A Philosophy for the Development of Kinetic Models in Predictive Microbiology

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submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy
University of Tasmania
Hobart
December, 1993

DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

T. Ross
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ABSTRACT

The development of predictive microbiology is reviewed and specific limitations relating to the generation of kinetic models identified. The issues of variability of response, lag time response, fluctuating environments and their effects, microbial interactions, choice of model for describing bacterial growth curves, and mechanistic *versus* empirical models are discussed and exemplified using experimental data. A philosophy for the development of reliable predictive kinetic models is developed and the appropriateness of that philosophy examined by simulations and reference to novel and independently published experimental data.

Specifically, the use of turbidimetric techniques is advocated for primary model development, methods of calibration to traditional (i.e. viable count) methods demonstrated, and the reliability of that calibration demonstrated. Using that methodology, models for the growth of several strains of *Staphylococcus aureus* and *Listeria monocytogenes* are generated. Novel indices of the reliability of models are developed, and used to assess the *S. aureus* 3b and the *L. monocytogenes* models, for constant environmental conditions, by comparison to published and novel data. An assessment of the three-parameter (temperature, water activity, pH) square-root model† is made using data for the growth of *L. monocytogenes*. A deliberately minimal experimental design was used to 'test to destruction' the proposed methodology, and revealed potential shortcomings of the lack of replication.

It is concluded that the experimental strategy proposed offers an efficient method for generating the quantity of data required for the development of reliable kinetic models from which to predict the growth of spoilage and pathogenic organisms of relevance to foods.

Technologies for the transfer of validated, laboratory-generated models to the food industry are demonstrated, and a mechanistic interpretation of the basis of the empirical square-root relationship developed.

† **McMeekin, T.A., Ross, T. and Olley, J.** 1992. Application of predictive microbiology to assure the quality and safety of fish and fish products. *Int. J. Food Microbiol.* **15**: 13-32.
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ACKNOWLEDGMENTS

I would like to acknowledge sincerely the contributions of:

my academic supervisors, Prof. T. A. McMeekin and Dr. J. Olley, for providing limitless support, stimulus, crisis intervention, and for providing the environment and opportunity;

The Rural Industries Research and Development Corporation for generous and gracious financial support;

David Ratkowsky for statistics and nonlinear regression expertise;

Dr. Kevin Sanderson, and Mr. Simon James for setting up and maintaining the computer facilities;

Glen McPherson for preparation of the Gompertz function fitting programme;

Dr. Fred Grau for *Listeria* cultures and collaboration;

Dr. Charles Jason and Dr. Peter Franzmann for insightful questions and comments;

all of my student colleagues, and staff of the Department of Agricultural Science (particularly Sally and Bill), for the help, good times, good ideas and just for being there;

and Wendy, Melanie and Morgan for understanding, tolerating and providing TLC throughout, and particularly when it was most needed.

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ABBREVIATIONS USED IN THIS THESIS

ABS	'true' absorbance of a sample after correction, if necessary, for high concentration deviation
ABS_{dil}	absorbance of a diluted sample
Abs_{obs}	observed absorbance (undiluted sample)
a_w	water activity
BHIB/A	brain heart infusion broth/agar
BPA	Baird-Parker Agar
CFU	colony forming unit
ΔG_{den}	Gibbs free energy change associated with protein denaturation
k^*	reciprocal of response time
MAFF	Ministry of Agriculture Food and Fisheries (UK)
MPD	maximum population density (of a bacterial culture in a given environment)
OLSA	Oxford Listeria Selective Agar
PCA	Plate Count Agar
pH_i	initial pH (of a broth culture)
pH_f	final pH (of a broth culture)
pH_{mid}	midpoint of pH_i and pH_f
ΔpH	change in pH of culture medium
RMS	mean of the sum of the squares of residuals
RSS	residual sum of squares
RH	relative humidity ($\approx 100 \times a_w$)
rsd	relative standard deviation
SD	standard deviation
SE	staphylococcal enterotoxin
t_G	mean generation time (of a bacterial population)
t_{Gmin}	minimum observed generation time (of a bacterial population during)
$t_{G\%T}$	t_{Gmin} estimate derived from %transmittance data
t_{GVC}	t_{Gmin} estimate derived from viable count data
T	temperature
TGI	temperature gradient incubator
TSB/A	tryptone soy broth/agar
Δ%T	change in %transmittance of a bacterial culture since inoculation
$\Delta\%T_{30}$	time required for %T of a culture to change by 30% from that at the $t = 0$
%T	% transmittance
μ_{max} *	maximum specific growth rate of a bacterial culture
USDA	United States Department of Agriculture
VC	viable count

* In the predictive microbiology literature, the symbol k has often been used to denote the reciprocal of microbial response times and, thus, to denote microbial growth rate. The designation of k as the 'growth rate constant', or specific growth rate, is a more established usage. The relationship between the generation time, GT, and specific growth rate is:

$$\textit{specific growth rate} = \log_e 2/GT = 0.693/GT$$

Within this thesis, response times rather than rates are frequently used. When used, the reciprocal of response times is denoted by k, while μ is used to denote the specific growth rate. This is not recommended usage, but is consistent with the literature, and is self-consistent within the thesis.

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"....the growth of bacterial cultures, despite the immense complexity of the phenomena to which it testifies, generally obeys relatively simple laws..... The accuracy, the ease, the reproducibility of bacterial growth constant determinations is remarkable and probably unparalleled, so far as biological quantitative characteristics are concerned."

(Monod, 1949).

INTRODUCTION

In 1983, at a Symposium of the Society for Applied Bacteriology, a panel of 30 expert food microbiologists using the Delphi technique of intuitive forecasting (Dalkey, 1967) predicted that the assessment of shelf life by computers, drawing on databases for the growth of spoilage organisms, had an 80% probability of being widely used by 1993. Nonetheless, at least 25% of the panel considered it improbable that such an approach would be accepted even at the beginning of the 21st century (Jarvis, 1983).

In 1992, the UK Ministry of Agriculture, Fisheries and Food launched 'Food Micromodel' - a food microbiology advisory service based on a database and mathematical models describing the growth response to environmental factors of food-borne pathogens. This service is a realisation of that earlier prophecy, and part of a new approach to the assurance of the microbiological quality and safety of food currently known as "Predictive Microbiology".

Predictive microbiology is based upon the premise that the responses of populations of microorganisms to environmental factors are reproducible, and that by considering environments in terms of identifiable dominating constraints it is possible, from past observations, to predict the responses of those microorganisms. In general, a reductionist approach is adopted and microbial responses are measured under defined and controlled conditions. The results are summarised in the form of mathematical equations which, by interpolation, can predict responses to novel sets of conditions, i.e. those which were not actually tested. Proponents claim that such an approach will enable:

i) prediction of the consequences, for product shelf life and safety, of changes to product formulation
ii) objective evaluation of processing operations and, from this, an empowering of the HACCP approach
iii) objective evaluation of the consequences of lapses in process and storage control
iv) the rational design of new processes and products, to achieve required levels of safety and shelf life.

In the decade prior to preparation of this thesis, the potential of predictive microbiology has attracted considerable research interest and funding, particularly in the United States of America and the United Kingdom, and also Australia and Western Europe. In the UK that interest has already borne fruit in the form of Food Micromodel. Interest in Europe is being developed through the FLAIR (Food Linked Agricultural and Industrial Research) program with ~30 laboratories in 10 EEC

countries acting collaboratively to examine growth responses of spoilage and pathogenic organisms in a wide range of natural products. In the United States of America predictive microbiology research is centred at the Microbial Food Safety Research Unit of the USDA in Pennsylvania, which has resulted in the preparation and release of software called the 'Pathogen Modeling Program'.

Earlier reviews of the subject include Farber (1986), McMeekin and Olley (1986), Baird-Parker and Kilsby (1987), Gibson and Roberts (1989), Gould (1989), Roberts (1989, 1990), Gibbs and Williams, (1990), Buchanan (1991a), and Baker and Genigeorgis (1993). A monograph on the subject has been prepared by McMeekin *et al.* (1993).

1.1 LITERATURE REVIEW

1.1.1 History

The use of mathematical models in food microbiology is not new. Baird-Parker and Kilsby (1987) point out that models for the thermal destruction of microorganisms by heat are well established in the literature and industry, e.g. the 'botulinum cook' (*see* Stumbo *et al.*, 1983). Mathematical modelling is also well developed in the fermentation industry. The application of mathematical modelling techniques to the *growth* and *survival* of microorganisms in foods, however, did not receive wide attention until the 1980's. McMeekin *et al.* (1993) suggest that two related trends contributed to the increased willingness to consider predictive modelling. The first was the marked increase in the incidence of major food poisoning outbreaks during the 1980's, which led to an acutely increased public awareness of the requirement for a safe and wholesome food supply. The second was the realisation by many food microbiologists, and clearly identified by Roberts and Jarvis (1983), that traditional microbiological methods to determine quality and safety were limited by the time needed to obtain results, and that the more rapid, indirect methods did not give a response until very large numbers of cells were present, i.e. they had little predictive value. Buchanan (1991b) points to another factor in the realisation of the concept: that of increased ready access to computing power.

Predictive microbiology appears to have been spawned from two separate lines of research. One of these was control of spoilage of fish, which is considered to spoil more rapidly than other flesh products (Olley and Ratkowsky, 1973a). This work had its origins at the Torry Research Station in the United Kingdom, with the publication of a model describing the effect of temperature on the rate of fish spoilage (Spencer and Baines, 1964). The problem of fish spoilage attracted continuing research interest and the modelling approach is clearly evident in the work of Olley and her colleagues at the now defunct CSIRO Seafood Technology Section (Olley and Ratkowsky, 1973a, b) and Nixon of the New Zealand Fishing Industry Board (Nixon, 1971). Olley had recognised the fundamental similarity of the response to

temperature of many spoilage processes, and also of bacterial growth, and in Daud *et al.* (1978) was able to apply the general spoilage model (Olley and Ratkowsky, 1973a) to the spoilage of chicken.

A second area of research dealt with the prevention of botulism, and other microbial intoxications. Genigeorgis' group at the University of California sought to go beyond the work of other researchers (e.g. Baird-Parker and Freame, 1967) on defining combinations of factors that would prevent pathogen growth and toxin formation. Their approach was to model the 'log reduction' of bacterial numbers due to intrinsic and extrinsic properties of foods, such as temperature, pH, NaCl concentration, etc. described as 'hurdles' by Leistner and Rödel (1976). The log reduction was then related to the probability of bacterial growth or toxin production (Genigeorgis *et al.*, 1971a).

At about the same time workers at the UK Ministry of Agriculture Fisheries and Food, were also involved in describing growth-controlling combinations of factors (Roberts and Ingram, 1973; Bean and Roberts, 1974), but did not begin to summarise their results as equations describing the probability of growth or toxin production until several years later (Jarvis *et al.*, 1979; Roberts *et al.*, 1981 a,b,c). Similarly, in the Netherlands, Kreyger (1972) had used a modelling approach to predict the mould-free storage life of cereals during ocean transport but presented results as diagrams only.

During the 1980's increased attention was given to modelling the *growth* of microorganisms of concern, with a number of groups publishing in this area. Genigeorgis' approach was applied to growth rate modelling (Metaxapoulos *et al.*, 1981a, b). Ratkowsky *et al.* (1982) and Ratkowsky *et al.* (1983) contributed simple and apparently universal models relating the growth rate of bacteria to temperature. Broughall *et al.* (1983) introduced a model relating the growth rate of bacteria to temperature and water activity which was built upon by Broughall and Brown (1984) to include the effect of pH also. Roberts group began growth rate modelling in 1987 (Gibson *et al.*, 1987). Their efforts led ultimately to an entirely different approach which has become the basis of programmes which resulted in Food Micromodel and the Pathogen Modeling Program.

Roberts and Jarvis (1983) paper may be viewed as the foundation stone of the rapid development of predictive microbiology in the subsequent decade. In that paper they challenged traditional methods of food quality and assurance testing, which they described as "an expensive and largely negative science", and advocated a more systematic and cooperative approach. Roberts (1990) recounts: "we have proposed the concept of 'predictive microbiology' within which the growth responses of the microbes of concern would be modelled with respect to the main controlling factors such as temperature, pH and a_w.... Models relevant to broad categories of foods would greatly reduce the need for *ad hoc* microbiological examination and enable

predictions of quality and safety to be made speedily with considerable financial benefit". This succinct statement of the need for such an approach places into perspective the vision of Scott (1937) who wrote: "A knowledge of the rates of growth of certain microorganisms at different temperatures is essential to the studies of the spoilage of chilled beef. Having these data it should be possible to predict the relative influence on spoilage exerted by various microorganisms at each storage temperature. Further it would be feasible to predict the possible extent of changes in populations during the initial cooling of sides of beef in the meatworks when the meat surfaces are frequently at temperatures very favourable to microbial proliferation".

1.1.2 Approaches and Potential Benefits

Predictive microbiology has to date usually been considered under two main headings. These are **kinetic** models, that is, modelling the extent and rate of growth of microorganisms of concern, and **probability** modelling: the construction of models to predict the likelihood of some event, such as a spore germinating or a detectable amount of toxin being formed, within a given time period .

The hypothesis underlying the kinetic modelling approach is that many perishable foods represent a 'pristine' environment open to exploitation by microorganisms, and that the growth of bacteria in this environment approximates a 'batch culture'. Typically, nutrients will not limit growth until spoilage has occurred or infectious dose levels are exceeded and, consequently, factors such as temperature, pH, water availability, gaseous atmosphere, preservatives etc. dictate the rate and extent of microbial proliferation. Thus, a detailed knowledge of the growth responses of microorganisms to those environmental factors should enable prediction of the extent of microbial proliferation in foods during processing, distribution and storage by monitoring the *environment* presented to the organism by the food during those operations. In the area of kinetic modelling two distinct approaches can also be identified. In one, growth rate is modelled and then used to make predictions in accordance with exponential population growth. This approach has been used by a number of groups (Broughall *et al.*, 1983; Smith, 1987; Blankenship *et al.*, 1988; Fu *et al.*, 1991; Dickson *et al.*, 1992; McMeekin *et al.*, 1993). In the second, a sigmoid function is fitted to the observed population growth curve and the effect of environmental factors on the values of parameters of that fitted sigmoid curve are modelled. This approach was introduced by Gibson *et al.* (1988). In both approaches models are constructed by following the increase in numbers or biomass of the organism, throughout the population growth curve, for a range of levels and combinations of environmental factors of interest. Thus, information is obtained about the lag phase duration, rate of growth, and maximum population density achieved under the conditions studied.

In the simplest case, when developing models to predict the probability of growth of pathogens or production of toxins, replicate samples of a known inoculum are observed under defined environmental conditions for a fixed period of time. At the end of the incubation period samples are examined for the presence of toxin, or detectable growth. The probability of detectable growth/toxin production within that incubation period can be calculated from the proportion of replicates positive for growth/toxin production. As this proportion is dependent upon the specific environmental conditions, a model relating the probability to those conditions may be derived. Typically, observations are made at a number of times, and the probability of detectable growth/toxin production is observed to increase with time. To date, the primary application of probability modelling has been to describe the effect of environmental factors on spore germination.

Gould (1989) considered that predictive microbiology would "encourage a more integrated approach to food hygiene and safety which will impact on all stages of food production, from raw material acquisition and handling, through processing, storage, distribution, retailing and handling in the home". In addition to the general benefits outlined above, modelling also provides a basis for comparison of data from diverse sources on the growth of microorganisms in foods, and should result in increased productivity by reducing the need for the time consuming and invasive microbiological testing procedures currently practiced. Genigeorgis (1981) noted that 'predictive microbiology' would provide a rational basis for the drafting of guidelines, criteria and standards pertaining to the microbiological status of food, and both McMeekin and Olley (1986) and Walker and Jones (1992) have pointed to the value of predictive microbiology, and devices based upon it, as educational tools for food workers and handlers. Davey (1992a) drew attention to major difficulties faced by food process engineers due to the poor understanding of the combined influences of environmental factors on the kinetics of bacterial growth and inactivation. He welcomed predictive modelling as a solution to that problem, and predicted that such models, coupled with indirect sensors and computers, would enable 'real-time' food process optimisation through automated in-line process control.

The potential benefits of the modelling approach are numerous but all derive from better understanding, and consequently control, of the microbial ecology of foods that predictive modelling represents.

1.1.3 Definitions and Rules for Modelling

Before commencing a detailed discussion of predictive microbiology it will be useful to introduce frequently used mathematical and statistical terms. The material in this section is derived largely from material prepared by the candidate and presented in Chapter 2 of McMeekin *et al.* (1993).

Independent, explanatory or **predictor** variables are those which it is believed will explain the type and magnitude of the response observed. In the case of models for food microbiology, these will typically be temperature, pH, water activity, and other agents in the modelled system which affect the rate of response of the modelled organism. **Response** or **dependent** variables are those properties of the system which are governed by the independent variables. In the case of predictive models in food microbiology, this will usually be the rate or duration of some microbial growth process or the probability of an event or condition arising within a given time.

A model is formulated to describe the known or assumed qualitative relationship between the predictor and response variables, but it is the **parameters** of the equation which quantify the relationship. Parameters, which are constants for a given set of experimental conditions, must be estimated from the data to give the best possible fit to the data. The usual means of estimation of parameters is the minimisation of the values of the residuals, which are the differences between the observed values of the response variable and those predicted by the fitted equation.

There is always some **error** inherent in any measurement of an observation. This error is the difference between the particular observation and the mean, or predicted, response. Accordingly, in any mathematical description of a data set there will also be some error between the predicted and observed values. The behaviour of the error can be investigated and described. For example it may, on average, be a constant value irrespective of the magnitude of the response. Alternatively it may be a function of the magnitude of the response. Description of this behaviour is an integral part of the modelling process and is expressed in the error, or **stochastic**, term of the model. The **deterministic** part of the model describes the relationship between the variables.

The error behaviour is particularly significant when fitting equations to data because those observations having larger error will be more influential in determining the parameter estimates when using residual minimisation techniques, e.g. least squares, for fitting. It is desirable that all observations are given equal weight in the fitting process, and for this reason one seeks to 'homogenise' the variance, i.e. to make the magnitude of the error independent of the magnitude of the observed response. Weighting may be applied to the data during the fitting process, so that those values with smaller residuals are given greater weight. An alternative is to transform the data mathematically, for example by taking the logarithm or some power of all the values, to thus homogenise the variance. The transformed data are then used in the fitting process.

Features of the mathematical expression of models are shown in Fig. 1.1. A model such as:

$$y = \alpha + \beta x + \gamma x^2$$

is classified as a linear regression model, since the parameters α, β, and γ appear linearly (i.e. as the *sum* of individual effects), although the relationship between the response variable y and the explanatory variable x describes a curved line. In nonlinear models the parameters do not appear linearly. The important differences between these two types of model are the manner in which they are fitted to data, and their estimation properties once fitted. The best estimates of parameters of linear models have explicit (algebraic) solutions. Best estimates of nonlinear models, however, do not have explicit solutions and are typically estimated by an iterative process in which approximate values of the parameter values are used as 'starting' values. Using these values a measure of the 'goodness-of-fit' of the fitted model is made. In the next step, new parameter values are sought which increase the 'goodness-of-fit' of the model to the data. This step-wise process is continued for a predetermined number of steps or until no further improvement in goodness-of-fit can be obtained. If at this stage the 'goodness-of-fit' meets some predetermined criteria of acceptability, this condition is known as 'convergence'. Not all data sets will achieve convergence nor does achievement of convergence guarantee that those parameter values are the best possible. Although nonlinear regression software is readily available, there is an element of expertise required when fitting data to nonlinear models.

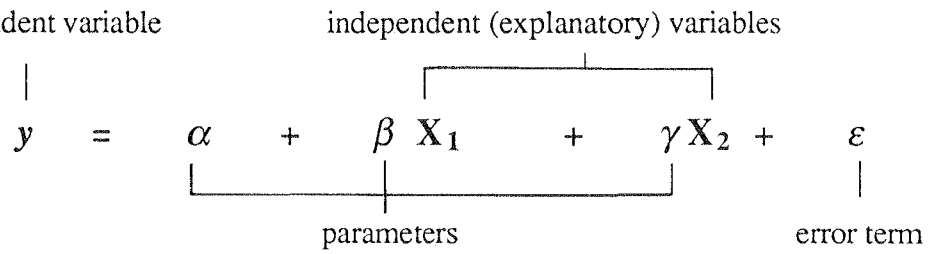

Fig. 1.1 An example of a mathematical model showing the nomenclature of the component terms. The values of the independent variables (X_1, X_2) are known or set before the response (y) is observed. The values of the parameters (α, β, γ) are determined by the data, and are fitted to minimise the difference, in some sense, between the observed response and that predicted by the model. The stochastic term (ε) indicates the extent to which the predicted response deviates from the observed response (*after* McMeekin *et al.*, 1993).

Ratkowsky (1983, 1990) has argued that it is a desirable goal of nonlinear modelling to search for models that are "close-to-linear", that is, ones that come close to achieving the properties that are attainable in linear models. 'Far-from-linear' models may have such highly biased estimators for at least one parameter that one can not have much faith that the estimates produced are sufficiently close to the true (but unknown) parameter values. Some practical considerations to which modellers should pay attention when engaged in nonlinear regression modelling are:

i) parsimony
ii) parameter estimation properties
iii) range of variables
iv) stochastic assumptions
v) interpretability of parameters

which are discussed in greater detail in Chapter 2 of McMeekin *et al.* (1993), Chapter 3 of Bates and Watts (1988) and Chapters 2 and 10 of Ratkowsky (1990).

