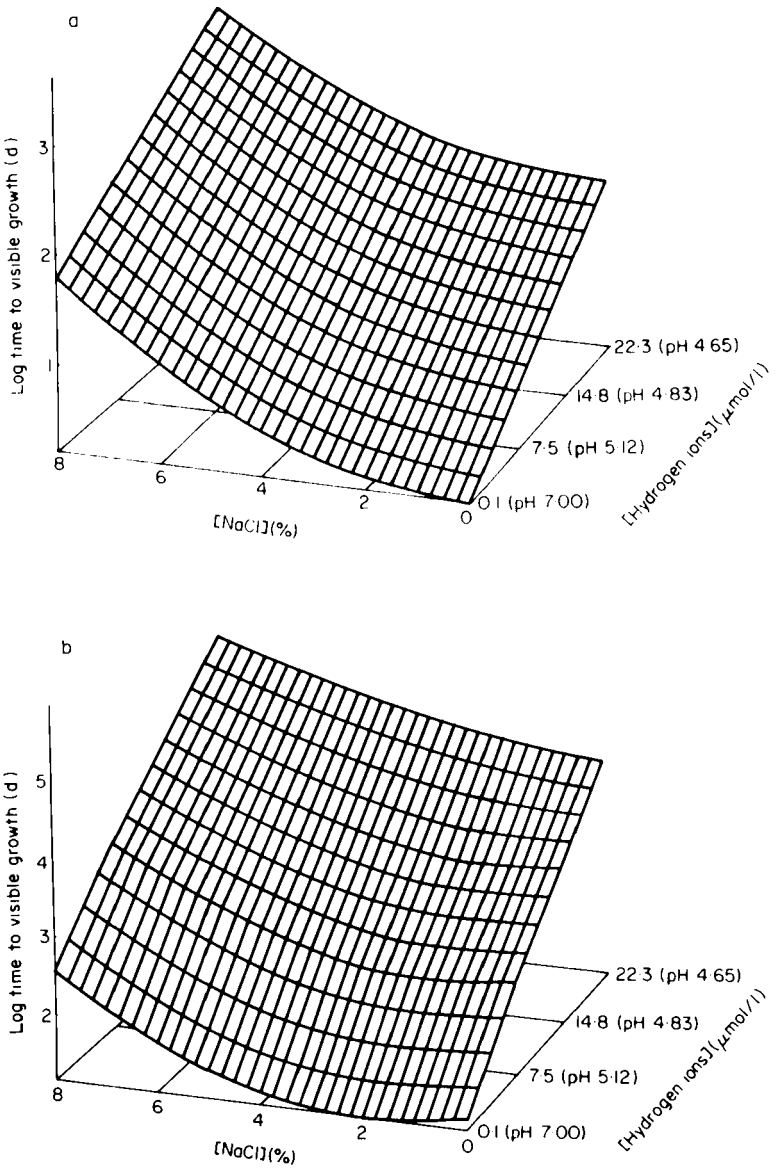

Fig. 2. Effect of salt and hydrogen-ion concentration on the $\log_e$ time to reach visible growth of *Listeria monocytogenes*. Response surfaces for the effect of salt (%, *x* axis) and hydrogen-ion concentration (μmol/l, *z* axis) on the $\log_e$ time to visible growth (days, *y* axis) representing at least a 100-fold increase in numbers of *Listeria monocytogenes* (a) at 30°C and (b) at 10°C. The response surfaces were derived from polynomial equations 1 and 2, from the analyses fully described in Materials and Methods.

Organic acids are generally more inhibitory to micro-organisms than inorganic acids due to their lipophilic nature (Corlett & Brown 1980). In this study pH was adjusted with a citrate/phosphate buffer which produced citric acid concentrations equivalent to 1·1% at pH 4·4 and 0·7% at pH 6·0. In addition to this, the chelating action of citric acid may also be relevant to growth inhibition at low pH values as reported in *Clostridium botulinum* (Graham & Lund 1986).

Previous observations in the study of microbial growth inhibition (Broughall & Brown 1984; Cole & Keenan 1987; Gibson *et al.* 1988)

Table 2. Statistical analysis of data

Table of analysis, 30°C (Equation 1)

Regression	DF	TYPE I SS	R-Square	F-Ratio	Probability
Linear	2	44·58	0·8005	284·37	0·0001
Quadratic	2	6·75	0·1213	43·08	0·0001
Crossproduct	1	0·04	0·0008	0·60	0·4427
Total regression	5	51·38	0·9226	131·10	0·0001
Residual	DF	SS	Mean square		
Total error	55	4·31	0·0783		

Table of analysis, 10°C (Equation 2)

Regression	DF	TYPE I SS	R-square	F-Ratio	Probability
Linear	2	14·15	0·8172	482·00	0·0001
Quadratic	2	2·73	0·1578	93·07	0·0001
Crossproduct	1	0·02	0·0012	1·43	0·2413
Total regression	5	16·90	0·9763	230·31	0·0001
Residual	DF	SS	Mean square		
Total error	28	0·41	0·0146		