It is intuitively apparent that a fundamental aspect of a predictive model is that it describes observed data accurately, but it is equally important that it can predict accurately responses to novel conditions. Unless a model has a proven mechanistic basis, predictions from that model should be based only on interpolation. Hence it is essential to have as full a range of the variables as possible. When reliable statistical analysis demonstrates that competing models predict equally well, other characteristics of the model such as parsimony; parameter estimation behaviour; parameter interpretability should be considered. A further, almost trivial, consideration in the selection of models is their ease of use, but it is only when all other criteria fail to provide discrimination between competing models that this be considered a basis for selection. The ease of use of a particular model will, of course, depend upon ones own environment, resources and background, and personal preferences.

Davey (1992b) suggested the adoption of 'express terminology' in predictive microbiology to clarify discussion of issues. His comments relate to the use of the terms to categorise and describe models unambiguously, and to seek a uniform usage of the terms 'interactive' in relation to environmental variables, and 'validated' in terms of the ability of models to predict responses to novel conditions, i.e. data not used to generate the model. Those comments were, in general, endorsed by Baranyi and Roberts (1992), who also suggested consistent terminology for describing rates of growth, and also by Whiting and Buchanan (1993).

1.1.4 Models

In kinetic models the response variable is expressed in units of time (i.e. a rate, or the time taken for a particular response). The evolution of kinetic models has been well

reviewed by Ratkowsky *et al.* (1991) and Heitzer *et al.* (1991). In this category are recognised four main model types which are:

Bělehrádek- or square-root-type models
Arrhenius-type models
Modified Arrhenius, or 'Davey' models
Polynomial, or 'Response Surface' models

There is less divergence in the form of probability models published to date, but slightly different forms have been adopted by the groups of Genigeorgis, Roberts and Lund.

1.1.4.1 Kinetic models

Bělehrádek-type models

Ratkowsky *et al.* (1982) introduced a simple model to describe the rate of microbial growth as a function of temperature. The model was based upon the observation of Ohta and Hirahara (1977) that the square root of the rate of nucleotide degradation in carp muscle is related to temperature. Olley (1986) describes how she and her coworkers found this relationship well described the growth rate response to temperature of many bacteria. The latter model has the form:

$$\sqrt{k} = b\,(T - T_{min}) \qquad (1.1)$$

where k is the rate of growth, T is the temperature, T_{min} is a notional minimum temperature for growth and b is a coefficient to be estimated.

Subsequently it was shown that the 'square root' model was a special case of the Bělehrádek (1930) equation widely used in other biological sciences (Ross, 1987). Ratkowsky *et al.* (1983) extended the applicability of their equation to include temperatures superoptimal for growth. This new model had the form:

$$\sqrt{k} = b\,(T - T_{min})(1-\exp(c(T - T_{max}))) \qquad (1.2)$$

where k, T , T_{min}, and b have the same meaning as above and T_{max} is a notional maximum temperature for growth analogous to T_{min} and c is a coefficient to be estimated.

Minor modifications to this model were advocated by Zwietering *et al.* (1991) and Kohler *et al.* (1991). This general form was further extended by McMeekin *et al.* (1987) who included a term for water activity:

$$\sqrt{k} = b\,(T - T_{min})\,\sqrt{(a_w - a_{w\,\min})} \qquad (1.3)$$

where k, T , T_{min} and b have the same meaning as above, a_w is the water activity and $a_{w\,\min}$ is a notional minimum water activity for growth

and by Adams *et al.* (1991) who incorporated a term for pH:

$$\sqrt{k} = b\,(T - T_{min})\,\sqrt{(pH - pH_{\min})} \qquad (1.4)$$

where k, T , T_{min} and b have the same meaning as above and $pH_{\min}$ is a notional minimum pH for growth.

The success of the Equations (1.3) and (1.4) led McMeekin *et al.* (1992) to conjecture that the effects on microbial growth of temperature, pH and water activity might be described by a simple four parameter model of the following form:

$$\sqrt{k} = b\,(T - T_{min})\,\sqrt{(a_w - a_{w\,\min})}\;\sqrt{(pH - pH_{\min})} \qquad (1.5)$$

where all parameters are as previously defined.

This equation, and a variation of it, introduced by Zwietering *et al.* (1992a), incorporating a pH term to cover the entire pH range was subsequently applied by Wijtzes *et al.* (1993) to describe data for the growth of *Listeria monocytogenes*.

Whilst Eqn. (1.1) may be expressed as a linear model, Eqns. (1.2) to (1.5) are nonlinear, and the parameters of those models must be estimated by iterative procedures.

Arrhenius-type models

The simplest form of 'Arrhenius-type' model in use in predictive microbiology is the 'classical' Arrhenius equation:

$$\ln k = \ln A - \frac{E_a}{RT} \qquad (1.6)$$

where, when applied to microbial growth, k is the rate of growth, A is a parameter to be fitted, R is the gas constant (8.314 J K^{-1} mol^{-1}), T is temperature, and E_a is sometimes interpreted as the activation energy of the growth-rate-limiting reaction or, to avoid this assumption, is simply referred to as a 'temperature characteristic'.

Although a number of researchers (Ingraham, 1958; James and Olley, 1971; Olley and Ratkowsky, 1973a; Mohr and Krawiec, 1980; Fu *et al.*, 1991) have attempted to apply this form of the Arrhenius equation, many (Scott, 1937; Daud *et al.*, 1978; Ratkowsky *et al.*, 1982; Stannard *et al.*, 1985; Phillips and Grifffiths, 1987) found it

was inadequate to describe the rate response to temperature of complex biological systems. More sophisticated models were developed to take into account the deviation, at high and low temperatures, from the rate predicted by the simple Arrhenius equation. A number of models of essentially similar form were developed by Johnson and Lewin (1946), Hultin (1955), and Sharpe and DeMichele (1977). The latter model was reparameterised by Schoolfield *et al.* (1981). Of the Arrhenius-type models, the model of Schoolfield *et al.* (1981) has received the most attention in the predictive microbiology literature, having been used by workers at Unilever Research in the UK (Broughall *et al.*, 1983; Broughall and Brown, 1984; Adair *et al.*, 1989). In its original parameterisation, this nonlinear model has the form:

$$\frac{1}{K} = \frac{\rho_{(25)} \frac{T}{298} \exp\left\{\frac{H_A}{R}\left(\frac{1}{298} - \frac{1}{T}\right)\right\}}{1 + \exp\left\{\frac{H_L}{R}\left(\frac{1}{T_{1/2_L}} - \frac{1}{T}\right)\right\} + \exp\left\{\frac{H_H}{R}\left(\frac{1}{T_{1/2_H}} - \frac{1}{T}\right)\right\}} \qquad (1.7)$$

where T is absolute temperature, R is the universal gas constant and, for modelling bacterial growth, the other parameters have been interpreted as follows: K is the response (e.g. generation) time, $\rho_{(25)}$ is a scaling factor equal to the response rate ($1/K$) at 25°C, H_A is the activation energy of the rate-controlling reaction, H_L is the activation energy of denaturation of the growth-rate-controlling enzyme at low temperatures, H_H is the activation energy of denaturation of the growth-rate-controlling enzyme at high temperatures, $T_{1/2_L}$ is the lower temperature at which half of the growth-rate-controlling enzyme is denatured, $T_{1/2_H}$ is the higher temperature at which half of the growth-rate-controlling enzyme is denatured.

For modelling in the suboptimal temperature region only, the model can be simplified by the deletion of terms relating to high temperature inactivation, i.e. the second exponential term in the denominator.

Broughall *et al.* (1983) further developed this temperature-rate model to include the effects of water activity by modelling the change in the parameters ln $\rho_{(25)}$, H_A, ln($-H_L$) and $T_{1/2_L}$ using expressions of the general form:

$$\text{parameter} = F + G(a_w - 0.95)$$

Broughall and Brown (1984) extended this approach to include the effect of pH. The effects of pH and water activity on the fitted values of the parameters ln $\rho_{(25)}$, H_A, H_L and $T_{1/2_L}$ were modelled using expressions of the form:

$$\text{parameter} = F + G(pH - pHS) + H(a_w - a_wS)$$

where pHS and a_wS are approximations of the mid-point of the range of pH and a_w respectively for which data were obtained. The resulting equation thus required the estimation of twenty parameters to describe the effect of temperature, pH and water activity on bacterial growth rate in the sub-optimal temperature region.

Adair *et al.* (1989) reparameterised the Schoolfield *et al.* (1981) model to give:

$$\ln(K) = A + (B/T) - \ln T + \ln\{1 + \exp[F + (D/T)] + \exp[G + (H/T)] \quad (1.8)$$

where K = lag or generation time, A = ln 298 - {[H_A/ 298 R)] - ln $\rho_{(25)}$; $B = -H_A/R$; $D = -H_L/R$; $F = -H_L/(T_{1/2_L} R)$; $G = -H_H/(T_{1/2_H} R)$; $H = -H_H/R$, and R and T are as defined above.

For modelling in the suboptimal temperature region, the model is simplified by the deletion of the 'exp [G + (H/T)]' term. This simplified model is another form of the Johnson and Lewin model (*see* Ratkowsky *et al.*, 1991).

Equations (1.7) and (1.8) are reparameterisations of the model of Sharpe and DeMichele (1977) introduced to overcome difficulties in fitting the model to data by nonlinear regression methods. The success of nonlinear regression fitting may be dependent upon the ability to obtain good initial parameter estimates, a process which may be enhanced by reparameterisation.

Davey/Modified Arrhenius models

Davey (1989a) introduced an Arrhenius-type model, for the effects of temperature and water activity, which was linear and thus allowed for explicit solution of the best parameter estimates. This model has the form:

$$\ln k = C_0 + \frac{C_1}{T} + \frac{C_2}{T^2} + C_3 a_w + C_4 a_w^2 \quad (1.9)$$

where k, a_w and T have the same meanings as previously, and C_0, C_1, C_2, C_3, C_4 are coefficients to be determined.

Davey (1989a) reported that the model well described seven data sets from the literature and subsequently demonstrated the ability of the model to describe lag phase duration also (Davey, 1991).

Polynomial/Response Surface models

Polynomial models represent a purely empirical approach to the problem of summarising growth rate responses. A polynomial-type approach was used by Metaxopoulos *et al.* (1981a, b) to describe the increase over time in numbers of *S. aureus* in salami as a function of numbers of lactic acid bacteria, pH, water activity

and $NaNO_2$. Einarsson and Erikson (1986) used a polynomial to model the increase in bacterial numbers as a function of time and temperature. Others had used polynomials earlier to construct probability models (Raevuori and Genigeorgis, 1975; Genigeorgis *et al.*, 1977; Jarvis *et al.*, 1979, Roberts *et al.*, 1981a, b, c). In this technique a linear model is constructed, which has the form of a polynomial function in the modelled parameters. Multiple linear regression is used to determine the best fit values for the parameters. The regression equations have the general form:

$$Y = a + b_1X_1 + b_2X_2 + ... + b_iX_i + ... b_nX_1^2 + ... + b_tX_i^2 + ... + b_vX_1X_2 + ... + b_zX_iX_j \quad (1.10)$$

where a, $b_{1,2,...z}$ are parameters to be estimated and $X_{1,2,.....i,j}$ are variables.

Cole and Keenan (1987) modelled the doubling and lag times of *Zygosaccharomyces bailii* in a model fruit-drink system. Doubling time was expressed as a polynomial, and lag time was modelled by the function:

$$\text{Lag} = e^x$$

where x was a polynomial expression.

Thayer *et al.* (1987) and Gibson *et al.* (1987) also used similar functions to model growth rate, but Gibson *et al.* (1988) introduced the approach of Jefferies and Brain (1984) in which the parameters of a sigmoid function, which describes the growth curve of the organism under study, are modelled as a function of the environmental variables. The model which has been most used to date is the so-called 'Gompertz function'. The appropriateness of this and other functions used will be discussed in 1.2 and 3.1. The effect of environmental factors on the parameter values of the fitted equations are then modelled by polynomial expressions. For example, for a constant initial inoculum, Buchanan and Phillips (1990) described the aerobic growth curve of *Listeria monocytogenes* Scott A, as a function of temperature (T), initial pH (P), sodium chloride concentration (S) and sodium nitrate concentration (N) by the expressions:

$$\begin{aligned}\ln M = {} & 37.657 + 0.0135T - 13.7331P + 0.4013S + 0.0713N + 0.00372T^2 + \\ & 1.9759P^2 - 0.000667S^2 - 0.00007051N^2 - 0.083TP + 0.0000842TS - \\ & 0.00241TN - 0.1155PS - 0.0167PN - 0.000125SN + 0.0000292T^2 - \\ & 0.0935P^3 - 0.00000328S^3 + 0.000286TPS + 0.0000315TPN + \\ & 0.00000014TSN + 0.0017PSN - 0.000384T^2P - 0.00000855T^2S - \\ & 0.00000043T^2N + 0.000731TP^2 - 0.0000441TS^2 + 0.00672P^2S + \\ & 0.000968P^2N + 0.000294PS^2 + 0.00000062PN^2 - 0.00000016S^2N\end{aligned}$$

and

$$\ln B = -47.709 + 0.1631T + 18.6861P - 0.33609S + 0.01N - 0.00161T^2 - 2.7074P^2 - 0.00623S^2 - 0.0000863N^2 + 0.0242TPS - 0.000906TS + 0.00594TN + 0.0671PS - 0.00715PN + 0.00337SN - 0.0000684T^3 + 0.1276P^3 - 0.000029S^3 - 0.00051TPS - 0.0000733TPN - 0.00000033TSN - 0.0000431PSN + 0.000189T^2P + 0.0000549T^2S - 0.00000047T^2N - 0.00222TP^2 + 0.0000459TS^2 - 0.0007781P^2S + 0.000777P^2N - 0.000872PS^2 + 0.0000112PN^2 - 0.00000038S^2N$$

where B and M are parameters of the 'Gompertz function'.

Discussion of the strengths and weaknesses of various kinetic modelling approaches may be found in Lowry and Ratkowsky (1983), Stannard *et al.* (1985), Phillips and Griffiths (1987), Adair *et al.* (1989), McMeekin *et al.* (1989), Davey (1989a), Davey (1989b), Kilsby (1989), Ratkowsky *et al.* (1991), Zwietering *et al.* (1991), Gill and Phillips (1990), Heitzer *et al.* (1991), Alber and Schaffner (1992), van Impe *et al.* (1992), and Ross (1993).

1.1.4.2. Probability models

Genigeorgis *et al.* (1971a) modelled the 'decimal reduction' in the number of *S. aureus* subjected to different environmental conditions. Using MPN methods, the primary response measured was the number of cells having initiated growth at times up to 20 days after inoculation. The probability, P, of a single cell initiating growth was calculated as:

$$P = R_G / R_I$$

where R_I is the number of cells inoculated into the system and R_G is the number having initiated growth.

The expression:

$$\log(R_I/R_G)$$

represents the number of decimal reductions of a population resulting from its exposure to a particular environment. The effect of environmental conditions on P was modelled by the polynomial expression:

$$\log(R_I/R_G) = a + b_1(\%NaCl) + b_2(pH) + b_3(\%NaCl)^2 + b_4(pH)^2 + b_5(\%NaCl)(pH)$$

One of Genigeorgis' motivations was to provide quantitative data, for factors other than thermal processing, to enable prediction of safe combinations of conditions to prevent pathogen growth and toxin formation (Genigeorgis *et al.*, 1971a). The use

of decimal reduction as a response variable is consistent with the description of the effects of thermal processing.

This model type was used for a number of other organisms and combination of environmental factors (e.g. Raevuori and Genigeorgis, 1975; Yip and Genigeorgis, 1981). A different form of model was adopted by Lindroth and Genigeorgis (1986). The probability, P, of one spore of *C. botulinum* to initiate growth and toxigenesis was defined as:

$$P(\%) = \frac{\text{MPN x 100}}{\text{inoculum}}$$

where MPN is the number of spores which have initiated growth and toxigenesis, and inoculum is the number of spores initially present. When no samples were toxic, P% was defined as 10^{-3}.

The probability of any event must have a value between zero and one. The following function:

$$P = \frac{e^{y}}{1+e^{y}} \qquad (1.11a)$$

or its reparameterisation:

$$P = \frac{1}{1+e^{-y}} \qquad (1.11b)$$

where P is the probability of the response and where 'y' is a function of the variables modelled by a polynomial,

describes this range of values and has been adopted by several probability modellers. Lindroth and Genigeorgis (1986) also recognised that the probability of growth detection within a given time was also dependent upon the lag time and initial inoculum density. They used the model, based on Eqn. 11a:

$$\text{Log}_{10}P(\%) = 5\left(\frac{e^{y}}{1+e^{y}}\right) - 3$$

where the effect of environmental variables is expressed in 'y' by the expression:

$$y = b_1 + b_2 * ((T) + b_3 * (S_t - LP) + b_4 * T * (S_t - LP) \qquad (1.12)$$

where b_1 -b_4 are coefficients to be determined, T is temperature, S_t the elapsed time, and where LP, the time to toxigenesis, was modelled by:

$$LP = a + b_5 T + b_6 (1/T) + b_7 (I)$$

where I is the inoculum concentration, and a, b_5 -b_7 are values to be determined.

Roberts' group developed a model for the probability of toxin production by *C. botulinum* based on more than 50 000 observations (Gibson and Roberts, 1989). The model was of the form of Eqn. 1.11b, where y was given by a linear polynomial expression.

Lund *et al.* (1987) introduced to predictive microbiology the model:

$$\begin{aligned} \text{Log}_{10}\text{P} &= A - S\,(X - T) && \text{for } T<X \\ &= A && \text{for } T>X \end{aligned} \qquad (1.13)$$

where P is the probability of growth, A is the maximum value of Log_{10}P, X is the time required for $\log_{10}$P to reach a maximum value, S is the slope of the line relating $\log_{10}$P to incubation time and T is time.

This model allows for asymptotic values other than 1, i.e. under some conditions, no matter how long one waits, not all samples will show growth/toxigenesis. In a second stage of the modelling process the parameters A, S and X were expressed as functions of the environmental variables by polynomial expression similar to those exemplified above.

So *et al.* (1987) presented a method to enable ecogram data, an earlier pictorial convention for expressing the probability of toxin production, to be summarised in models of the form:

$$Y = \beta_0 \left[1 - e^{-\beta_1 (T - T_0)}\right]$$

where Y = % of samples becoming toxic, T is the storage temperature, and β_0, β_1, and T_0 are parameters to be estimated.

T_0 was interpreted as the temperature below which no toxin is produced. The parameters β_0, β_1, and T_0 were expressed as polynomial functions of NaCl and $NaNO_2$ concentrations. By interpreting the results of the vast literature on challenge study data for *Clostridium botulinum* as probabilities of growth and toxigenesis, Hauschild (1982) was able to compare results from divergent challenge studies and discern new information regarding factors influencing the safety of cured meat products.

The response measured by probability modellers is dependent upon the time for a response to be detectable, which is a function of the time required for germination or lag resolution, the rate of growth of the organism, and the number of cells initially present which commence growth. The probability of detectable growth,

when plotted as a function of time, is a sigmoid curve which has an upper asymptotic value representing the maximum probability of growth given infinite time. As will be detailed in a later section, Ratkowsky *et al.* (1991) highlighted the increasingly variable nature of the bacterial growth responses under conditions stressful to the organism, and showed that the growth rate becomes increasingly variable as a function of generation time. Thus, upon closer analysis, the distinction that has traditionally been made between probability and kinetic models is an artificial one. One may now interpret the sigmoid shape of the probability curve as a reflection of the range of growth/lag-resolution rates, the fastest rates resulting in the earliest detection of growth. Whilst probability models indicate the absolute likelihood of an event occurring given sufficient time, they also include information about the variability of rates of growth as recognised by Baker *et al.* (1990): "The rate of P increase ..... expresses the growth rate ....".

The two types of models may be considered as the extremes of a spectrum of modelling needs, and research from both 'ends' is now converging. At near growth limiting conditions the kinetic modeller must consider the probability of a predicted growth rate, or growth at all. Similarly, the probability modeller must include some kinetic considerations. In a situation where no growth of an organism of concern is tolerable, one would use a probability model to ensure that the chance of lag resolution or spore germination is insignificantly low. At the other extreme, in a product which must be handled under conditions for which the probability of growth of spoilage organisms is unity, one would need only a growth-rate estimate for shelf life prediction. The two approaches converge in situations where growth up to some threshold is acceptable, but for which the environmental conditions are such that the responses are highly variable. Buchanan (1991b) identified one of the problems involved in implementation of probability based models as the translation of probabilities into values that can be used to set safe shelf lives, and noted that this issue was being increasingly addressed by integration of kinetics and probability based models.

1.1.5 Potential Problems and Solutions

It should be remembered that predictive microbiology is perhaps more a philosophical approach than a specific method or technology, and while it may be successfully applied to many problems, it cannot solve all. Despite weaknesses and limitations, predictive microbiology has been successfully applied. Limited commercial acceptance was realised in the mid-1980's, with some companies in Canada, the United States, England, and Europe using predictive modelling in their food operations (Farber, 1986). In a personal communication Dr. A.C. Baird-Parker (1986) wrote: "We [Unilever] routinely use Autologs for the collection of time/temperature data during distribution and use this data for predicting the safety and shelf-life of chill stored

products. This is based on our internal data base (pathogens and spoilage organisms) and the use of the nonlinear Arrhenius equation for predictive mathematical modelling." An 'expert system' has been developed by Unilever (Adair and Briggs, 1993). This, and other commercial applications of predictive microbiology, are usually considered proprietary. Nonetheless, there are reports in the literature detailing successful applications of the concept. Some criticisms of predictive microbiology will be considered here, evidence presented to counter those arguments and strategies introduced to overcome some perceived limitations. A complementary defence of predictive microbiology is given in McMeekin and Ross (1993).

Scepticism exists that a model derived in an experimental system can reliably predict the growth of the modelled organism in a food. Evidence to support the hypothesis underlying the modelling approach (i.e. that growth rates are determined by environmental constraints which can be simulated in model systems and that such models do lead to reliable predictions in foods), may be found in the kinetic modelling literature (Daud *et al.*, 1978; Gill, 1984; Pooni and Mead, 1984; Muir and Phillips, 1984; Smith, 1987; Gibson *et al.*, 1988, Reichel *et al.*, 1991; Ross and McMeekin, 1991; Wijtzes *et al.*, 1993). The results of Gibson *et al.* (1988) and Wijtzes *et al.* (1993) are particularly convincing. Gibson *et al.* (1988) compared the predictions of their model for the growth of Salmonellae in tryptone soya broth (TSB) to independently derived literature values for *Salmonella* growth in foods. Similarly, Wijtzes *et al.* (1993) compared growth rates of *Listeria monocytogenes* in TSB as a function of temperature, pH, and water activity, to independently derived literature values. They concluded that the predictions from the model compared to those reported in the literature were very good. Notably, in most cases the growth rates in TSB were *not* slower than those reported in foods. Gibson *et al.* (1988) concluded that "there was good agreement between predicted generation times and those published, with the exception of *S. typhimurium* inoculated into blended mutton at 10°C". This exception, and the observations of Gibson *et al.* (1987) that at temperatures approaching those limiting growth *C. botulinum* appeared to grow better in meat products than in laboratory medium, may also have contributed to mistrust. A possible explanation for these observations may lie in the extraordinary sensitivity of growth rates to small temperature changes at near growth-limiting temperatures, as commented on by Muir and Phillips (1984). This temperature sensitivity may also be appreciated by consideration of the simple square root model, and may *partially* explain the increasing variance of growth rate estimates as incubation temperature is reduced from the optimum for growth.

Genigeorgis *et al.* (1971b) and Raevuori *et al.* (1975) reported also that *S. aureus* and *B. cereus* grew better in actual foods or food homogenates than in laboratory media, and subsequently used food homogenates as their model systems. Ikawa and Genigeorgis (1987) concluded the close agreement between predictions

from their model system, and those observed in rockfish fillets, was confirmation of the reliability of that experimental design and methodology.

Gill (1986) identified three problems in the practical application of temperature function integration[1] yet, ironically, Gill and his colleagues have subsequently been more energetic in finding practical solutions to those problems than any other group. The first problem identified by Gill, and others (e.g. Pooni and Mead, 1984) is the dependence of useful predictions (e.g. time to spoilage, time before statutory levels are exceeded) upon the initial number of microorganisms present. In some cases it may be feasible to enumerate the organisms of concern. In many situations, however, economic factors dictate that the product can not be 'held up' for the time taken to obtain results from traditional (viable count-based) methods. In those situations two obvious strategies emerge. One is to use a rapid method to enumerate the initial microbial load, but there is still no method sufficiently specific and rapid or cost effective. The second is to base predictions upon an assumed starting inoculum. The value chosen may be based upon that which is achievable using GMP, or, in less controlled circumstances, upon a 'worst case'. The latter strategy was adopted by Gill and Phillips (1990). They concluded that, for regulatory purposes, a process is defined by the product of poorest hygienic condition that the process yields, rather than the average product condition, and developed strategies based on this realisation (Gill *et al.*, 1988a). For foods produced to a consistent level of quality, that level may be used as the baseline for predictions. Chandler and McMeekin (1989a), using an electronic device based on the concept of relative rates, were able to predict the time of spoilage of commercial milk products on the basis of temperature history and a model for *Pseudomonas* growth. Similarly, Gill and Harrison (1985) were able to obtain good predictions for the proliferation of *E. coli* on offal during cooling in cartons, based on an assumed initial inoculum, consistent with good slaughter technique.

In many situations it may not be necessary to have knowledge of absolute growth rates under each set of conditions: predictions may be based on relative rates. In this approach, models are used to predict the growth rate under a particular set of storage conditions relative to that under conditions for which the shelf life of product is known. For example, milk is typically spoiled by pseudomonads, and has a shelf life at 4°C of 8-10 days. If the temperature of storage is found to be 10°C the shelf life at 10°C can calculated without reference to the absolute growth rate for those organisms under those conditions. Models for *Pseudomonas* show that its growth rate at 10°C is ~ 1.5 times that at 4°C. Thus the shelf life at 10°C is (8-10 days)/1.5, i.e. ~ 5-7 days. One can also integrate relative rates, a technique that has been

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[1] Temperature Function Integration (Nixon, 1971): the summation of the temperature history of a product and it's interpretation in terms of potential bacterial growth. See also 6.1.2.

incorporated into several predictive microbiology devices (Ross and McMeekin, 1991).

The second problem Gill experienced was the identification of a mathematical model relating bacterial growth to temperature and which facilitated ready integration. The many models available have already been discussed: Gill's group adopted a Bělehrádek-type model.

Gill's third objection, echoed by Riemann (1992), is that the bacterial/food system is complex and incompletely understood. In this regard other potential difficulties are apparent, e.g. how reproducible is the lag time of bacteria in such systems and how accurately may it be predicted; what, if any, is the effect of interactions among the bacteria present. In addition to these problems is that of the heterogeneity of some foods and the distribution of microorganisms within them, and also the possibility of micro-environments. The problem of heterogeneity was considered by Gill *et al.* (1991a) and overcome by a 'worst case' strategy. i.e. to find the slowest cooling part of a carcass which was contaminated with spoilage or pathogenic organisms, and to base predictions on that worst case. In many cases it may not be necessary to predict the end result of a temperature history, but simply use that history to predict that an event could not have happened.

An aspect of the system's complexity is that of microbial interactions and the differential effects of temperature ranges on the components of the microbiota as envisaged by Scott (1937) cited earlier. Available evidence (e.g. Nderu and Genigeorgis, 1975; Gill and Newton, 1980; Mackey and Kerridge, 1988; Ross and McMeekin, 1991) suggests that microorganisms do not greatly affect the growth of one another, except where population densities are very high. Metaxapoulos *et al.* (1981a,b) modelled the growth of *S. aureus* in fermented meats under commercial conditions and obtained good agreement between the predicted increase in numbers of *S. aureus* in the product and that observed. From the point of view of predictions of interest in food microbiology, such population densities occur only after spoilage, toxigenesis or infectious dose levels are reached. This suggests that, for many applications, the effect of the environmental history on each component of the microbiota may be modelled and calculated independently without the need to consider interactions. Nonetheless, one can envisage situations where microbial interactions may occur, especially with *Lactobacillus* strains which produce bacteriocins or other microbial compounds and, if these affect the growth rate of the organisms of concern, this factor would have to be included in the model development. This emphasises the need to understand the microbial ecology of the product (McMeekin *et al.*, 1993).

Another objection relates to 'dynamic' environments, i.e. those in which environmental conditions change during the life of the product, with several writers (Gibson, 1985; Mackey and Kerridge, 1988; Gibbs and Williams, 1990) pointing out that models derived from experiments performed under static conditions may not be

applicable to fluctuating conditions. The factor most obviously likely to fluctuate is temperature and, although one can envisage situations in which changes in pH, gaseous atmosphere and water activity may occur, it is temperature which has been most investigated. Walker *et al.* (1990), Fu *et al.* (1991), and Buchanan and Klawitter (1991) hypothesised that incubation conditions would subsequently affect the rate of growth of microorganisms and, therefore, that it would be necessary to know the previous history of the organism in order to accurately predict its rate of growth in a particular environment. Fu *et al.* (1991) termed this possibility a 'temperature history effect'. Microbial cultures, when shifted abruptly from one temperature to another, may exhibit a transient growth rate before assuming the growth rate expected at the new temperature (Ng *et al.*, 1962; Shaw, 1967; Araki 1991). Fu *et al.* (1991) observed a similar effect and concluded that there was a temperature history effect. Several investigations (Walker *et al.*, 1990; Buchanan and Klawitter, 1991; Hudson, 1993, Li and Torres, 1993a) found no significant temperature history effects on growth, but effects on lag time duration were suggested. These conclusions are consistent with those of Neumeyer (1992), who also presented data which suggested the effect of temperature shifts on lag times might be related to the *magnitude* of the temperature shift. Critical analysis of the results of Fu *et al.* (1991) suggest that other interpretations are possible, and that temperature history effects need not be invoked to explain their observations. Nonetheless, if transitions between temperature are abrupt and frequent, i.e. if transitional rates represent a large part of the storage history, predictions from the current generation of models may be unreliable. In addition to those cited above, there are now many reports (Langeveld and Cuperus, 1980; Gill, 1984; Smith, 1987; Blankenship *et al.*, 1988; Dickson *et al.*, 1992; Spencer and Baines, *unpublished* in McMeekin *et al.*, 1993) based on a range of products, which indicate that predictions from models based on data generated under constant conditions can reliably predict growth under dynamic temperature conditions.

The potential problems considered above relate to limitations in the amount of information available from which to make predictions based on models. A more fundamental and limiting problem is suggested by the results of Ratkowsky *et al.* (1991) who began to quantify the inherent variability of the growth responses of microorganisms, as did Muir and Phillips (1984). Similar observations were alluded to by Gill (1984), and Mackey *et al.* (1980). Using the limited amount of replicated published data concerning growth rate estimates under varying environmental conditions, Ratkowsky *et al.* (1991) concluded that those responses became increasingly variable at slower growth rates. The following equation characterised the relationship between the variance in a response and its magnitude:

$$\text{variance} = 4 \times (\text{response time})^3 \times \text{variance}(\sqrt{k}) \qquad (1.14)$$

where k is the reciprocal of the response time

In the data presented by Ratkowsky *et al.* (1991) var$\sqrt{k}$ is a constant. McMeekin *et al.* (1993) subsequently showed that this relationship was dictated by the distribution of response times which, for the data sets they considered, was inverse Gaussian. Alber and Schaffner (1992) showed that for a strain of *Yersinia enterocolitica* (serotype 08) the variability in response times is better described by a Gamma distribution for which logarithmic transformation better homogenises the variance. Ratkowsky (1992) presented the following general relationship between the variance in growth response times and the mean of those responses for a range of possible distribution types:

$$V = c\alpha^n \qquad (1.15)$$

where α is the mean of the probability distribution, V is the variance of the probability distribution, n is an integer exponent having values 0, 1, 2, or 3, corresponding to the normal, Poisson, Gamma and Inverse Gaussian distributions respectively, and c is a constant.

Table 1.1, abridged from McMeekin *et al.* (1993), illustrates the consequences of Eqn. 1.14 for the confidence limits of the predictions of such models. The table gives predicted generation times, θ_0, for the case of *S. aureus* 3b, which should be used for predictive modelling to achieve a certain level of safety. For example, one in ten thousand *S. aureus* 3b have generation times, θ, at 12.5°C of less than 69 minutes so that predictions of response times based on this value should only *over*estimate the response time once in ten thousand events, i.e. the prediction will fail one time in 10 000. A high level of confidence requires that more products are predicted to be unsafe than actually are, however, so that unnecessary wastage may be a consequence. At 12.5°C the difference in the response times corresponding to 95% and 99% confidence levels is quite large (nearly a factor of 2) whereas at 30°C, because the variability is less, the difference is of the order of 10% of the estimate. At 30°C the rates are much faster and the generation times are much smaller, so that risks of 10^{-3} and 10^{-6} require generation times of 23.5 and 17.75 minutes, respectively.

Similar exercises can be carried out with data that are distributed in other ways, to produce bounds that attempt to take account of the variability of estimates of time-based quantities such as generation time and lag time. A general approach to modelling taking into account the nature of the distribution of responses was presented by Ratkowsky (1992).

Table 1.1 Values of Generation Time, θ (minutes), Required to Achieve Minimum Selected Probabilities $P[\theta < \theta_0]$ of Prediction Failure for *S. aureus* 3b. [Inverse Gaussian Distribution with Scale Parameter c = 0.000817. Data of Neumeyer (1992)].

12.5 °C		30 °C	
θ_0	$P[\theta < \theta_0]$	θ_0	θ_0
46	0.000001	17.75	0.000001
69	0.0001	21.0	0.0001
136	0.01	27.0	0.01
203	0.05	30.5	0.05
2356	0.95	56.25	0.95
3782	0.99	63.75	0.99
6038	0.999	73.0	0.999

Finally, a more philosophical criticism was levelled by Hedges (1991). He stated that he was unconvinced that many of the papers in the predictive microbiology literature represent a real contribution to science because of the empirical nature of many of the models published. He considered that such contributions do not help to elucidate underlying processes but merely describe a set of observations. Despite that other workers claim to have presented a more scientific (Ross and McMeekin, 1991) or mechanistic (McMeekin *et al* , 1993, Chapter 10) approach, the criticism has some basis. In a defence, however, Cole (1991a) asserted that researchers in the field of predictive microbiology are working towards mechanistic models but that currently there are none able to deal with the many parameters of interest. He concluded: "Therefore, in the meantime, we will continue to develop our predictive models .....because of the power that models provide for the food microbiologist in day-to-day decision making."

1.1.6 Technology

There are a variety of technological applications based on the predictive microbiology concept, ranging from simple, disposable, chemical time-temperature integrators, to complex 'expert systems' incorporating predictive models amongst the database(s) used for decision making.

Chemical monitors, which are intended to mimic the response to temperature of bacterial growth were reviewed by Olley (1978), McMeekin and Olley (1986), Wells and Singh (1988a,b), Taoukis and Labuza (1989a,b), Fu *et al.* (1991), Labuza and Taoukis (1991), Sherlock *et al.* (1991), Taoukis *et al.* (1991), and McMeekin *et al.* (1993) who concluded: "There are currently no cheap visual monitor strips available which simulate bacterial growth or food spoilage over a wide enough temperature range. One type, marketed until recently by the I-POINT ® company, showed promise and it is hoped that their technology can be rehabilitated. Alternatively, in the future, enzymes specifically selected to mimic the bacterial growth-rate-temperature relationship .....could be incorporated into enzyme-substrate strips".

Previous discussions of electronic devices include Olley (1978), McMeekin and Olley (1986), and Ross and McMeekin (1991). McMeekin *et al.* (1993) discussed comprehensively the existing electronic and chemical devices, their history, and the potential for further development and concluded that [semiconductor-based] 'technology already exists to develop devices able to monitor many of the environmental parameters in foods which affect the rate of microbial growth and thus, using microprocessor technology, to yield estimates of food quality and safety".

The potential of monitoring devices relies, however, upon data relating growth of food-borne microorganisms to environmental conditions. Buchanan (1991b) considered that, despite the many published predictive models for microbial growth and toxigenesis, there had been "relatively little transfer of this technology to food microbiologists involved in non-research aspects of the field" and further "that the limited acceptance of predictive modeling techniques seems to be due in large part to a lack of application software that reduces to routine operations the often complex mathematical manipulations associated with the use of the models". A similar opinion was expressed by Stecchini *et al.* (1993). Earlier in this review a range of such systems were alluded to, including the Pathogen Modeling Program and Food Micromodel. Another example is the applications software associated with the Delphi Temperature Logger developed by Gill and his colleagues for assessment of meat processing operations. The former two systems were described in Buchanan (1991b) and Walker and Jones (1992), respectively. Cole (1991b) reviewed the use and potential of databases, including expert systems, in food microbiology and concluded that the most significant developments were in the area of predictive microbiology. A number of expert systems are being developed in various institutions around the world. As part of the Food Micromodel project, the UK government is developing a relational database to allow direct remote access to the models base by industrial users of that system (Jones, 1993). Agriculture Canada is developing software to assess product safety and to define production system critical control points. MKES (an acronym of Microbial Kinetics Expert System) Tools simulates the growth and

survival of pathogenic organisms when subjected to many different environmental conditions. The responses predicted are used to estimate the significance of the individual factors in that situation (Voyer and McKellar, 1993). The Unilever company has established a food microbiology expert system. The system incorporates a set of rules based on the knowledge and 'decision-making' processes of an expert food microbiologist, as well as a database of knowledge of chilled food manufacturing and food-borne microorganisms, and is linked to a spreadsheet providing predictions based on microbiological growth models (Adair and Briggs, 1993). Zwietering *et al.* (1992a) described the basis of an expert system which models bacterial growth in food production and distribution chains.

1.1.7 Summary

Predictive food microbiology may be seen as providing a rational framework for understanding the microbial ecology of food, and as a large step towards making food microbiology "more science, than art". Though many food systems are too complex to model in detail, in many cases a number of strategies can be applied to simplify the problem, and to allow useful predictions to be made. The potential advantages of predictive microbiology are numerous and, although predictive modelling systems are already in use on a commercial basis, the full realisation of that potential will depend upon a conscientious and rigorous approach to data gathering and modelling, as well as ingenious solutions and strategies for the application of those data and models, and willingness on the part of the food industry to trial the approach and think in terms of the premises upon which predictive microbiology is based.

1.2 CONTEMPORARY RESEARCH

Contemporary research issues in predictive microbiology are reflected in the session titles at the 1992 International Workshop on the Application of Predictive Microbiology and Computer Modeling Techniques to the Food Industry, namely: Probability Models; Kinetic Models; Inactivation/Survival Models; Applications Software and Expert Systems; Application of Modeling Techniques to Food Safety; Quality and Production, Data Collection and Design of Experiments. Many of these have already been discussed in this review, but there remains a number of areas of ongoing research worthy of comment.

To return to an earlier point, Gibson *et al.* (1987) are generally credited with having introduced sigmoid functions to describe the bacterial growth curve objectively and reproducibly. In fact, Stannard *et al.* (1985) fitted their data to a sigmoid function and noted: "In comparing data for analysis, the use of a computer program was an advantage in fitting the best growth curves and calculating useful parameters of the fitted curve". This was an important step. Prior to that work, most growth curves which had been used to derive kinetic data for predictive models had been fitted 'by

eye' to data considered to represent the exponential phase, although Broughall and Brown (1984) refined this by a sophisticated and reproducible routine to determine the points that were most likely to represent the exponential phase.

The choice of the most appropriate function to describe the growth curve was discussed by Jennison (1935) and continues to generate research effort. Whilst Jason (1983) provided compelling evidence that the logistic model was a good model to describe bacterial batch growth, Bratchell *et al.* (1989) concluded that the modification of the Gompertz function due to Gibson *et al.* (1987) was more reliable than a modification of the logistic function which Gibson *et al.* (1987) had also trialed. This finding was supported by Zwietering *et al.* (1990) who compared the performance of five sigmoid functions for describing the bacterial growth curve. The use of fitted sigmoid functions also allowed unambiguous definitions and analyses of the lag phase and exponential phase duration to be made, based on the fitted parameters (Gibson *et al.*, 1988, Buchanan and Cygnarowicz, 1990; Garthright 1991; Zwietering *et al.*, 1992b).

Draper (1988) considers that mechanistic models are preferable to empirical ones because they usually contain fewer parameters, fit the data better, and extrapolate more sensibly. Baranyi *et al.* (1993) wrote "it is a reasonable aim to choose models which can be connected to generally accepted mathematical descriptions of nature". The modified-Gompertz function is an empirical application of a mechanistic model which was initially derived for actuarial purposes, and there is a growing recognition that the modified-Gompertz function may not be the most appropriate model to describe bacterial batch culture growth (Whiting, 1992; McMeekin *et al.*, 1993; Baranyi *et al.*, 1993). The deterministic model of Jason (1983) is limited because it requires a subjective assessment of the end of lag phase to be made. In consequence, a number of new mechanistic models have been published.

Dalgaard (1993) used simply the logistic function, after logarithmic transformation of both sides of the equation and inclusion of an additive parameter to model the lag phase empirically, and in so doing stated he was able to retain the original interpretation of parameters. Baranyi *et al.* (1993) developed a model which also described lag, exponential and stationary phases but had the added advantage that the lag and exponential growth stages were treated independently, so that one was not influenced by the other in the fitting process. Whiting and Cygnarowicz-Provost (1992) included the death phase also in their model, as did van Impe *et al.* (1992), who presented a very comprehensive treatment of growth/survival/death modelling. The latter authors pointed out that the Gibson *et al.* (1988) modelling approach is valid only for constant conditions, and that conditions which fluctuate over time can lead to discontinuous predictions. Accordingly they developed a model able to deal with time-varying temperatures over the whole temperature range for growth and inactivation.

Interest in the variability of growth rate responses arose from questions regarding the most appropriate form of kinetic model (Adair *et al.*, 1989). The results of Ratkowsky (1992), described earlier, emphasised that the stochastic behaviour cannot be overlooked in modelling, and that failure to correctly deal with the stochastic behaviour can lead to incorrect conclusions and unreliable models. Equally importantly, this work addressed the limits of confidence one can have in predictions from models.

1.3 OBJECTIVES OF THIS THESIS

In keeping with the theme of Roberts and Jarvis (1983), there prevails a largely cooperative spirit within the international predictive microbiology 'community'. The rapid increase in interest and research output in predictive microbiology has, however, resulted in a rather *ad hoc* development of methodologies for data generation, analysis and summary. There is consensus, though, that replicated data of good quality are fundamental to the development of reliable predictive models for microbial growth in food (Bratchell *et al.*, 1989, McMeekin *et al.*, 1993). Traditional methods of growth curve generation are extremely tedious and labour intensive and researchers are attempting to develop methods and experimental designs which will maximise the data available from experiments (McMeekin *et al.*, 1992). Various automated systems have been trialed. Buchanan's group, who generate kinetic data using viable count methods from broth cultures, use spiral platers and automated plate reading equipment. Other groups have advocated the use of turbidimetric methods (Mackey and Kerridge, 1988; McMeekin *et al.*, 1993; Dalgaard *et al., in* press), particularly automated instruments such as the Bioscreen[2] (McClure *et al.*, 1993) or automated conductimetric methods (Borch and Wallentin, 1993). In another approach, efforts are being made to make compatible the results of diverse groups and laboratories. A protocols document has been prepared (Anon., 1990) for laboratories participating in the MAFF program in the UK. On an even larger scale, efforts are underway to combine existing databases and coordinate the modelling efforts at the international level, (R.L. Buchanan, 1993, pers. comm.), as is already happening in the FLAIR program operating in Western Europe.