DF, degrees of freedom; TYPE I SS, contribution of each independent variable to the regression sum of squares; R-Square, coefficient of determination; F-Ratio, ratio of type I mean of squares over the residual mean square; Probability, probability of obtaining an F-Ratio this large or larger at corresponding degrees of freedom; Mean square, Mean square error, sum of squares divided by the degrees of freedom.

have consistently demonstrated that as pH decreases, relatively small changes in growth rate and lag take place initially but as pH values approach limiting values, much more significant changes occur. These observations, together with the fact that the pH scale is equal to the negative log of the hydrogen-ion concentration, have led to consideration of hydrogen-ion concentration rather than pH in these trials. The experiment was therefore designed with equal increments of hydrogen-ion concentration rather than equal pH steps. Examination of the regression table of the models presented clearly shows that the choice of experimental design was justified. The effect of hydrogen-ion on growth (Equations 1 and 2) can now be described by a simple linear term. The quadratic term for hydrogen-ion concentration is insignificant at the 95% confidence level and can be left out of the predictive equations (Equations 1 and 2) with little consequence. If pH rather than hydrogen-ion concentration had been modelled then the mathematical description of the effect of pH would have been much more complex. Of more importance, the resulting fit of the models presented indicates that if the physiological effects of pH are to be fully understood then they should be investigated in terms of hydrogen-ion concentration rather than pH value.

A similar transformation can also be considered for the time to visible growth, as in most multidimensional growth models near optimum growth conditions there is little difference between the lag times under one condition compared with another, and most growth occurs quickly. As the limits for growth are approached lag period increases exponentially, with long periods of time where no growth is recorded (Broughall & Brown 1984; Cole *et al.* 1987; Gibson *et al.* 1988). Hence in the model presented the time to visible growth is expressed as a natural log. With this transformation the relationships between the log time to visible growth, hydrogen-ion and salt concentration can be expressed much more simply than if time to visible growth itself were used. In contrast to the effect of hydrogen-ion concentration the effects of salt concentration require a higher order quadratic relationship to describe them. This indicates that there may be an optimum salt concentration for the growth of *L. monocytogenes*. In a similar manner to the way that the protective effect of salt on survival was emphasized at lower temperatures; the optimum for growth also becomes more noticeable at

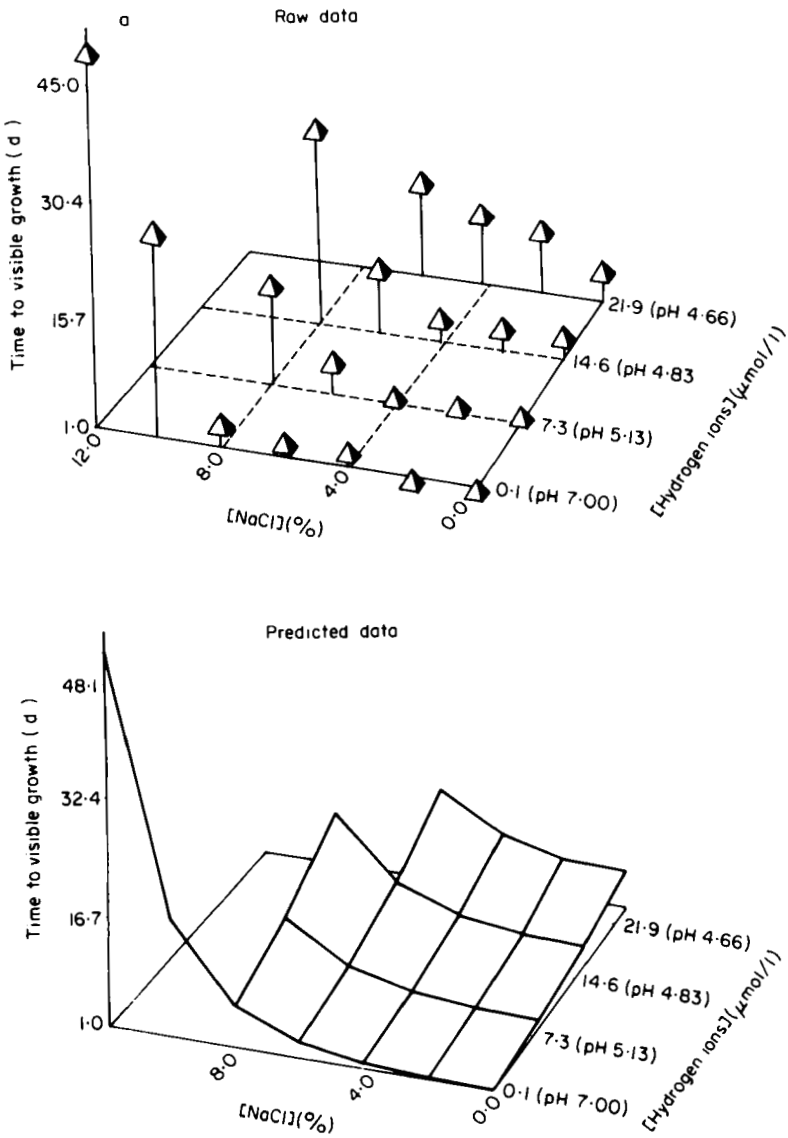

Fig. 3. Effect of salt and hydrogen-ion concentration on the time to reach visible growth of *Listeria monocytogenes*. Three-dimensional scatter plot for the effect of salt (%, *x* axis) and hydrogen-ion concentration (μmol/l, *z* axis) on the time to reach visible growth (days, *y* axis) representing at least a 100-fold increase in numbers of *Listeria monocytogenes* (a) at 30°C and (b) at 10°C. The mean actual values are compared with predicted values determined from polynomial equations 1 and 2.