Whiting and Buchanan (1993) proposed that three levels of model could be recognised in predictive microbiology. They suggested that models which measure the response of microorganisms to a single set of conditions over time be classified as 'primary' models. Models which describe the response of one or more of the parameters of the primary model should be classified as 'secondary' models, and that applications of one or more secondary models which make predictions of those

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[2]BIOSCREEN: Labsystems, U.K. Ltd., Basingstoke, Hants, U.K.

models available to 'non-modellers' be called tertiary models. These classifications provide a convenient framework to describe this thesis.

The work reported in this thesis is concerned primarily with the development of a labour efficient strategy for the derivation of kinetic models applicable to foods. Two primary models to describe the bacterial growth curve are evaluated and a robust turbidimetric method developed. Using the proposed strategy, secondary models to predict the growth rate of *Staphylococcus aureus* in response to temperature and water activity,and of *Listeria monocytogenes* in response to temperature, water activity (due to [NaCl]) and pH are generated.

To evaluate the reliability of the methodology, a deliberately minimalistic experimental design is used to simulate the conditions under which smaller organisations may be likely to develop models. A 'test to destruction' philosophy is adopted and no attempt is made to 'fine tune' the results to suit a particular hypothesis. Predictions from models developed in this manner are compared to novel and published data for the growth of those organisms in foods, and indices by which to evaluate the reliability of models are introduced. Technologies for the application of predictive models in the food industry, i.e. tertiary models, are also described.

The work was also concerned with the interpretation of the physiological mechanisms underlying the responses of microorganisms to temperature and the elucidation of a mechanistic interpretation of the square-root relationship between growth rate and temperature.

2 KINETIC MODEL GENERATION: HYPOTHESES

2.1 INTRODUCTION

A spectrum of needs and strategies exists for developing predictive models for food microbiology. There are different types of problem (toxin formation, spoilage, pathogen growth, death kinetics); models of different types (e.g. probabilistic, kinetic); various methods for collecting data (optical density, viable counts, conductimetry, metabolite assays); several mathematical expressions used to describe what is essentially the same type of data; and there have been a number of indices suggested for evaluating models (Adair *et al.*, 1989; Bratchell *et al.*, 1990; Zwietering *et al.*, 1990). Underlying this diversity are fundamental elements common to all model development, and which have been recognised by various authors (Draper and Smith, 1981; Farber, 1986; Baird-Parker and Kilsby, 1987; Roberts, 1990; Ross and McMeekin, 1991). These are:

i) Planning
ii) Data collection and analysis
iii) Mathematical Description and
iv) Validation and Maintenance

This chapter presents a discussion of the modelling process in terms of these stages as the basis for the development and rationalisation of the modelling philosophy employed and evaluated in this thesis. The philosophy strives to be rational, rigorous, yet labour efficient, and to yield reliable and accurate models. Although the discussion is from the perspective of the development of kinetic models, some of the discussion will also be relevant generally to other model types. Much of the material in this chapter is drawn from that presented by the candidate in Chapter 2 of McMeekin *et al.* (1993).

2.1.1 Empirical *vs* Mechanistic Models

Causton (1987) states that the essential purpose of mathematical models is to describe succinctly a set of acquired data. It is more correct, however, to consider the model as describing an underlying process which generates data (McMeekin *et al.*, 1993). Two major types of model are recognised. Empirical models are derived from an essentially pragmatic perspective, and simply describe the data in a convenient mathematical relationship. Consequently, they often give little insight into the underlying process. Mechanistic, or deterministic, models are built up from theoretical bases, and if they are correctly formulated, may allow the interpretation of the response in terms of known phenomena and processes.

In building regression models to describe natural processes, it is likely that the appropriate model will prove to be a nonlinear one. This is because physical, chemical and biological models are often solutions of differential equations, and such solutions are rarely of the form that the model is linear in its parameters. (McMeekin *et al.*, 1993). Draper (1988) also states that mechanistic models are often nonlinear in their parameters and more difficult to fit and evaluate and that, consequently, appropriate experiments are more difficult to design.

Empirical models usually take the form of first or second degree polynomials. Polynomial models of higher order are rarely fitted partly because of the difficulty in interpreting the form of the fitted response surface which, in any case, produces predictions whose standard errors are higher than those from lower order models (Draper, 1988). Draper (1988) considers that mechanistic models are preferable to empirical ones because they usually contain fewer parameters, fit the data better, and extrapolate more sensibly, further noting that polynomial models often extrapolate poorly.

2.2 PLANNING

Factors that need to be considered when designing experiments to gather the data upon which the model is based are: the variables to be included; whether there are interactions between variables; the range of values of the variables; the amount of data required; the experimental model used and the response to measure and model.

2.2.1 Independent Variables

When developing models to describe the growth rate response as a function of several independent variables, one is modelling a 'response surface' in n dimensions where n is the number of independent variables. A response surface design is simply an arrangement of experimental points representing possible combinations of variables in that n~dimensional space. The choice of response surface design is thus one of selecting a set of suitable combinations according to some preselected criterion of goodness (Draper, 1988). Desirable features of such a design are given in Table 2.1.

2.2.2 Interactions

If it is necessary to model the effect of a number of variables acting simultaneously, it may be useful to do a factorial experiment first to determine whether there are any interactions among the factors. Typically this is done by having each variable either present at a single level or absent, and testing the response at each combination of the variables, that is, using 2n test conditions where 'n' is the number of variables. Factorial experiments may also be designed to include many values of the independent variables. The analysis of factorial experiments is fairly straightforward

and is discussed in texts on statistics or experimental design (for example Cochran and Cox, 1957).

2.2.3 Range of Values

The range of the variables to be included in the experimental design will depend upon the range expected to be encountered in the product. It is generally accepted that it is unwise to extrapolate beyond the range of the data used to generate the model so that the full range expected must be tested. It is also sound practice to use as full a range of the independent variable as possible to obtain as full a range of the response as possible. Even in situations where the expected range of values of the independent variable is small, using a wider range may reveal behaviour that could not be resolved over a narrower range, and provide greater confidence in predictions within the region of concern.

Statistical principles should also be considered when determining the number of levels of independent variables to be tested. When many independent variables are being evaluated the number of test conditions rapidly multiplies, so that decisions have to be made regarding the feasibility of conducting all experiments concurrently. This must also be considered in relation to the method used to measure the response. Various strategies have been proposed to deal with this problem. Some workers use a central composite design to reduce the number of experimental points that are tested on the response surface (Gibson *et al.*, 1988; Buchanan and Phillips, 1990; Palumbo *et al.*, 1991). The central composite design is described by Draper (1988) who comments that it has many of the desirable features referred to in Table 2.1. For some applications, however, one may be more interested in the response at conditions far removed from the centre of the range. For example, many food processors will be more interested in the responses of microorganisms at near growth-limiting conditions, as these are the conditions which will extend the shelf life and safety of their products. In this situation most observations are required near the extremes of the range, where growth rate is retarded, rather than the centre of the range. This limitation has recognised by some workers, and has been addressed by the use of augmented, or supplemental, central composite designs (Buchanan *et al.*, 1993a; Benedict *et al.*, 1993).

2.2.4 Preparation of Inocula

Reports on the growth responses of microorganisms have drawn attention to the fact that the condition of the inoculum may have an effect on the subsequent growth kinetics, particularly the lag phase duration. Consequently, it is necessary to take steps to use inocula that are, as far as possible, identical.

Kinetic models attempt to explain the time taken for a specified growth response in terms of environmental variables such as temperature, water activity,

pH, etc. As discussed in 1.1.5, recent reports have suggested that growth responses (particularly lag time duration) may also be dependent upon the prior temperature history of the inoculum used for kinetic experiments, whilst other reports suggest that exponential growth rates are independent of temperature history.

One means to avoid the problem is to perform all experiments simultaneously using a single culture for the inoculum. In this situation it may still be necessary to take precautions such as reducing or stopping the growth of the inoculum, (for example by reducing the temperature to that at which the growth rate is negligible), prior to and during the inoculation procedure so that the inoculum density does not increase during the time taken to inoculate all cultures. In many cases, however, it is not possible to perform all determinations simultaneously and special precautions must be taken to prepare standardised inocula.

Table 2.1 Desirable Features of Response Surface Designs

The response surface design should:

1. generate a satisfactory distribution of information about the behaviour of the response variable throughout a region of interest, R
2. ensure that the fitted value at x, $\hat{y}$ (x), be as close as possible to the true value at x, $\eta(x)$.
3. give good detectability of lack of fit
4. allow transformations to be estimated
5. allow experiments to be performed in blocks
6. allow designs of increasing order to be built up sequentially
7. provide an internal estimate of error
8. be insensitive to wild observations and to violation of the usual normal theory assumptions
9. require a minimum number of experimental points
10. provide simple data patterns that allow ready visual appreciation
11. ensure simplicity of calculation
12. behave well when errors occur in the settings of the predictor variables
13. not require an impractically large number of predictor variable levels
14. provide a check on the constancy of variance assumption

[*After* Draper (1988)].

2.2.5 Inoculum Size

The possibility that inoculum size has an effect on the growth responses of a culture such as lag time, exponential growth rate or maximum population density has also been addressed. In general there appears to be no effect of inoculum size on exponential growth rate or maximum population density (Jason, 1983; Buchanan and Phillips, 1990). It is important to distinguish between the detection time of a response and the lag time. All practical bacterial enumeration methods have a lower limit of detection. If the inoculum density used is below this level then the time taken for an observable change in bacterial numbers is a combination of the lag time and the time taken for the population size to increase to a detectable level.

2.2.6 Mixtures of Strains and Species

Some workers (e.g. Gibson *et al.*, 1988; Blankenship *et al.*, 1988; Baker *et al.*, 1990, Benedict *et al.*, 1993; Hudson and Mott, 1993) prefer to use a mixture of strains of the modelled organism, arguing that it is more representative of the situation that is likely to pertain to contamination of real foods. Such mixtures are sometimes referred to as 'cocktails'.

An alternative is to model several strains independently and to choose from those the strain that represents the worst case, i.e. that strain which grows fastest under the conditions of most interest (McMeekin *et al.*, 1993).

2.2.7 Response Variable

The primary response measured is usually the change in bacterial population density over time. The rate of change is then typically expressed as lag phase duration, generation time, or time to reach some specified level or condition, although the response is often modelled with rate (i.e. 1/response time) as the dependent variable. For reasons discussed below, the response variable is often mathematically transformed. For example, the response variable of the square root model is $\sqrt{\text{rate}}$ and of Arrhenius based models is usually ln (rate).

In addition to the obvious response variables of lag and generation rates, other response variables which have been modelled include the square root of the reciprocal of the time taken for the culture to achieve a 30% change in optical density (McMeekin *et al.*, 1987; Adams *et al.*, 1991); the logarithm of the reciprocal of the time taken for the culture to reach the most rapid rate of growth, given by the Gompertz M parameter (*see* 3.1) (Gibson *et al.*, 1987; Buchanan *et al.*, 1989); various transformations of the time taken to achieve a specified increase in population numbers, for example; an increase by a specified number of $\log_{10}$ cycles (Stannard *et al.*, 1985; Gibbs and Williams, 1990), the time taken to reach a 'specified spoilage level' (see 6.1) indicated organoleptically or by a specified microbial load (Daud *et al.*, 1978; Gibbs and Williams, 1990), organoleptic scores

(Bremner *et al.*, 1987), metabolite levels (Olley and Ratkowsky, 1973a) and time to reach a specified point on the modelled growth curve (Phillips and Griffiths, 1987). Lag phase duration and doubling time of a food spoilage yeast were modelled by Cole and Keenan (1987). Adair *et al.* (1989) used the natural logarithm of response time. For probabilistic models the response variable is the probability (as a function of the independent variables of storage conditions, product parameters and time) that some event such as growth initiation or formation of toxin will occur within a certain time.

The form of the response variable chosen will depend upon the nature of the problem under investigation (growth rate, probability) and the model chosen to describe the response. For example, the use of logarithm of rate originally stemmed from the adoption of the Arrhenius model to describe bacterial growth responses. Prior to the development of computers and nonlinear regression software, the parameters of this model were best estimated from a graph of logarithm of rate against the reciprocal of absolute temperature. Similarly, for the square root model the fitting process is simplest when the data are expressed with square root of rate as the response variable as this leads to a 'straight-line' relationship between the response and explanatory variables.

With the advent of computer software which can perform complex fitting processes, one can be more objective about the selection of appropriate response variables. The more important issues relate to the stochastic properties of the selected response. Stated succinctly, to obtain the best fit of the model to the data, the error in the estimate of the selected response must be independent of the value of the response or, if there are compelling reasons for the use of a response variable which does not have this property, the error behaviour must be compensated for by a weighting in the fitting process to give each datum point equal influence in the estimation of parameters.

2.2.8 Experimental Model

The experimental system used in the development of predictive kinetic models will always be based on a growth medium. In practice this will usually be a laboratory medium or some type of food. Determination of growth responses in liquid media allows a greater range of detection methods. In particular, indirect methods can be used which permit many more determinations to be made for a given amount of effort compared to direct enumeration methods. The organism is also more likely to be uniformly distributed than on a solid medium. A criticism of liquid laboratory media, however, is that they may not accurately model conditions in real foods, so that the model will subsequently have to be validated in the food product to which it is intended to be applied.

Conversely, using real foods is both costly and logistically very difficult, and in general limits the method of measurement to viable (plate) counts or most probable number (MPN) methods. There are also difficulties in applying the test organism to the food in a consistent and representative manner. Furthermore, all available test methods are invasive. Accordingly, unless a liquid food is used, an extra element of variability is introduced from the growth medium as each determination must be undertaken on a unique sample of that food. Food-based experimental models may also lead to complications due to the presence of normal spoilage biota, and may generate models specific for the particular food substrate used.

Some of these problems were encountered and discussed by Gibson *et al.* (1987, 1988) particularly in relation to obtaining sufficient reliable data to model the growth curve so that objective measurements of growth parameters could be derived.

2.2.9 Quantity of Data

An absolute lower limit to the amount of data required to fit a particular model is the number of parameters of that model, but for reliability of the estimates there must be many more data points than parameters to be estimated. Bratchell *et al.* (1989) demonstrated the effects of insufficient measurements in a region of interest, but were unable to quantify those consequences. As a rule of thumb McMeekin *et al.* (1993) suggested that, to fit most of the kinetic models in contemporary use reliably, ten to fifteen data points are required per independent variable.

2.3 MATHEMATICAL DESCRIPTION

2.3.1 Introduction

The data obtained by experiment must be further processed to fit the data to the model type chosen and evaluate the model's parameters. Generally speaking, the selection of a function to describe a particular response is a statistical exercise, as one is dealing with estimates of the true response and must consider the probability of the response observed being an accurate indication of the true or general response. This point is further discussed later. In contrast, the actual fitting of data to a chosen function is largely devoid of statistical considerations and is, rather, an exercise in algebra.

2.3.2 The Principle of Least Squares

The actual process of fitting a function to a data set, that is, to determine the parameter values that best fit the model to those data, is based on the principle of least squares. This criterion aims to derive parameter values that minimise the sum of the squares of the differences between observed values and those predicted by the fitted model, i.e. the residuals. The application of the least squares principle is not

always straightforward, however, and there is now an extensive literature on function estimation (Causton, 1987).

While the principle of least squares is in itself largely devoid of statistical concepts, to use it appropriately to fit functions to data one must consider some statistical aspects of the response being modelled. In addition, analysis can be applied to the data to determine the statistical properties of the parameter estimates, e.g. confidence intervals. Statistical theory shows that, for independent and identically distributed normal error, least squares estimation is appropriate. When the errors are not normally distributed, then least squares estimation is devoid of statistical 'underpinning' (Ratkowsky, pers. comm., 1993).

2.3.3 Stochastic Behaviour

Earlier the deterministic and stochastic parts of the model were differentiated. The stochastic part is included to account for the random variation in the response and, if fully specified, indicates the probability of the measured response falling within a particular range of the response predicted by the deterministic part of the model.

In fitting functions to data by least squares it is generally assumed that the error in the data is homogeneous, that is, the error has about the same magnitude irrespective of the value of the observation. The validity of this assumption can only be tested by having estimates of the error over the range of response variable values. The principle of least squares aims to minimise the sum of the squares of the residuals. Consequently, if the error is not homogeneous, more weight will be given to those points in which the magnitude of the error is large because the greatest reduction in the RSS (sum of squares of the residuals) will be achieved by more closely fitting to those points.

There are a number of methods available to overcome the problem of unhomogeneous errors. One is to use weighted least squares regression analysis. In this method a weighting is applied to individual data points, in inverse proportion to the magnitude of the error variance, for the purposes of estimating the least squares parameter values. Another is to transform the model mathematically into a form in which the response variable does display homogeneous error. Transformation of the model may also be used to improve other properties of the model, such as better parameter estimation in the case of nonlinear models. Causton (1987) argues against the use of weighted regression analysis, however, on the grounds that with modern computing facilities the model can be fitted without undue difficulty in the transformation in which the variance in the response variable is homogeneous, and that "any fudging with weighted regressions is just not worthwhile." The important issue is not the difficulty of fitting the model, however, but that the error behaviour is investigated and correctly specified when fitting the model.

2.3.4 Regression Analysis

The usual steps involved in fitting any function to data by least squares are to:

(i) formulate an expression for the sum of the squares of the residuals
(ii) partially differentiate the expression obtained with respect to each of the parameters, in turn, of the fitted function
(iii) equate the resulting partial derivatives to zero
(iv) solve the resulting set of (simultaneous) equations

These steps are followed for all functions, but the complexity of calculations increases rapidly as the number of terms in the equation increases. Even for the case of a simple linear regression, which may be performed on many pocket scientific calculators, the calculations would normally be performed by a computer with appropriate software.

As alluded to in 1.1.3, when the partial derivatives of nonlinear equations are calculated and equated to zero, the resulting equations cannot be explicitly solved to generate a single optimal value for each parameter. Intrinsically linear (or non intrinsically nonlinear) models can be transformed into a linear form which can be used for parameter estimation purposes. As indicated above this may be an inappropriate strategy if it leads to an unhomogeneous error, or non-normally distributed error, in the transformed response variable. A number of techniques are available for fitting nonlinear functions to data including the linearisation method (also called the Taylor series or Gauss-Newton method), and its various modifications, and the steepest descent method. These are briefly introduced by Draper and Smith (1981). Software is available commercially that incorporates these methods and which can be tailored to suit a particular modelling application but it is necessary to be aware of limitations of the methods and the software.

The ability of nonlinear regression to achieve convergence rapidly depends, in part, on good initial parameter estimates. The means by which parameter estimates are obtained will be aided greatly by having a model or parameterisation for which the parameters are interpretable. If good initial parameter estimates are not used the iteration process may converge slowly, or not at all, even though a solution may be available. Another potential problem with iterative procedures is that they may converge to a local, but not overall, minimum sum of squares value. A further problem, associated with the use of commercial software packages is that the standard errors of the parameter estimates are usually based upon so-called 'large sample-theory', but in most cases in biology and microbiology the sample size is not sufficiently 'large'. This may result in considerable bias in the parameter estimates, underestimation of the true variance in those estimates and, consequently, false confidence in the predictions of the fitted model.

2.4 VALIDATION AND MAINTENANCE

2.4.1 Validation Techniques

The value of a model ultimately rests on how well it can predict microbial responses under novel conditions that were not specifically tested for in the derivation of that model, i.e. how well it works in the real world. Methods to assess the predictive ability of a model using the data used to generate it, and also to compare the fit of different models to the same data set are discussed by Draper and Smith (1981). These methods may be used to validate a model based on data obtained at one time. If the data are collected over a longer time span the parameter estimates may change in time. This can be tested by constructing models from data taken over shorter time spans and considering the values of the fitted parameters as a function of time.

2.4.2 Laboratory to Field

The validation methods referred to above are useful for comparing 'goodness of fit', but the ability of laboratory generated models to predict accurately the behaviour of microorganisms of concern in foods in commercial preparation, storage and distribution must also be demonstrated before predictive microbiology models can be applied practically with confidence. Kilsby (1990, pers. comm.) expressed concern at the potential consequences of the premature use commercially of a predictive model. From the perspectives of consumer safety and confidence, and industry acceptance of the predictive microbiology concept, the result of failure of an invalidated model would be serious, and could lead to dismissal of the predictive microbiology concept as unreliable.

For models based on data derived from non-food systems, assurance that the model is applicable to food systems is essential, i.e. after the model has been developed it must be tested against observations in real foods. This has been approached in several ways.

Both the MAFF and USDA groups have tested their models' predictions against data from the literature (Buchanan, pers. comm.; Roberts, pers. comm.; Gibson *et al.*, 1988). Gibson *et al.* (1988) also tested predictions of a model based on the growth of Salmonellae in laboratory media against growth in slurries of minced defatted pork.

2.5 RATIONALISATION OF EXPERIMENTAL PROTOCOL

The following discussion outlines and explains the experimental strategy adopted in this study.

2.5.1 Response Variable

Section 1.1.5 addressed perceived limitations of predictive microbiology, including factors affecting lag times, the apparent need to know the initial inoculum, the

complexity of food systems, etc. A standard strategy to deal with the complex is to adopt a reductionist approach. In this work only the growth rate of the organisms of interest is modelled intensively. (Stochastic considerations will determine the transformation of the generation time/rate used as the response variable for the secondary and tertiary models). Factors, particularly history effects, which may potentially affect the duration of the lag phase of a microbial contaminant on a food are numerous, and may be specific to individual situations. Consequently, it is proposed to develop initially models quantifying growth rate responses only. Ultimately, lag phase data may be derived and incorporated. In the interim, for the development of tertiary models, provision will be made to allow the inclusion of lag phase information where it exists. Similarly, no assumptions are made about the inoculum levels in foods, but provision will be made to permit the inclusion of such information in tertiary models. It is proposed that this approach, used with strategies outlined in 1.1.5 will provide useful tertiary models in the short term, and a robust database which can be readily augmented when more information is available. To further simplify the work the range of the independent variables was restricted to the region between the lower limit and the optimum for growth.

2.5.2 Axenic or Mixed Culture ? Evaluation by Simulation

To resolve whether to model axenic or mixed strain cultures, the following simulation was undertaken. This treatment is concerned only with the simple case of a number of strains or species, each of which is growing exponentially.

The expression:

$$\text{relative growth rate} = \frac{\text{d(log[cell numbers])}}{\text{d(time)}}$$

is used for any bacterial population.

Consider the situation of two organisms, each with a unique specific growth rate, in a given environment. Based on the assumption of exponential growth, one can derive an expression for the apparent relative growth rate of the mixed population:

$$\text{relative growth rate} = \frac{\log N_{(t\,+\,\Delta time)} - \log N_{(t)}}{\Delta time}$$

substituting into the equation expressions for the increase in numbers of the sub-populations:

$$\text{relative growth rate} = \frac{\log\left(N_0^{\mathrm{A}}.2^{\Delta time/GTA} + N_0^{\mathrm{B}}.2^{\Delta time/GTB}\right) - \log\left(N_0^{\mathrm{A}} + N_0^{\mathrm{B}}\right)}{\Delta time}$$

where N_o^A and N_o^B are the initial densities at some time, *t*, of organism A and B respectively, GT_A and GT_B are the generation times in the hypothetical environment during exponential growth of organism A and B respectively, and $\Delta time$ is the time interval over which the growth rate is being determined.

Rearranging:

$$\text{relative growth rate} = \frac{\log\left(\frac{N_o^A.2^{\Delta time/GT_A} + N_o^B.2^{\Delta time/GT_B}}{N_o^A + N_o^B}\right)}{\Delta time}$$

$$\text{relative growth rate} = \frac{\log\left(\frac{N_o^A}{N_o^A + N_o^B}.2^{\Delta time/GT_A} + \frac{N_o^B}{N_o^A + N_o^B}.2^{\Delta time/GT_B}\right)}{\Delta time}$$

$$\text{let} \quad \frac{N_o^A}{N_o^A + N_o^B} = \rho^A \text{ and } \frac{N_o^B}{N_o^A + N_o^B} = \rho^B$$

then

$$\text{relative growth rate} = \frac{\log\left(\rho^A.2^{\Delta time/GT_A} + \rho^B.2^{\Delta time/GT_B}\right)}{\Delta \text{time}}$$

Using an analogous procedure to the above one may derive a general equation for the observed growth rate of a mixed culture containing n strains or species:

$$\text{relative growth rate} = \frac{\log\left(\rho^A.2^{\Delta time/GT_A} + \rho^B.2^{\Delta time/GT_B} + \ldots\ldots + + \rho^n.2^{\Delta time/GT_n}\right)}{\Delta \text{time}}$$

where ρ^A, ρ^B,.....ρ^n are the initial proportions of organism A, B,....., n, and GT_A, GT_B,.. .. ..,GT_n are the generation times during exponential growth of organism 1, 2,...., n, respectively, Δtime is the time interval over which the growth rate is being determined, and where

$$\rho^k = \frac{N_o^k}{N_0^A + N_o^B + \ldots\ldots\ldots\ldots + N_o^n}$$

where k = A, B,....., n

Thus, the observed rate of growth of the mixed population is a combination of the individual rates and the ratio of the sizes of the sub-populations. The ratio of the

sizes of the sub-populations will change with time, however, because those organisms with faster growth rates will increase as a proportion of the total population as a function of time.

Consider the situation of two organisms, A and B initially present in equal numbers in a given environment. Let them have generation times in that environment of 20 and 40 minutes, respectively. Table 2.2 shows how the apparent generation time of the mixed population calculated over successive 20 minute intervals falls toward that of the fastest growing strain.

This simple treatment can be extended to predict the behaviour of cocktails of strains/species under a variety of environmental conditions by substitution of appropriate models for the GT terms into the equation. As a general rule the initial rate observed is a weighted mean value of the individual rates under the conditions specified, but rises to that of the fastest growing strain as the duration of incubation increases. This hypothesis is supported by results of Hayward (1990) for the growth of six strains of *Aeromonas hydrophila*. Superimposition of the plots of square root models derived from axenic culture of each strain gave rise to a square root plot very similar to that for the growth of a culture containing all six strains initially in equal proportions. In practice, of course, there is a limitation to the amount of growth that can occur.

Table 2.2 The Apparent Generation Time for a Population Comprising Two Sub-populations with Generation Times of 20 and 40 Minutes Respectively

$\Delta time$ (min)	Numbers of A	Numbers of B	ρ^A	ρ^B	Total Population Size	Apparent GT (min)
0	1000	1000	0.50	0.50	2000	
10	1414	1189	0.54	0.46	2603	26.3
20	2000	1414	0.59	0.41	3414	25.9
40	4000	2000	0.67	0.33	6000	24.6
60	8000	2828	0.74	0.26	10828	23.5
80	16000	4000	0.80	0.20	20000	22.6
100	32000	5656	0.85	0.15	37656	21.9
120	64000	8000	0.89	0.11	72000	21.4
140	128000	11313	0.92	0.08	139313	21.0
160	256000	16000	0.94	0.06	272000	20.7
200	1024000	32000	0.97	0.03	1056000	20.3

Where mixtures of strains are used the fastest growing strain may be expected to dominate the population, i.e one might ultimately be modelling the growth of a single strain only. Thus, one attempts to model the 'worst case' under each set of conditions. It is a pragmatic approach but may produce meaningless results. Unless the preparation of mixed cultures is meticulously controlled both within and between experiments, an additional source of variability is introduced because a composite growth curve is generated. The contribution from each of the strains present will depend on the relative numbers present initially, their growth rate, and their lag time. Consequently, modelling of mixtures of strains is less likely to provide good resolution of fundamental microbial responses to environmental factors.

From the above it is concluded that the development of models from the study of axenic cultures is more consistent with the general reductionist philosophy being advocated, and will provide more fundamental data. It will be possible to predict the growth of mixed populations from models for the growth of the component species. The converse may not be possible.

2.5.3 Response Surface Design

A reductionist approach is advocated also for the experimental design. Reports (Chandler and McMeekin, 1989b, c; Davey, 1989a; Adams *et al.*, 1991) indicate that, for the independent variables considered in this work, interactions are insignificant. It is hypothesised that, in the absence of interactions the growth rate response in any dimension is affected only quantitatively by the values of the variables in the other two dimensions. That is, the qualitative nature of the response in any dimension is identical at all combinations of the other dimensions. (An obvious, but not trivial objection, to this assumption is that temperature, pH and water activity may act synergistically at the point where growth is prevented, *cf* those combinations where the rate is merely reduced). It follows, that by defining the response in any dimension at fixed levels of the other variables, it may be defined at all other levels simply by multiplication with a scaling factor. The value of the scaling factor is a function of the other variables. Further, it is proposed that the shape of growth response surface for the effects of pH, temperature and water activity may be unambiguously described by the product of the individual (qualitative) responses and that its position in n-dimensional space may be fixed by a scaling factor. This possibility is supported by the form of the square-root and Davey models in which the rate is a product of functions of temperature and water activity and/or pH and a constant (Chandler and McMeekin 1989b, c, Davey 1989a, Wijtzes *et al.*, 1993).

This possibility was recognised by Ross and McMeekin (1991). They considered that the first step in the construction of a model was the evaluation of the

effect of temperature and other environmental factors on the growth rate of the organisms of interest, and that this was best achieved by measuring rates at close intervals of the primary factor under consideration and for a number of levels of the second. They further speculated (McMeekin *et al.*, 1992) that, in the absence of significant interactions between these three variables, the combined effect of those factors may be described simply by accurate determination of three independent parameters.

These hypotheses will be investigated in this thesis, i.e. rather than adopting a set of variable combinations that are evenly distributed over the response surface, or concentrated in a particular region (e.g. the central composite design), the response to each factor will be described by determination of rates at many levels of each factor at a few levels of the other factors. Despite that this is a non-classical approach to the description of the response surface, it does fulfil many of the desirable properties of response surface designs listed in Table 2.1 and, when coupled with the prior knowledge of the non-interaction of the factors, does not appear to contravene any of them. Additionally, it is proposed that this approach will provide a better foundation for the interpretation of response mechanisms.

2.5.4 Experimental Model

The quantity of data required for the development of reliable models was considered in 2.2.9. Ross and McMeekin (1991) advocated the use of indirect methods so that the quantity of data could be maximised and that, for validation, this data be compared subsequently to a smaller data set obtained in real products for validation. It was also pointed out in 2.2.8 that broth systems offer several advantages for the construction of primary models.

The response variable chosen for primary modelling in this study is optical density of broth cultures. Optical density is a function of cell density, and the rate of change of optical density is thus a function of growth rate. Optical density may be rapidly and non-invasively determined with equipment readily available in most laboratories. These features reduce the variability between determinations, and permit many determinations to be made on the same culture, i.e. the system is data, but not labour, intensive. A number of potential difficulties arise in the use of indirect methods, however, which are discussed and addressed in 3.2.

2.5.5 Other Factors

The preparation and handling of inocula are described in Chapters 4 and 5. Consistent with the proposal of Ross and McMeekin (1991), it is considered that validation to novel data must be undertaken. Evaluation of laboratory derived models will be by comparison with novel data obtained by inoculation of the modelled organisms into a variety of food products under a range of storage

temperatures. To augment this validation process, model predictions will also be compared to data from the literature.

3 PRIMARY MODELLING

3.1 SELECTION OF A SIGMOID FUNCTION TO DESCRIBE THE RESPONSE

3.1.1 Introduction

Sigmoid functions fitted to bacterial growth curves provide an objective and reproducible means to analyse those data. Recent developments in the quest for suitable functions were reviewed in 1.2. The functions may be manipulated mathematically to yield formulae which describe the growth rate and lag phase duration in terms of their traditional interpretation, i.e. the fastest rate is based on the steepest tangent to the growth curve, and the lag time is taken as the x-coordinate (i.e. time) of the point on this line at which the y coordinate ($\mathrm{Log}N_{(t)}$) is equal to $\mathrm{Log}N_{(0)}$. These interpretations are illustrated in Fig. 3.1. The origin and application of sigmoid functions used in predictive microbiology were discussed in detail by the candidate in McMeekin *et al.* (1993) Chapter 2.

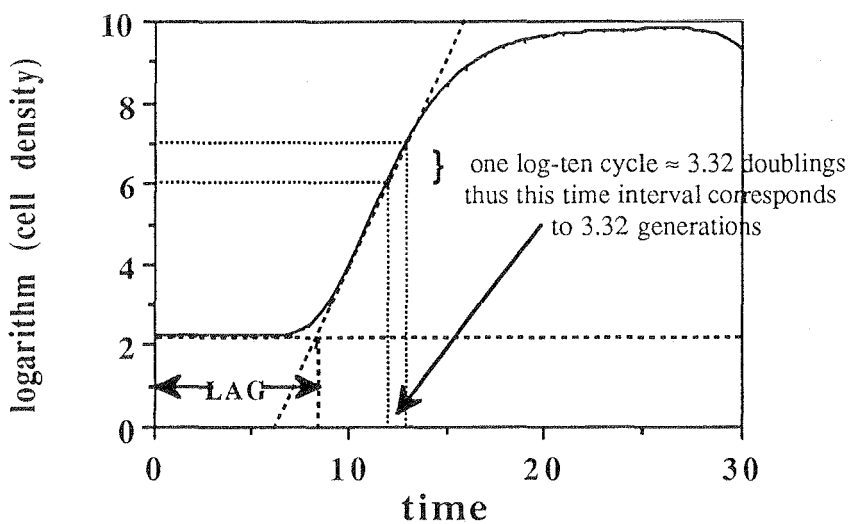

Fig. 3.1 A graphical method for the estimation of generation and lag time from a bacterial population growth curve. The slope of the tangent to the steepest part of the curve estimates exponential growth rate. The generation time can be calculated from this tangent as the time for a 0.301 unit increase in log(cell density), i.e. a doubling of the population. The intercept of this tangent with the initial inoculum level (i.e. $\log N_{(0)}$) is taken as the end of the lag phase.

The function which has gained most prominence is the modification of the Gompertz (1825) function (modified-Gompertz) introduced by Gibson *et al.* (1987), although recent reports indicate shortcomings of this model (Garthright, 1991; Whiting and Cygnarowicz, 1992; Baranyi *et al.*, 1993; McMeekin *et al.*, 1993; Dalgaard *et al.*, in press).

Of other models proposed to describe the bacterial growth curve the logistic function has most appeal because of its mechanistic basis. It has also been shown to describe well the growth of bacterial populations assessed by conductimetric methods (Jason, 1983) and radioassay of respired CO_2 (Boonkitticharoen *et al.*, 1989). A practical limitation of the logistic function is that it describes the exponential and stationary phases of growth only, i.e. it does not model a lag phase. This problem was addressed by Dalgaard (1993), who reparameterised it to enable the lag phase to be modelled also. Mechanistic models to describe the entire bacterial growth curve have been presented (Whiting and Cygnarowicz, 1992; Baranyi *et al.*, 1993) but, because of technical difficulties in their implementation and routine use, were not considered in this comparison.

This section describes the results of a study in which generation time estimates obtained by fitting the modified-Gompertz and Dalgaard's logistic function to simulated VC-based growth curve data were compared.

3.1.2 Theory

3.1.2.1 Modified-Gompertz function

The modified-Gompertz function introduced by Gibson *et al.* (1987) may be written as:

$$\text{Log}\, N_{(t)} = A + D\,(\exp(-\exp(B(M - t)))) \qquad (3.1)$$

where Log refers to Log_{10}, A is value of the lower asymptote [i.e. $\text{Log}N_{(-\infty)}$], D is the difference in value of the upper and lower asymptote [i.e. $\text{Log}N_{(\infty)} - \text{Log}N_{(-\infty)}$], B is a parameter related to the slope of the curve and M can be shown to be the time at which the tangent to the curve is steepest, and which corresponds to $\text{Log}N = A + D/e$.

The fitted function can be manipulated to yield the kinetic parameters of the culture such as doubling time, lag time or time to reach a specified level (*see* McMeekin *et al.*, 1993, p. 80). From the first and second derivatives of the equation, expressions for the maximum exponential growth rate may be derived, and from this the minimum generation time.

$$\text{steepest tangent to Eqn. 3.1} = BD/e \qquad (3.2a)$$

$$\Rightarrow \quad \text{minimum generation time}(t_{G_{min}}) = e \log 2/BD$$

$$= 0.8183/BD \qquad (3.2b)$$

3.1.2.2 The Dalgaard (1993) modification of the logistic function

A mechanistic basis for the logistic function is described by Causton (1977) and was derived again *de novo* by Jason (1983) using impedance data for the growth of *E. coli*. It may be written:

$$N_{(t)} = \frac{\text{MPD}}{1+\exp\{\mu_{\max}(\text{M}-t)\}}$$

where $N_{(t)}$ is the number of cells at time t, MPD is the maximum population density, $\mu_{\max}$ is the maximum specific growth rate, and M is the time at which the number of cells equals MPD/2.

The specific growth rate, μ, of the culture is observed to decrease as the population approaches the maximum population density (MPD). Consequently μ must have its maximum theoretical value, $\mu_{\max}$, when the population is at infinite dilution, i.e. at $t = -\infty$. The change in the observed rate, μ_{obs}, is modelled by the expression:

$$\mu_{obs} = \mu_{\max}\left[\frac{\text{MPD}-N_{(t)}}{\text{MPD}-N_{(0)}}\right] \qquad (3.4)$$

To use the logistic function to describe bacterial growth it is necessary first to determine the end of the lag phase and then to apply the function to the data representing growth after this time.

Dalgaard (1993) sought to modify the logistic function to enable it to model the lag phase also, by inclusion of an additive parameter to represent $N_{(0)}$. His parameterisation may be written:

$$\text{Log}N_{(t)} = \text{Log}\left[\text{A}+\frac{\text{D}}{1+\exp\left\{\frac{0.693}{t_{G\min}}(\text{M}-t)\right\}}\right] \qquad (3.5)$$

where Log refers to Log_{10}, A and D are as previously defined, M is a parameter to be estimated, and $t_{G\min}$ is interpreted as the minimum generation time of the culture.

No mechanistic development was given for this modification, hereafter called the modified-Logistic. The consequences for the original interpretation of the logistic function parameters due to this reparameterisation are not described but it is questionable whether the original mechanistic basis and parameter interpretation remain valid.

The logistic function describes the increase in absolute numbers of cells. The error in estimates of cell numbers is homogenous in Log*N* (Jarvis, 1989, p.36; Dalgaard *et al.*, in press). Accordingly, both sides of Eqn. 3.5 are log-transformed for fitting to data.

Because of literature reports (*see* 1.2) comparing the slope estimate from the modified-Gompertz function to other sigmoid functions which are based on exponential growth, it was also considered desirable to estimate the steepest slope to Eqn. 3.5. In a manner analogous to that used to determine growth rate expressions based on fitted modified-Gompertz curves, Eqn. 3.6 based on Dalgaard's logistic function was derived.

$$slope_{\max} = \frac{\log_{10} e\left(\left(\frac{\ln 2}{t_{G_{\min}}}\right)\times\left(D\times\sqrt{\frac{A+D}{A}}\right)\right)}{\left(A+D+\sqrt{\frac{A+D}{A}}\right)\times\left(1+\sqrt{\frac{A+D}{A}}\right)} \qquad (3.6)$$

3.1.3 Methods

An idealised bacterial growth curve (Log(cfu) *vs.* time) was devised. For calculation purposes the curve comprised ten points: three points at equal time intervals representing the lag phase, six points at equal time intervals representing the exponential phase, and three points at equal time intervals representing the stationary phase (the points representing the intercept of the exponential and lag phase, and the exponential and stationary phases were common to two growth phases). To obviate assumptions about the shape of the growth curve in the acceleration and deceleration phases, the intercept points were omitted from the data set used for curve fitting. The basic data set is represented in Fig. 3.2. Simulated datasets based upon this pattern were used to test the effect of the range of values of Log*N* (i.e. D) and the ratio of the duration of the exponential and lag phases (exp./lag). In all data sets the slope of the rising part of the curve was one. In consequence the 'true' generation time is 0.30103, i.e. $\text{Log}_{10}2$. Eqns. 3.1 and 3.5 were fitted to the datasets by Ultrafit[1] nonlinear regression software without data weighting. The parameter estimates and their 95% confidence limits were recorded, and the steepest slope and generation time estimates of each model calculated based on the fitted values.

3.1.4 Results

The datasets used are presented in Appendices 1.1-20, and the fitted results summarised in Appendix 1.21. Parameter estimates of subsets of those data in which i) exp/lag is held constant (= 4) while D varies, and ii) D is held constant (= 8) while exp/lag varies are presented in Tables 3.1 and Table 3.2 respectively.

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[1]Ultrafit: BIOSOFT® 49 Bateman St., Cambridge, CB2 1LR, UK.

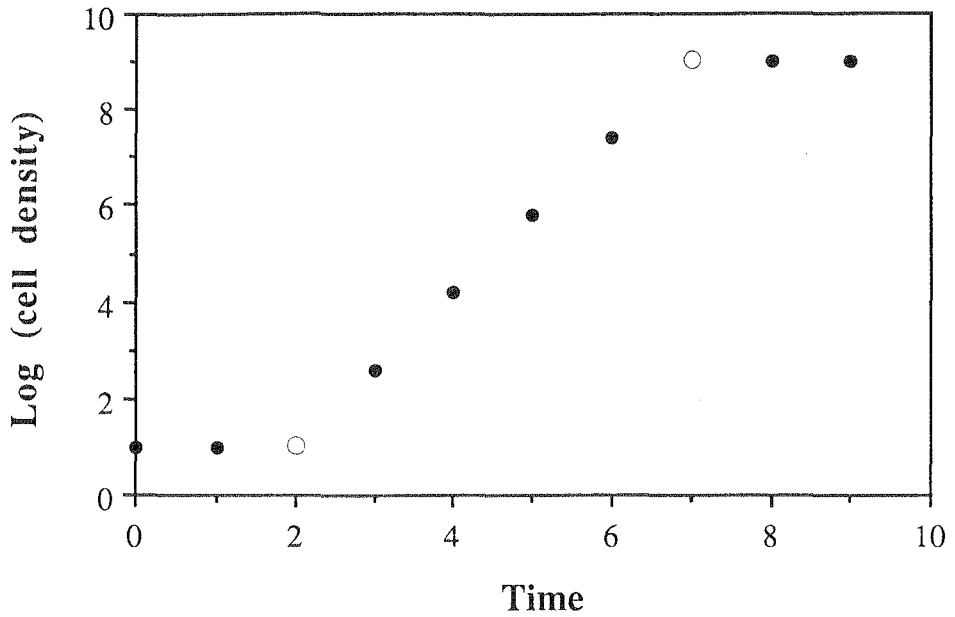


Fig. 3.2 Pattern of data used to generate simulated growth curves. The points (●) comprised the dataset; the 'imaginary' points (○) were used for calculation purposes only, but indicate the end points of the lag and exponential phases.

Table 3.1 The Effect of the Relative Duration of Lag and Exponential Phases on Growth Rate Estimates Derived from the Fitted Parameters of Eqns. 3.1 and 3.5, (D = 8).