10°C than at 30°C (Fig. 3). The optimum salt concentration for growth of *L. monocytogenes* at 10°C would appear to be around 2–2·5%.

The resulting model can also be used to determine the nature of the interaction between salt concentration and hydrogen-ion concentration on the growth of *L. monocytogenes*. The tables of analyses for growth at both 30°C and 10°C clearly show that there is no significant interaction of these two factors, the F-ratio for the crossproduct terms at both temperatures is low (0·6, DF = 1 and 1·43, DF = 1) indicating that the interaction is insignificant at the 95% confidence level. In other words hydrogen-ion

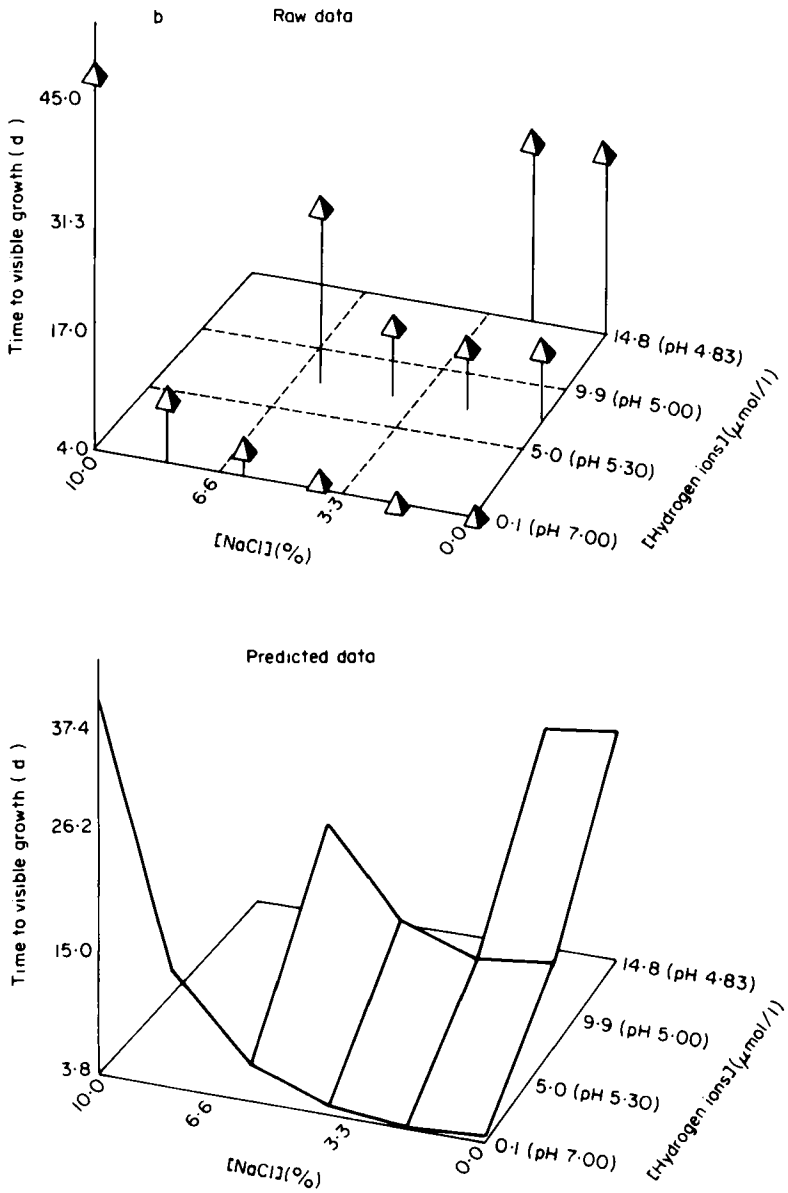

Fig. 3—*(continued)*

concentration and salt concentration are acting in a completely additive manner and there is no synergistic effect between them. This is also clearly demonstrated in Fig. 2. Although the model presented here is a simple one based on visual determination of growth (representing at least a 100-fold increase in numbers), the experimental design has allowed the combined effects of the factors to be quantified. The resulting polynomial equations (Equations 1 and 2) may be used to predict the time to visible growth by interpolation within the limits set by the design of any pH and salt concentration at 10°C and at 30°C even at values where time to visible growth has not been measured. For example, at 10°C in the presence of added 2% salt the model would predict a time to visible growth of 16 d at pH 5·0 but only 6 d at pH 5·5. The

results are of particular significance in highlighting relationships between the three stress factors under study.

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