Exp./Lag	Generation Time		Steepest Tangent	
	Eqn. 3.1	Eqn. 3.5	Eqn. 3.1	Eqn. 3.5
13.333	0.318	0.298	1.054	1.010
4.000	0.335	0.299	1.111	1.005
1.330	0.345	0.300	1.144	1.005
0.400	0.347	0.300	1.151	1.004
0.040	0.357	0.300	1.152	1.004

Fig. 3.3 shows that there is a systematic variation in the estimation of generation time when using the modified-Gompertz equation to describe the growth curve. There is also slight variation in the estimates derived from Eqn. 3.5, the estimates being close to the true value of 0.301. Eqn. 3.2b consistently overestimated the generation time. Similar results are obtained if one considers the slope estimates (i.e. Eqns. 3.2a and 3.6).

Table 3.2 and Fig. 3.4a demonstrate the effect of the range of the response variable on estimates of generation time derived from Eqns. 3.2b and 3.5. Generation time estimates derived from the modified-Logistic function are significantly affected by the extent of growth of the culture. Figure 3.4b demonstrates the effect of the range of the response variable on the slope of the steepest tangent to the curve modelled by the two functions. Although the steepest slope of the modified-Logistic function is closer to the true value, the variation in estimates of that slope is greater than that for estimates of the slope of the modified-Gompertz function. Data sets with extreme values of D and exp./lag were deleted from the full data set to create a data set more representative of a range of 'typical' growth curves. The slope and generation time estimates of the reduced set are shown in Table 3.3. The means and standard deviations in that table show that estimates from Eqn. 3.6 are closer to the true value, but that the variability of the estimates is greater than that obtained by Eqn. 3.2a. Eqn. 3.2a consistently overestimates the true slope by approximately 11 - 15%, whereas Eqn. 3.6 gives estimates close to the true value.

Table 3.2 The Effect of the Extent of Growth on Growth Rate Estimates Derived from Fitted Parameters of Eqns. 3.1 and 3.5, (Exp./Lag = 8).

D	Generation Time		Steepest Tangent	
	Eqn. 3.1	Eqn. 3.5	Eqn. 3.1	Eqn. 3.5
8.0	0.335	0.299	1.111	1.005
5.0	0.335	0.292	1.111	1.025
3.0	0.335	0.268	1.111	0.918
1.0	0.335	0.154	1.111	1.015
0.1	0.335	0.018	1.111	0.978

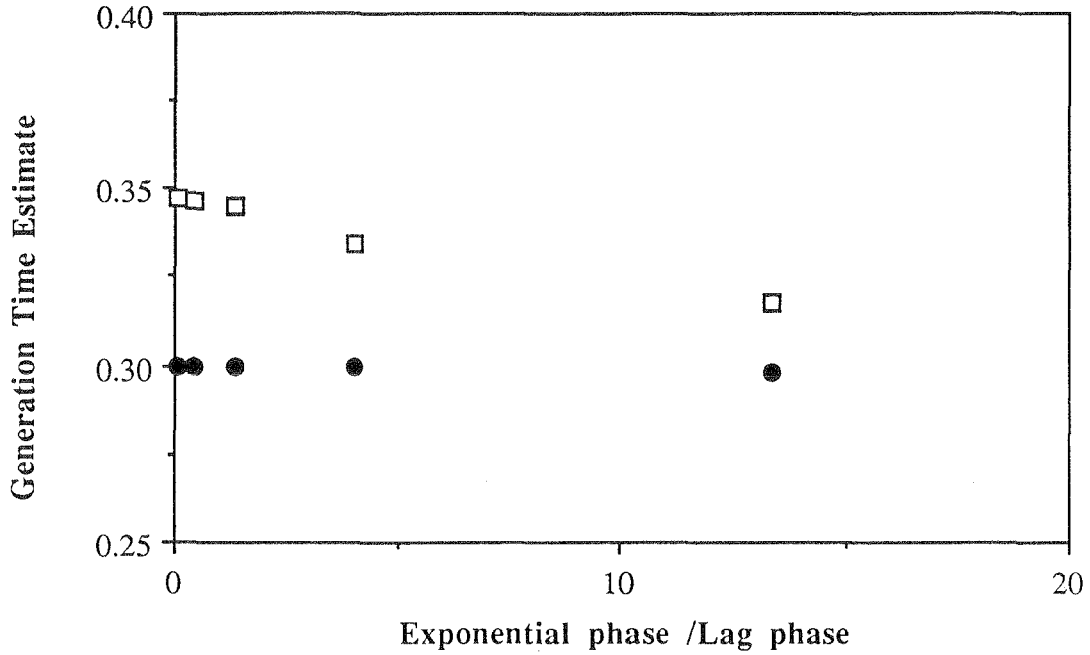


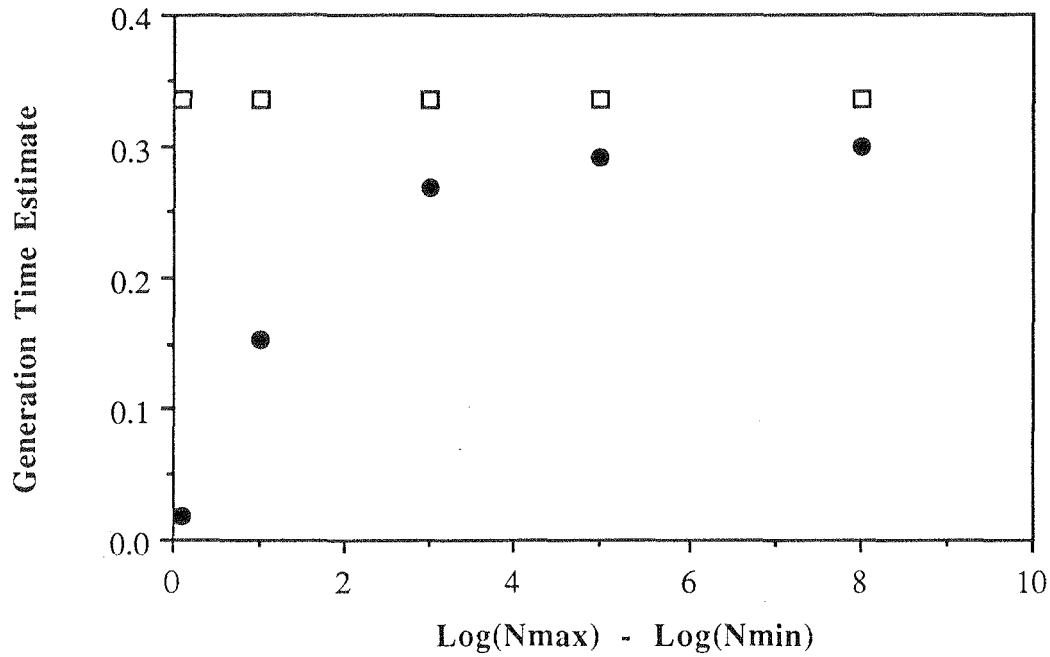
Fig. 3.3 The effect of the relative lengths of the lag and exponential phase on estimates of generation time derived by Eqns. 3.1 (□) and 3.5 (●).

It was noted (results not shown) that, for all data sets, goodness-of-fit indices and the behaviour of residuals suggested that Eqn. 3.5 fitted the data better than Eqn. 3.1. In addition, the upper and lower asymptotic values of the data sets were consistently estimated more accurately by the modified-Logistic function (results not shown).

3.1.5 Discussion

From an empirical perspective Eqn. 3.5 better describes the idealised bacterial growth curves used in this simulation than does Eqn. 3.1. Earlier experience had shown it to fit more closely experimental data sets for bacterial growth curves also, but inconsistencies were detected between the estimates of t_G from Eqn. 3.5 and those derived by the traditional methods described in 3.1.1, namely that the $t_{G\min}$ estimate decreased as the growth range decreased. These inconsistencies were commented upon by Dalgaard *et al.* (in press).

a.



b.

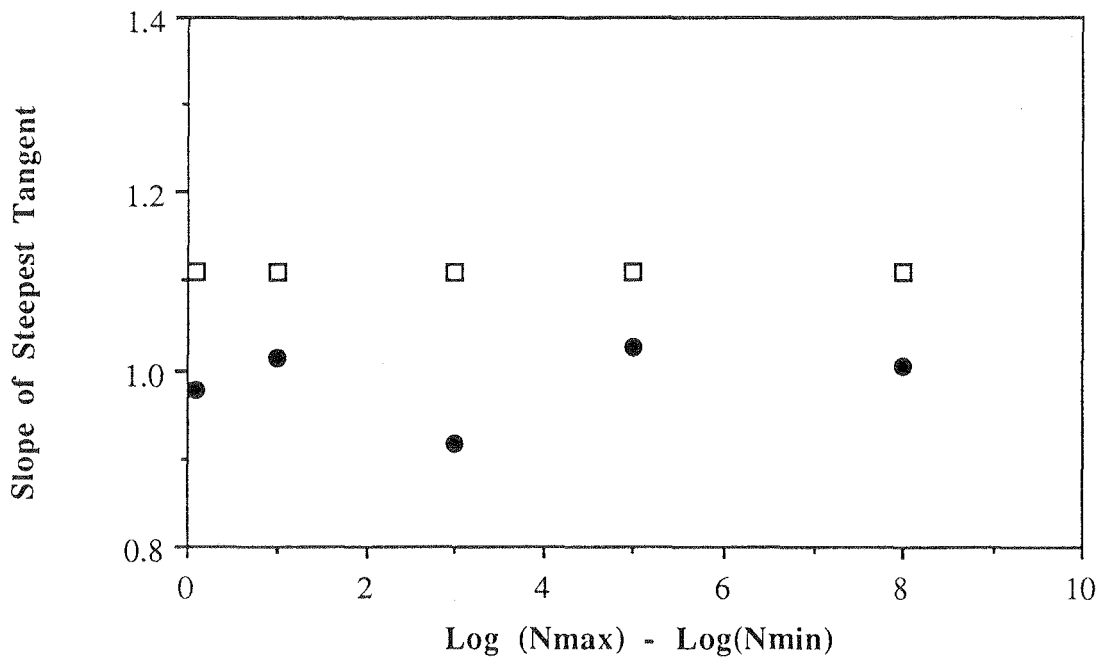


Fig. 3.4 The effect of the extent of growth, D (= LogN_{max} - LogN_{min}) on kinetic parameters based on fitted values of Eqns. 3.1 (□) and 3.5 (●). a) generation time; b) slope of steepest tangent.

Table 3.3 The Effect of the Relative Duration of Lag and Exponential Phases on Kinetic Estimates Based on Eqns. 3.1 and 3.5.

D	Exp./Lag	Generation Time		Steepest Tangent	
		Eqn. 3.2b	Eqn. 3.5	Eqn. 3.2a	Eqn. 3.6
8.0	4.000	0.335	0.299	1.111	1.005
8.0	1.330	0.345	0.300	1.144	1.005
8.0	0.400	0.347	0.300	1.151	1.004
8.0	0.040	0.347	0.300	1.152	1.004
7.0	3.500	0.337	0.298	1.116	1.008
6.0	3.000	0.338	0.296	1.121	1.014
5.0	2.500	0.339	0.293	1.127	1.022
4.0	2.000	0.342	0.285	1.134	1.036
3.0	1.500	0.344	0.267	1.142	1.058
2.0	1.000	0.346	0.227	1.147	1.085
5.0	4.000	0.335	0.292	1.111	1.025
3.0	4.000	0.335	0.268	1.111	0.918
			Mean:	1.131	1.015
			SD:	0.0163	0.0396

This comparison of the two models has confirmed those inconsistencies but the results presented in Table 3.3 indicate, *prima facie*, that the parameter interpretations of the logistic model are invalidated by Dalgaard's reparameterisation. It is possible, however, that in those data sets representative of small growth ranges the data may not have reflected the true shape of the growth curve, i.e. such growth curves may not be observed in nature. This possibility was addressed by reference to Eqn. 3.4, which was used to calculate ranges of population densities above which the slope of the growth curve would be expected to differ by more than 5% from μ_{max}. Some of the simulated data sets did include values which would not be expected in nature, i.e. the simulated exponential phase extended beyond that which would be consistent with a logistic response. Even when these values were removed and replaced with values that fall within the expected 'straight line' region of growth, the $t_{G_{min}}$ estimates were still less than that obtained by assessment of the steepest slope. This observation, and consideration of the implications of the fitted values for the shape of the growth curve, suggests that the

inclusion of the A parameter affects the estimation of $t_{G\min}$ of Eqn. 3.5 so that the estimate represents the fastest specific growth rate only if the extent of growth represents many log cycles.

Consistent with the 'worst case' philosophy advocated in Chapter 2, the primary objective for this work is to identify a reliable method for the determination of the fastest growth rate and to derive kinetic models from that basis. Both Eqns. 3.1 and 3.5 provide good estimates of the slope. It was concluded that the modified-Gompertz function was preferable, i) because of its slightly lower variation, and ii) for pragmatic reasons, i.e. the modified-Gompertz is widely used and manual calculation of the expression describing the maximum slope is simpler. The consequences of the overestimation of the true slope by the modified-Gompertz function must, however, be addressed.

It is proposed that Eqn. 3.2a be modified by the inclusion of a factor to compensate for the overestimation. Other workers have reported that this overestimation is of the order of 10-20% (Whiting and Cygnarowicz-Provost, 1992; Baranyi *et al.*, 1993), based on experimental growth curve data. Dalgaard *et al.* (in press) found that Eqn. 3.1 produced slope estimates on average 12% greater than Eqn. 3.5. From the results in this chapter a value of 1.131 is found. Consequently, the following expression is proposed to describe the true steepest slope of a growth curve from the fitted parameters of Eqn. 3.1:

$$slope_{\max} = \frac{\mathrm{BD}}{1.13e} \tag{3.7}$$

Thus, for Log(CFU) data:

$$\begin{aligned} generation\ time &= \frac{e\log 2 \times 1.13}{\mathrm{BD}} \\ &= \frac{0.925}{\mathrm{BD}} \end{aligned} \tag{3.8}$$

Without this correction the true generation time will be underestimated, and will lead to falsely high predictions of microbial loads when generation time estimates are used to calculate population increases according to models based on exponential growth. Whilst this establishes a 'fail safe' rather than 'fail dangerous' situation, it may result in unnecessary product wastage.

These results also have significance for the 'response surface' modelling approach, in which the entire curve is modelled as a function of predictor variables. It is appropriate, thus, to employ a model which accurately fits the entire growth curve. The pattern of residuals of fitted modified-Gompertz models shows systematic deviations. Eqn. 3.6 provided a consistently better fit to simulated and experimental

data (results not shown). The results of Dalgaard *et al.* (in press) and Baranyi *et al.* (1993) indicate that models based on exponential growth better describe genuine growth curve data than does Eqn. 3.1. The systematic lack of fit of the modified-Gompertz function was also commented upon by Whiting and Cygnarowicz-Provost (1992). Eqn 3.5 may be an appropriate basis for 'response surface' models.

Another undesirable consequence of the overestimation by Eqn. 3.1 of the steepest tangent to the growth curve is that it leads to biased estimates of lag phase duration. If the steepest tangent to the curve is overpredicted, the lag phase will be overpredicted (*see* Fig. 3.1). The significance of this error will depend upon the duration of the lag phase: if the lag phase is long, the error may be small relative to the lag phase duration. Conversely if the lag phase is short, the magnitude of the error could equal or exceed that of the true lag phase duration. This error will have particular significance in modelling approaches in which lag phase duration is modelled discretely. This observation may also account for some of the reported variability in lag phase estimates. A 'corrected' lag phase expression is now derived.

In McMeekin *et al.* (1993) the candidate developed an expression for the lag phase duration based on an interpretation consistent with that depicted in Fig. 3.1. The lag time is given by the time coordinate of the point on the steepest tangent which corresponds to $\mathrm{Log}N_{(0)}$:

$$Lag\ \ time = \mathrm{M} - \frac{1}{\mathrm{B}}(1 - \exp(1 - \exp(\mathrm{BM}))) \qquad (3.9)$$

The full derivation of this equation is given in McMeekin *et al.* (1993), Appendix 2A.8, from which it can be seen that the analogous expression, corrected for the overestimation of the rate, is:

$$Lag\ \ time = \mathrm{M} - \frac{1.13}{\mathrm{B}}(1 - \exp(1 - \exp(\mathrm{BM}))) \qquad (3.10)$$

Eqn. 3.9 differs from the expression presented by Gibson *et al.* (1987) and subsequently presented by other workers (Buchanan *et al.*, 1989; Buchanan and Klawittter, 1991; Garthright, 1991). Eqn. 3.9 is a more general and correct expression for the lag time. When $\mathrm{A} = \log N_{(0)}$, Eqn. 3.9 reduces to the expression presented by Gibson *et al.* (1987). The use of Eqn. 3.9 eliminates the problem of negative values of lag time that may occur using the earlier expression (Buchanan and Cygnarowicz, 1990; Garthright, 1991). Negative lag times are only predicted by that earlier expression when $\mathrm{Log}N_{(-\infty)}$ is not well approximated by A, i.e. when there is no initial period during which the population density is static. For those cases Garthright (1991) suggested that the lag time be interpreted as zero, yet the lag time estimated by the traditional method always includes some period during which the

population is increasing. A novel interpretation of the lag phase is now proposed to support the more traditional approach.

The lag time is generally taken to represent a period of adjustment by cells to a new environment. If all cells in the population resolved the lag phase simultaneously one would expect an abrupt transition between lag phase and the exponential growth phase. An abrupt transition is seldom observed. If the resolution of lag phase does not occur synchronously, it follows that there will be a distribution of times required for the resolution of lag by individual cells within the population. Thus, for a population, the observed 'lag time' may be interpreted as the average response of the entire population.

The consequences of a distribution of lag times on the observed increase in numbers, and the effect on the observed rate of growth, may be appreciated by reference to the discussion and simulations presented in 2.5.2. It is apparent that even if a subpopulation were replicating at the μ_{max} of that organism, the observed growth rate of the *population* would be much lower due to the presence of non-replicating cells. Similarly, the exponential growth of the cells which resolve the lag phase most rapidly will significantly affect the observed lag time of the population because they will come to dominate that population. Thus, if the deviation of lag times about the mean is wide, the measured lag time may be shorter than the mean lag time of the population initially present.

It follows that the lag phase duration of a population will have an uncertain relationship, and physiological interpretation, to that of individual cells within the population. In this interpretation the 'lag time' of a population is affected by the mean time for lag resolution of the individual cells, the rate at which the cells grow once the lag phase is resolved, and the range of the distribution of responses. This interpretation of the lag phenomenon is consistent with the approach of McMeekin *et al.* (1993) and that taken here to develop Eqn. 3.10 and also offers some insight into the observed variability of lag time responses.

3.2 DEVELOPMENT OF A TURBIDIMETRIC METHOD FOR DOUBLING TIME DETERMINATION

3.2.1 Introduction

The advantages of turbidimetric methods for growth rate determination were described in 2.2.8. There are, however, a number of realised and potential problems inherent in their use. Firstly, for measurements on dense cultures there is a deviation from the response predicted by Beer's Law[2] resulting in falsely low estimates of cell density. This curvilinear response requires that, in order to obtain accurate estimates of cell density, samples must be diluted to ABS<0.3 (Koch, 1981), or that the

[2] Beer's Law: that absorbance is proportional to concentration.

observed responses be 'corrected' by reference to some function relating the observed to the true absorbance. Secondly, the lower sensitivity limit of turbidity measuring devices is usually such that they are unable to detect bacterial populations at densities less than ~10^7cfu/ml. If, at those high cell concentrations, the specific growth rate is significantly less than that observed at lower cell densities more sensitive methods, such as viable counts, might yield higher estimates of growth rate.

Koch (1981) stated that "the important point about optical density methods is that there is no set procedure" and further that "one uses the equipment at hand and seeks to obtain useful results". Although absorbance is the usual response variable used to monitor culture growth by turbidimetry, percent transmittance (%T) is measured on a linear scale, and is easier to read when using analogue instruments (i.e. "the equipment at hand"). This section describes the development of an objective and robust method for the determination of growth rates from the change in %T of growing cultures, and following Koch's pragmatic advice.

3.2.2 Theory

Absorbance is by definition related to %T as:

$$\text{Absorbance} = 2 - \log_{10}(\%T) \tag{3.11}$$

Over a finite range, absorbance is proportional to concentration. Thus, generation time may be determined from the time taken for the absorbance of a culture to double. Consequently it is easier to plot log(absorbance) against time and determine generation time from the time taken for log(absorbance) to increase by log(2), i.e. 0.301.

The relationship between log(absorbance) and %T is shown in Fig. 3.5. In the range 20% to 60% transmittance (Absorbance 0.70 to 0.22), the relationship between log(absorbance) and %T is well described ($r^2 > 0.999$) by the straight line:

$$\log(\text{absorbance}) = 0.089525 - 0.012293\ \%T$$

From the fitted line it is possible to derive a relationship between generation time and the rate of change of %T in the range 20 - 60 %T:

$$\text{slope of regression line} = -0.012293$$
$$= \text{change in log(absorbance)/change in \%T}$$

Thus, for one generation:

$$\text{change in \%T/generation} = -0.301/0.012293$$
$$= -24.5 \tag{3.12}$$

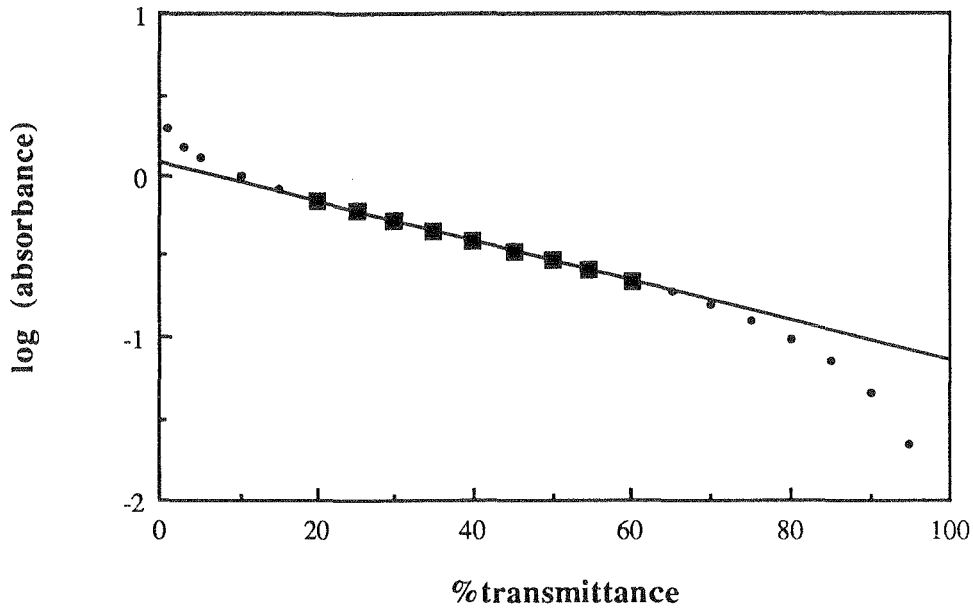


Fig. 3.5 Relationship between log(absorbance) and %T (●). In the region indicated by (■) the relationship is:
log(absorbance) = 0.089525 - 0012293 (Δ%T) [$r^2 > 0.999$].

i.e. in the specified range a population doubling corresponds, in theory, to a 24.5% decrease in transmittance.

The %T of a growing culture decreases with time and produces a sigmoid curve when plotted as a function of time. Choosing change in %T (%T of the uninoculated culture vessel prior to inoculation *minus* %T observed at time *t*) as the response variable produces a rising sigmoid curve. In 3.1 it was demonstrated that a Gompertz-like function could be used empirically to describe the steepest slope of a sigmoid curve. In this section the ability of a Gompertz-like function to describe the bacterial growth curve based on change in %T (Δ%T) data is assessed.

By analogy with Eqn. 3.1 it is hypothesised that the following function:

$$\Delta\%T_{(t)} = A + D\,(\exp\,(-\exp(\,B(M - t)))) \qquad (3.13)$$

where $\Delta\%T_{(t)}$ is the change in %T after time t; A is the value of the lower asymptote [i.e. $\Delta\%T_{(-\infty)}$]; D is the difference in value of the upper and lower asymptote [i.e. $\Delta\%T_{(\infty)} - \Delta\%T_{(-\infty)}$)], and M is the time at which the tangent to the fitted curve is steepest.

may be used to describe the Δ%T growth curve. By analogy with Eqns. 3.2 a,b, and by reference to Eqn. 3.12, the generation time at M is given by the expression:

$$\text{generation time}_{(\Delta\%T)} = 24.5e/BD \qquad (3.14)$$

3.2.3 Methods and Materials

3.2.3.1 Relationship between change in %T and population density

The %T (540nm) of doubling dilutions of a stationary phase (>2d @ 37°C, BHIB) culture of *Staphylococcus aureus* 3b, prepared in 0.1 % peptone and in BHIB with varying levels of added salt, were prepared. The %T (540nm) of dilutions of a stationary phase culture (1d @30C + 10d @20°C) of *Listeria monocytogenes* Scott A, prepared in TSB, were also recorded. All diluents and cultures were kept in an ice water slurry for the duration of the determinations. The reagent blank (100% transmittance) value was that of the relevant diluent.

3.2.3.2 Deviation from Beer's Law

Listeria monocytogenes Scott A was grown in TSB at 30°C until at stationary phase, and held at room temperature for a further 10 days. The culture was placed in an ice slurry and aliquots withdrawn and diluted with chilled TSB. Absorbance (540nm) of the dilutions was recorded, and absorbance of the undiluted sample (ABS) calculated as the product of the absorbance of the diluted sample (ABS_{dil}) and the dilution factor. Corresponding values of corrected %T were calculated from ABS using Eqn. 3.11.

3.2.3.3 Relationship between wavelength and generation time estimate

Listeria monocytogenes Scott A in TSB was incubated in side-arm flasks in a shaking water bath (Haake) at 31 ± 1°C. During growth the %T of the culture was recorded at 620, 580, 540, 500, 460 and 420 nm. The full scale deflection and zero of the spectrophotometer (Spectronic 20D) were adjusted before each reading to an uninoculated flask containing TSB of identical absorbance to that in the culture. Generation times at each wavelength were determined from fitted values of Eqn. 3.13.

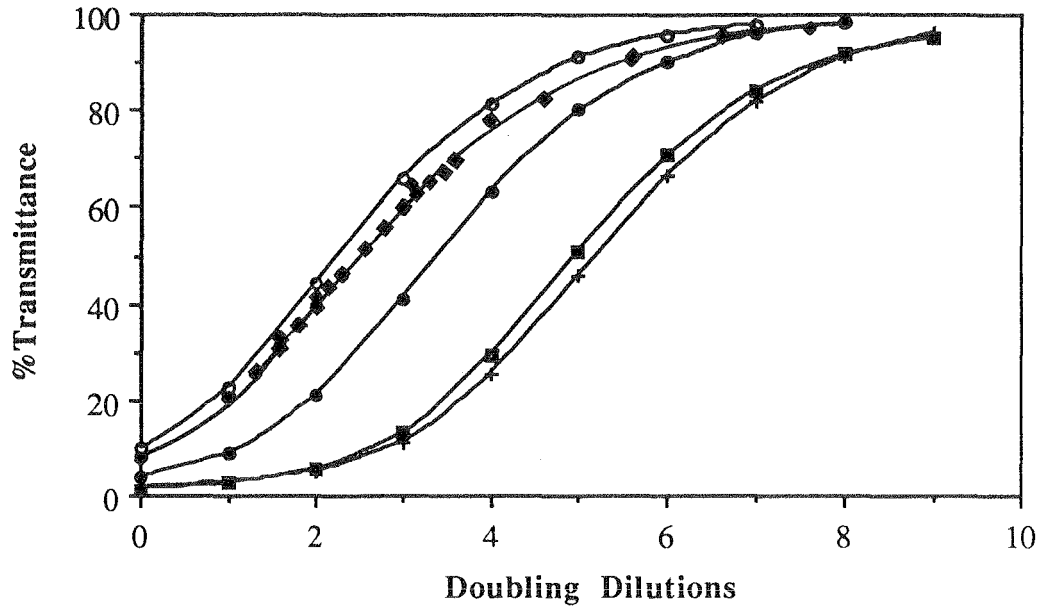


Fig. 3.6 Relationship between change in cell density and optical density, and showing the effect of medium composition. Data for *Staphylococcus aureus* 3b. ○: grown and diluted in BHIB + 16%w/w NaCl; ●: grown and diluted in BHIB + 13%w/w NaCl; ■ grown and diluted in BHIB; +: grown in BHIB and diluted in 0.1% peptone. Data for *Listeria monocytogenes* Scott A: ◆. Lines drawn through points are by interpolation using Cricket Graph[3]

3.2.3.4 Evaluation of Eqn. 3.13

Growth curves (n = 288) for *Staphylococcus aureus* 3b, grown in BHIB supplemented with up to 20%w/w NaCl and at temperatures from 10 - 40°C, were derived as described in 4.1. Data were manually plotted and the generation time estimated visually from the steepest tangent to the curve as described in Fig. 3.1. Eqn. 3.13 was fitted subsequently to the same data and the generation times estimated from fitted values. Estimates from both methods were compared. In addition, the slope estimated from Eqn. 3.13 was compared to that estimated by linear regression of points within the 20-60%T range for a number of *Listeria monocytogenes* Scott A growth curves.

[3] CA-Cricket Graph™ 1.3.2. Computer Associates Intl., Malvern, PA, USA.

3.2.4 Results

Dilution curves, presented in Fig. 3.6, confirm that in the range 20% T to 60%T there is a direct proportionality between cell doubling and Δ%T. For the five datasets, however, in that range a doubling of cell numbers corresponds to an ~20.5% (±0.5% SD) increase in transmittance compared to the theoretical value of 24.5% (Eqn. 3.13). The data also show that the relationship is independent of the composition of the suspending medium, or bacterium.

Fig. 3.7 shows both the raw and corrected values for %T derived from *Listeria monocytogenes* Scott A. The slope of the data in the 20 - 60%T range is 19.67 (r^2 = .997) i.e. a generation in that range corresponds to a decrease of 19.67 in raw %T. For the corrected data, a population doubling was found to correspond to a 24.32% change in transmittance.

Table 3.4 shows that there is no effect of the wavelength of light used for measurement of %T on the estimates of generation times obtained by fitting Eqn. 3.13. It was noted, however, that if a reagent blank of different optical density to the growth medium were used, the predicted generation times were significantly and systematically affected by the wavelength of light used for the determinations. A correction factor, based on the ratio of the optical densities of the two uninoculated media, was sufficient to overcome the apparent wavelength effect. The effect results simply from the different Δ%T which corresponds to a doubling of concentration if the full scale deflection of the spectrophotometer, based on the reagent blank, is set at other than 100%T.

Fig. 3.8 compares estimates of the generation time of cultures determined from Δ%T evaluated by manual methods and using Eqn. 3.14. The results are presented as the ratio of the estimates by the two methods. The mean value of the ratio is 0.993 ± 0.136 (SD), and regression analysis of the data in Fig. 3.8 indicated no significant relationship (r^2 = 0.0021) between the ratio of the two estimates as a function of generation time. Table 3.5 shows the slope estimated from Eqn. 3.13 and that estimated by linear regression of points within the 20-60%T range for *Listeria monocytogenes* Scott A growth curves. Consistent with observations in 3.1, the slope of the %T growth curve is overestimated by the Gompertz-like function, and by a similar amount. The increase in Δ%T in that range is well described by a straight line, whereas the residuals plots indicated a systematic lack of fit of Eqn. 3.13 to Δ%T data.

3.2.5 Discussion

The results show that %T data can be used to estimate the generation time of a bacterial culture, and that description of the Δ%T data by a Gompertz-like function provides an objective means of growth rate estimation from that data. The theoretical

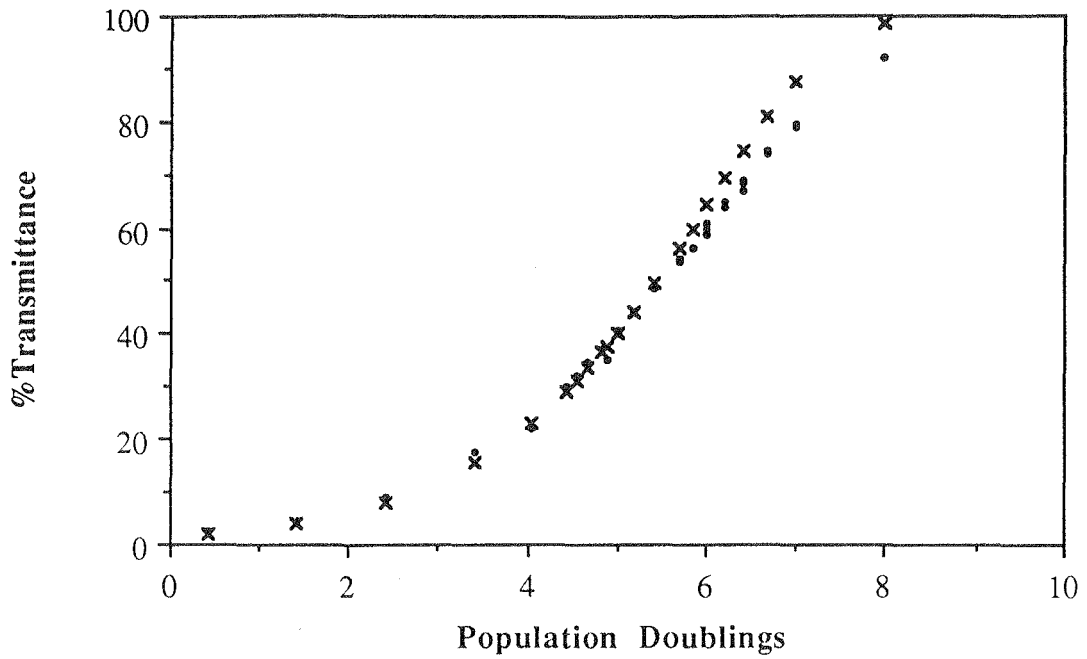


Fig. 3.7 The relationship between %T and population doublings showing the effect of the disproportionality of the response at ABS > 0.3 (~50 %T). • raw values; x: corrected values

Table 3.4 Effects of Wavelength of Light on Generation Time Estimates from %T Data

Wavelength (nm)	Generation Time (h)
620	1.04
580	1.05
540	1.06
500	1.06
460	1.01
420	1.08

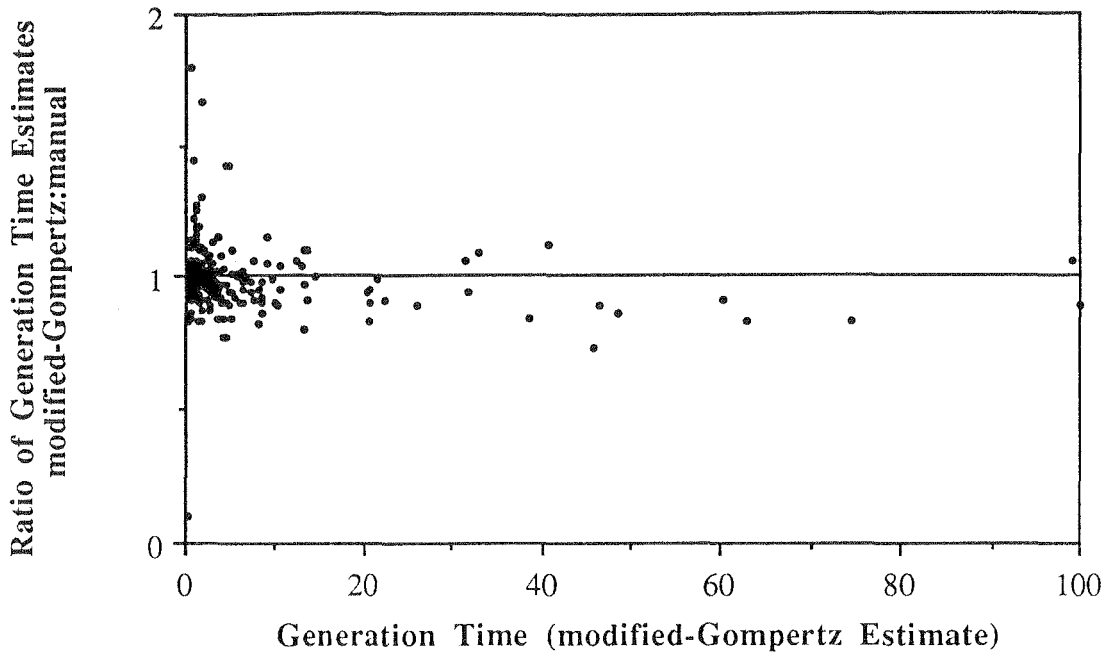


Fig. 3.8 The ratio of generation time estimates (min) derived from Δ%T data using manual (graphical) methods and by fitting Eqn. 3.13, plotted as a function of the estimated generation time.

Δ%T corresponding to one population doubling, in the linear part of the curve, agrees well with experimental determinations after the data have been corrected for deviation from Beer's Law and suggests that the theoretical derivation is sound. The theoretical development is based on increase in concentration only and is thus completely general, i.e. the calibration factor is not specific to a particular suspending medium, strain of organism etc., and is consequently robust. Due to the deviation from Beer's Law, a slightly different calibration factor is appropriate to uncorrected %T data. Provided that the turbidity of the culture is measured against an appropriate reagent blank, the deviation from Beer's Law is neither medium nor organism dependent (Dalgaard *et al.*, in press). Use of the experimentally determined calibration factor of 20.5% with uncorrected %T data obviates the additional data processing steps necessary to correct the data.

The use of Δ%T, rather than %T, as the response variable has no effect on the absolute values of the fitted parameters of Eqn. 3.13, and results only in a change of sign of the parameter, B. For the ideal situation (blank value = 100%T) the linear

Table 3.5 Comparison of Growth Rate Estimates Derived from Eqn. 3.13 or by Linear Regression of Data in the Range 20-60%T.

Estimated Slope			Ratio
Eqn. 3.13	Linear Regression	(r^2)	
0.427	0.385	0.998	1.111
0.372	0.377	0.995	0.986
0.394	0.361	0.999	1.090
0.327	0.336	0.999	0.973
0.090	0.082	0.983	1.091
0.083	0.073	>0.999	1.136
0.039	0.037	0.997	1.053
0.047	0.041	0.972	1.149
0.027	0.024	0.993	1.139
0.130	0.118	0.995	1.109
0.129	0.117	0.995	1.095
0.183	0.167	0.994	1.093
0.189	0.174	>.999	1.083
0.161	0.156	0.994	1.033
0.153	0.171	0.996	0.891
0.193	0.166	0.999	1.164
0.203	0.166	0.991	1.220
		Mean:	1.083
		SD:	0.079

range is 40 - 80Δ%T, although the deviation from linearity over the range 35 - 80 Δ%T is of the order of a few percent only. The fitted value of M fell outside of the times corresponding to this range in only a few of the several hundred growth curves fitted. Provided that the slope in this region is well estimated, a reliable estimate of generation time may be obtained. Table 3.5 shows that the slope is overestimated by Eqn. 3.1 and that, as for Eqn. 3.2, further correction is needed for Eqn. 3.14. The true minimum generation time of the %T growth curve can now be calculated from the fitted parameters of Eqn. 3.13:

$$\text{Generation time} = \frac{1.08 \times 20.5 \times e}{B \times C}$$
$$= \frac{60.2}{BC} \qquad (3.15)$$

Some workers (McClure *et al.*, 1993) have used turbidimetric methods to develop kinetic models but have first related turbidity to cell numbers by calibration. Absorbance per cell is reported to vary according to strain (Squiggens *et al.*, 1990) and growth rate (Neidhardt *et al.*, 1990, p.437). In addition, the optical density response has a narrow range of sensitivity. It is insensitive to change in density at high concentrations and is characterised by high relative error at low concentrations. These limitations require that a separate calibration be prepared for each condition and organism to be modelled, and seem to preclude the use of turbidimetric methods. The methodology presented here, however, is general for a range of organisms and media (Dalgaard *et al.*, in press). The method assumes that the optical density of the medium itself is not significantly affected by the growth of the bacterium and that the cell mass does not alter significantly as a function of population density within the range of measurement. The methodology presented may be used to determine doubling times directly but is unable to provide information regarding the MPD of the culture. In many situations however, this is unnecessary. Due to the disproportionality of the %T-log(cell density) response below ~20%T, determination of the lag phase could be unreliable by this methods. If the determination of lag times were desirable, a high inoculum density in the region of proportional response would have to be used.

3.3 COMPARISON OF TURBIDIMETRIC AND VIABLE COUNT METHODS FOR GENERATION TIME ESTIMATION

3.3.1 Introduction

In 3.2.1 two potential problems relating to the use of turbidimetric methods for kinetic modelling were identified. Firstly, because viable count methods remain the standard method of enumeration in food microbiology, it is necessary to demonstrate the equivalence of results derived from indirect methods and those from VC methods. This section explores the relationship between growth rate estimates from the turbidimetric method developed in 3.2 and those obtained from viable counts. Secondly, the effects of inoculum density are also investigated.

3.3.2 Methods and Materials

S. aureus 3b was grown overnight in BHIB without agitation at 35 ± 2°C. *Listeria monocytogenes* Scott A was incubated in TSB for 12 - 15 hours at 30 ± 2°C without

agitation. The inocula were placed in an ice-water slurry for 30 - 45 minutes before inoculation or further manipulation. In one set of experiments, serial dilutions were prepared in chilled (4°C) growth medium and a range of dilutions used as inocula for duplicate determinations. In a second method, two inoculum levels were used, without replication, at a range of incubation temperatures. Equivalent volumes of inoculum were added to L-tubes, containing 15 ml BHIB or TSB for experiments involving *S. aureus* and *L. monocytogenes* respectively, and incubated with agitation (40 ± 20 rpm) on a temperature gradient incubator (TGI). The %T of the cultures was monitored and, at appropriate intervals, 0.2 ml aliquots withdrawn and serially diluted in 0.1% peptone. Viable counts were determined by spreading 0.1 ml of appropriate dilutions on PCA. Plates were incubated at 35°C (*S. aureus* 3b) or 30°C (*L. monocytogenes* Scott A) for 48 ± 6 h. Colonies were counted 'manually' and CFU values calculated as described in Appendix 2. Data were fitted to Eqns. 3.1 and 3.15 as appropriate and generation times derived from fitted parameters for VC data and %T data using Eqns. 3.8 and 3.15 respectively.

3.3.3 Results

Table 3.6 details the generation times derived from the complementary %T and VC data sets and details the temperature and initial inoculum conditions employed for each organism. The ratio data are graphed in Figs. 3.9 a,b as a function of inoculum density and generation time respectively. There are no trends evident in the data. The mean value of the ratios is 1.245 ± 0.159. The mean of the ratios calculated on species basis, or experimental block basis, were not significantly different from the grand mean. The average difference in coefficient of variation (ratio of the standard deviation to the mean), $CV_{(VC)}$ - $CV_{(\Delta\%T)}$, is 0.0067 ± 0.046 based on all duplicate pairs, or 0.0011 ± 0.062 if the isothermal, non-replicated, *L. monocytogenes* data sets are treated as duplicates.

The data also permit consideration of the effect of inoculum density on the subsequent growth rate of the culture. To allow comparison of all the data, relative generation times were calculated (observed value/mean value at that temperature). The results for %T and VC based estimates are presented in Figs. 3.10a,b.

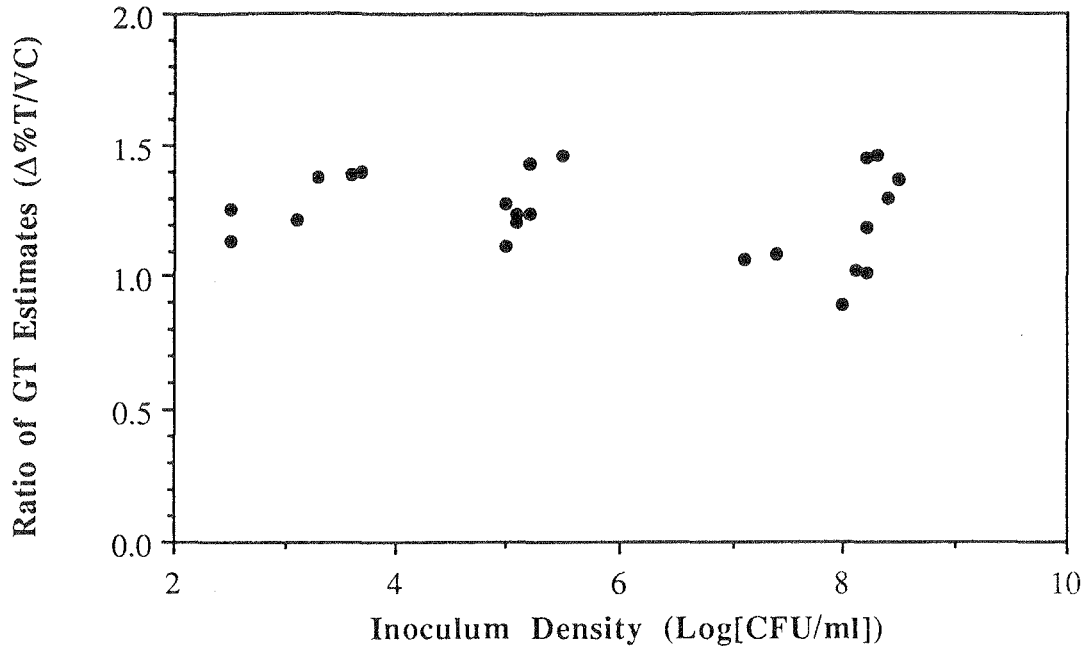
3.3.4 Discussion

The average difference in coefficient of variation, $CV_{(VC)}$ - $CV_{(\Delta\%T)}$, indicates that bacterial growth rate estimates derived from turbidimetric data are at least as precise as those obtained from viable counts, and confirms the results presented in 3.2. Though the estimates of generation time obtained from VC data are smaller than those from the %T method, the relationship is constant. Consequently a simple calibration factor can be used to equate results from the two methods.

Table 3.6 Comparison of Generation Time Estimates Derived from Turbidimetric and Viable Count Data from the Same Population

Organism	Inoculum Log(CFU/ml)	Temperature (± 0.5°C)	Generation Time (h) (%T data)	(CFU data)	Ratio $[t_{G\,(\%T)}/t_{G\,(VC)}]$
S. aureus 3b	7.1	27	0.89	0.83	1.07
	7.4	27	0.88	0.81	1.09
	5.5	27	0.98	0.67	1.46
	5.2	27	1.08	0.76	1.43
	3.1	27	0.81	0.66	1.22
	3.3	27	0.95	0.69	1.38
L. monocytogenes (Scott A)	8.5	19.5	2.83	2.07	1.37
	8.4	19.5	2.87	2.21	1.30
	8.1	19.5	2.02	1.96	1.03
	8.0	19.5	1.96	2.21	0.89
	3.6	19.5	2.29	1.65	1.39
	3.7	19.5	2.42	1.73	1.40
	2.5	19.5	1.91	1.51	1.26
	2.5	19.5	1.82	1.60	1.14
	8.2	28	0.88	0.86	1.02
	5.0	28	1.00	0.78	1.28
	8.2	27	0.95	0.80	1.19
	5.1	27	1.15	0.95	1.21
	8.3	13	4.17	2.86	1.46
	5.2	13	4.53	3.64	1.24
	8.2	9	9.54	6.58	1.45
	5.1	9	8.00	6.46	1.24
	5.0	6.5	11.27	10.07	1.12

a.



b.

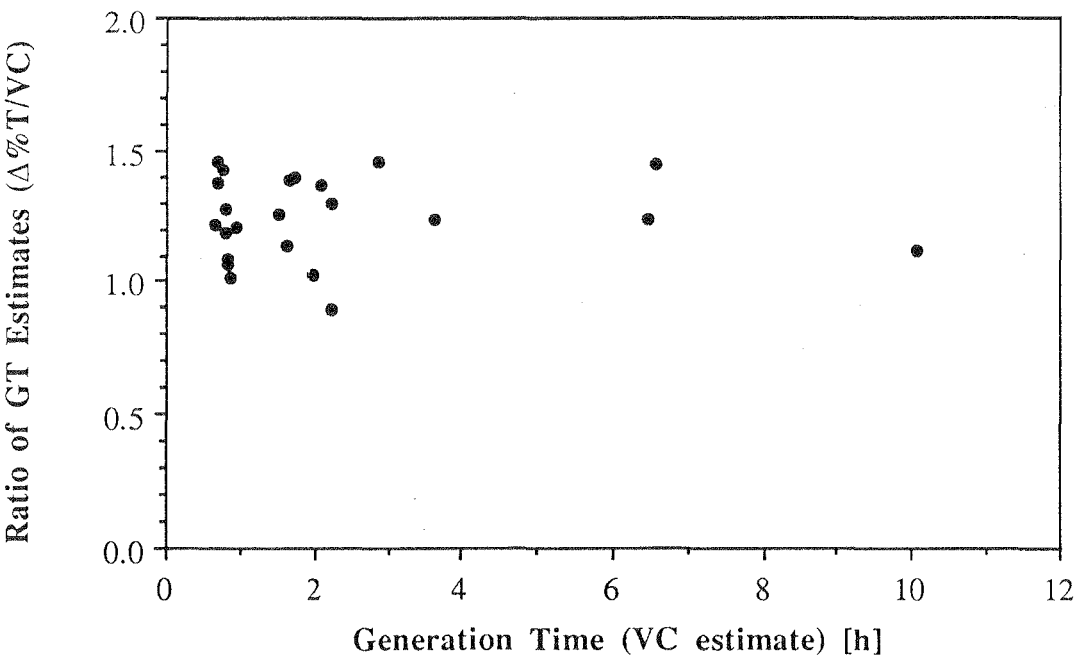
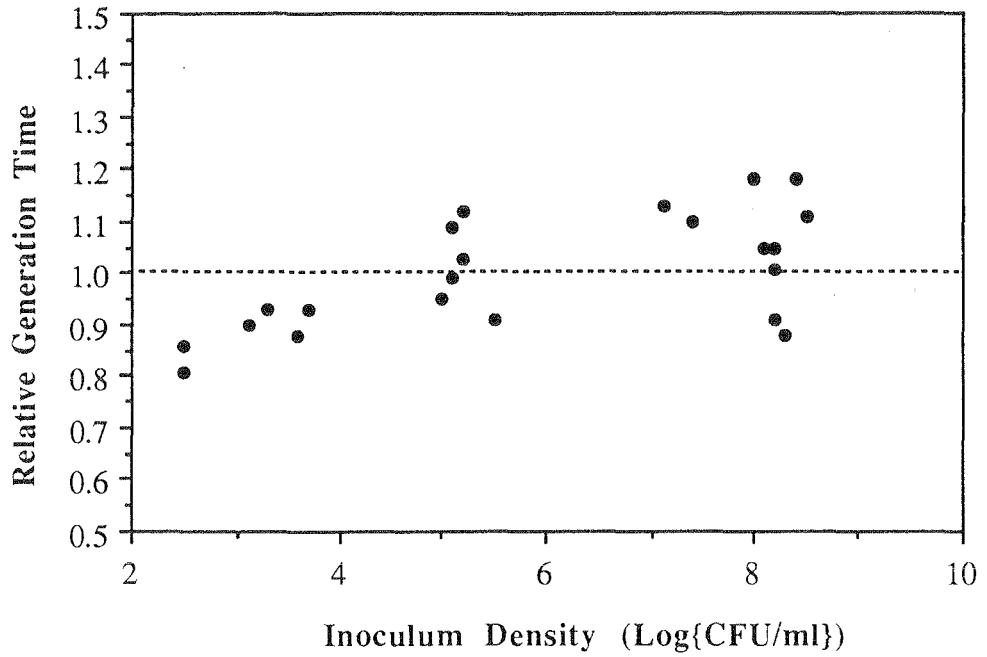


Fig. 3.9 Dependence of the ratio of generation time estimates by %T and VC methods on a) inoculum density; b) generation time.

a.



b.

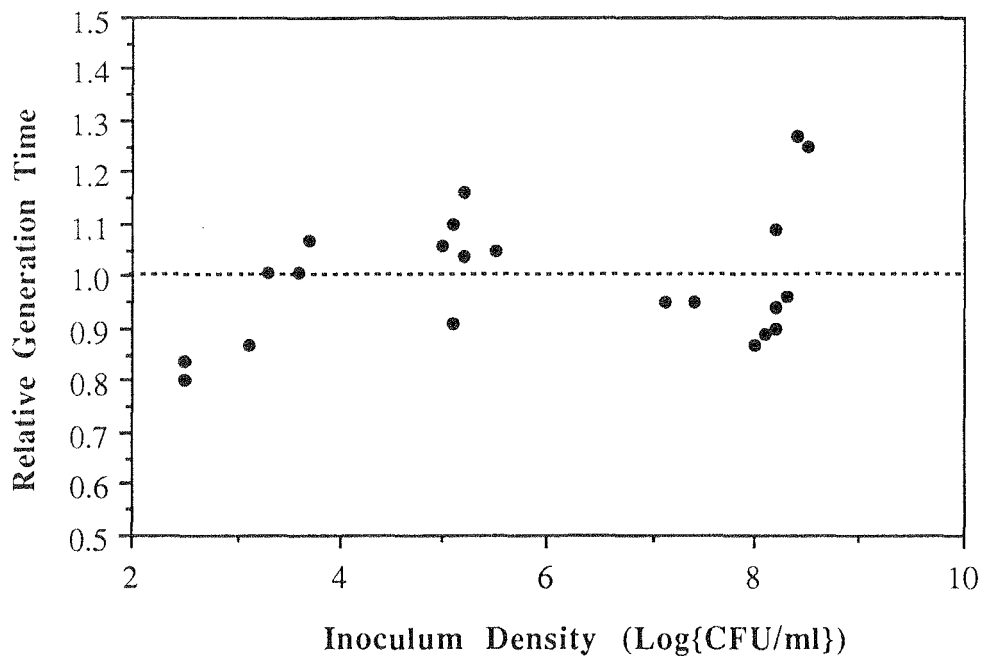


Fig. 3.10 The effect of inoculum density on generation time estimates (standardised to the mean estimate at each temperature) derived from a) Eqn. 3.8 (VC data) and b) Eqn. 3.15 (Δ%T data).

The results indicate that Δ%T-based estimates of generation time must be divided by 1.25 to yield the 'true' generation time, as assessed by viable counts. Essentially identical conclusions were drawn by Dalgaard *et al.* (1993) in a study incorporating the data presented here and data for *Pseudomonas* spp., *Shewanella putrefaciens* and *Photobacterium phosphoreum*. The results presented here are based on a slightly different approach and yield a lower calibration factor.

The basis of the difference in growth rate estimates from the %T and VC methods is unclear. The results in Table 3.6 and Figs. 3.10a and b suggest that the %T method is insensitive to initial inoculum densities but that growth rates determined from VC methods are faster for lower inoculum densities. With turbidity based methods, the times on the growth curve at which the fastest growth rates are estimated will always occur at high population densities. At this time the growth rate may already be declining due to increasing population density. For the VC based growth curves this will occur only where the initial population density is high. If this were the basis of the difference the ratio $[t_{G(\%T)}/t_{G(VC)}]$ would be expected to be closer to one at high population densities. Although this behaviour is evident in the *S. aureus* data in Table 3.6, it is not in the *L. monocytogenes* data. Consequently, the basis of the difference in growth rate estimates by the two methods remains unresolved.

The apparent relationship between generation time and $N_{(0)}$ is not supported by the results of other workers (Jason, 1983; Buchanan and Phillips, 1990; Buchanan *et al.*, 1993a) who found no evidence of an effect of inoculum size on growth rate.

3.4 CONCLUSIONS

Bratchell *et al.* (1989) and McMeekin *et al.* (1993) stressed the importance of the quality and quantity of data used for model generation in predictive microbiology. Bratchell *et al.* (1989) suggested that, to generate reliable models, 15 - 20 data points per growth curve were required. McMeekin *et al.* (1993) considered that 10 - 15 data per secondary model parameter are necessary. Thus, to generate a model of even moderate complexity using viable count methods would require the preparation and interpretation of thousands of culture plates. Turbidimetric methods enable rapid, inexpensive and non-invasive data generation and the results presented in this chapter show that, provided a rigorous methodology is followed, models derived from turbidimetric data are at least as reliable as viable count data for the generation of kinetic models. Thus, the many advantages of turbidimetric methods can be realised in predictive modelling. Simple calibration factors are required, however, to reconcile turbidimetry-based rate estimates with those obtained from viable counts, but appear to be general for a range of organisms and growth conditions.

The method presented is 'self calibrating' and obviates the need to relate optical density measurements to cell numbers by reference to calibration curves.

There are some limitations to the methodology, which can not be used to model the full bacterial growth curve. Information concerning MPD cannot be obtained because the response becomes increasingly less sensitive to changes in population density at levels greater than ~$5x10^8$cfu/ml. Using high inoculum densities lag time information could be obtained, but the use of high inoculum densities may be unrepresentative of the situation in foods. A better alternative in this situation would be to monitor the change in absorbance, which is linearly related to cell numbers at lower population densities than %T.

The original reasons for the use of %T as the response variable were pragmatic. The use of absorbance as the response variable is preferable, because of its greater sensitivity. Given access to digital spectrophotometers and spreadsheet software, absorbance data can be readily derived and manipulated. The data can be objectively fitted and interpreted using Eqn. 3.5 and generation times estimated using Eqn. 3.6.

The robustness of the method presented has been demonstrated for the determination of growth rates in laboratory broth media. In subsequent chapters, the utility of the method for the generation of kinetic models applicable to foods will be considered.

4 SECONDARY MODELLING

4.1 INTRODUCTION

Several groups, independent of the originators of the square root-type models, have reported that Bělehrádek-type models describe the effects of temperature on the rate of microbial growth at least as well as other existing models (Zwietering *et al.*, 1991; Kohler *et al.* 1991, Alber and Schaffner, 1992; Duh and Schaffner, 1993). Bělehrádek-type models for the combined effects of temperature, pH and/or water activity on bacterial growth rate were described in 1.1.4.

The results of McMeekin *et al.* (1987) and of Chandler and McMeekin (1989b,c) suggest that the parameter T_{min} is an intrinsic property of an organism. From this observation they proposed that, to generate a Bělehrádek-type model for the effects of temperature and water activity for a given bacterium/humectant system, "it is necessary simply to determine a MINa_w [i.e. $a_{w_{min}}$] value for growth of the organism of concern and combine this with the predicted T_{min} value" (Chandler and McMeekin, 1989b). A similar strategy was proposed by McMeekin *et al.* (1992) for the combined effect of temperature, pH and water activity. They reasoned that if the responses to temperature, water activity and pH are independent, then it should follow that the parameters T_{min}, $a_{w_{min}}$ and pH_{min} are independent. They hypothesised that it should be possible to construct a model by three, 'one-variable', experiments to determine accurately these parameter values, and the replication of a one or a few sets of conditions to determine the variance in the response. From that variance, the variance under all other conditions may be estimated by reference to the equations presented by Ratkowsky (1992). Further, the mean value of the rate under those replicated conditions should be a representative value, and thus enable the determination of the scaling factor, 'b' in the square-root-type equations. Thus, a complete temperature-water activity-pH model could be generated from as few as 40 - 50 rate determinations.

These hypotheses are tested in this chapter through the development of complementary Bělehrádek-type models based on the approach proposed above and the more usual approach based on nonlinear regression of all data simultaneously. Using the methods described in Chapter 3, models are developed for the growth rate of *Staphylococcus aureus* in response to temperature and water activity (NaCl), and *Listeria monocytogenes* growth rate as a function of temperature, pH (lactic acid as acidulant), and water activity (NaCl). Other topics briefly addressed in this chapter include strain to strain variation in responses, the effect of humectant on $a_{w_{min}}$, and environmental history effects.

4.1.1 *Staphylococcus aureus*

Staphylococcus aureus is a major cause of food borne illness, as well as being an opportunistic pathogen of clinical significance. There is a vast literature on both aspects of its significance to human affairs. On a world scale, staphylococcal intoxication remains a common cause of food poisoning (Genigeorgis, 1989). Comprehensive reviews of its significance in food-borne illness include Smith *et al.* (1983), Eyles, (1989), Genigeorgis (1989), Halpin-Dohnalek and Marth (1989), and Bergdoll (1989), and general information will be found in any reputable microbiology text. It is of particular interest in this study because of its tolerance of low a_w, with many reports indicating a lower a_w limit for growth of 0.86.

Staphylococcus aureus causes food-borne intoxications due to the production of a range of thermostable, protein enterotoxins which have an emetic effect. Seven toxins of similar molecular weight are currently recognised, namely staphylococcal enterotoxins A, B, C_1, C_2, D, E and F, which are abbreviated as SEA, SEB, etc. respectively. From a food safety perspective, it is the ability of a given strain of *S. aureus* to produce SEs that is of direct concern but, until the advent of ELISA-based methods, detection and identification of toxins has been laborious. The ability to produce SEs is correlated with a number of other, more readily assessable, characteristics of *S. aureus* such as the ability to clot blood plasma (coagulase), production of thermostable DNase, and production of haemolysins. These characteristics, particularly the production of coagulase, have been used as presumptive identifications of toxigenic strains. Hence, the term 'coagulase positive *S. aureus*' is taken to indicate an enterotoxigenic strain. The production of toxin is closely linked to the growth of the organism but the relationship between growth and toxin production varies for different SEs, and under different growth conditions (Smith *et al.*, 1983). Whereas SEA and SED are produced under nearly all water activities at which growth is possible, production of other SEs appears to be more sensitive to water activity (Ewald and Notermans, 1988). Also, production of enterotoxins appears to be confined to narrow temperature limits within the temperature range for growth (Schmidt *et al.*, 1990). In general, where growth of *S. aureus* is possible, there is a risk of SE production. In the absence of clearer patterns of production of SEs, both Leistner and Rödel (1979) and Halpin-Dohnalek and Marth (1989) concluded that control of the *growth* of enterotoxigenic *Staphylococcus aureus* was the most appropriate strategy to control staphylococcal food poisoning. Accordingly, in the current state of knowledge, a model for *S. aureus* growth rate will have equal utility to a model for the rate of toxin production.

4.1.2 *Listeria monocytogenes*

Despite that *Listeria monocytogenes* was not considered to be a food-borne pathogen until the early 1980's, there now exists a vast literature concerning the ecology and physiology of this organism and its significance in food-borne disease. Though it is still not regarded as a common cause of food borne illness, it is important because it can cause abortion and fatal infections, particularly of the immunodeficient. The organism has been reported to grow at 0°C, can grow at water activities as low as 0.92, and growth in laboratory media at pH less than 4.5 has also been reported (Walker, 1990). These characteristics enable *Listeria* to overcome several of the key strategies used in food preservation.

Listeria monocytogenes was recognised as pathogenic for animals since its description by Gray *et al.* in 1926, but was considered to be an uncommon disease of humans (McLaughlin, 1987). It is a low grade intracellular pathogen, and first came to the attention of the food industry in 1981 as a result of an epidemic in the Maritime Provinces of Canada over the period March to September involving 41 cases with 42% mortality. Other outbreaks were reported in Massachusetts in 1983 (49 cases, 14 deaths), California in 1985 (≥ 86 cases, 29 deaths), Switzerland from 1983-1987 (22 cases) and Philadelphia in 1987 (45% mortality). In Australia, an outbreak of Listeriosis occurred in 1990 in which commercially prepared pâté was strongly suspected of being the vehicle of transmission (Watson *et al.*, 1990). Ten cases in pregnant women were identified, with a case-fatality rate of 55% among the foetuses. Todd (1989) estimated the cost of *Listeria*-related outbreaks and product recalls to the US to be $313 million per annum.

The high cost, both in human and economic terms of food-borne *Listeriosis*, demands that action be taken to minimise its incidence. The National Advisory Committee on Microbiological Criteria for Foods (NAC) of the USA recommended a number of control strategies for *Listeria*, including minimisation of the presence, survival and multiplication of the pathogen in foods (NAC, 1991). A number of characteristics of *Listeria* suggest that this may be a formidable task. Listeriae are non-fastidious organisms and are considered to be ubiquitous in the environment, particularly in soils, and associated with plant material. There is also evidence that there may be a significant reservoir in the intestinal tracts of humans and other animals. Thus, it may be impossible to eliminate *Listeria* completely from foods and an equally important strategy may be the use of predictive models to identify, and assess the safety of foods at risk from this organism.

4.1.3 Existing Predictive Models

Predictive models for the growth of *Staphylococcus aureus* have been published both prior to undertaking this project and during it (Broughall *et al.*, 1983; Broughall and Brown, 1984; Buchanan *et al.*, 1993a). Models for the growth of *Listeria*

monocytogenes have also been published during the preparation of this thesis (Buchanan and Phillips, 1990; Cole *et al.*, 1990; Wijtzes *et al.*, 1993). These models will provide a useful baseline upon which to assess the hypotheses presented in this chapter, and the models evaluated in the subsequent chapter.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Details of strains and their sources, reagents and media, and equipment used are presented in Appendix 2.

4.2.2 Methods

4.2.2.1 Inoculation procedures

Inocula of *S. aureus* were grown for 12 - 18 h in 50 mls BHIB at 35°C with shaking (50 ± 10 rpm). Inocula of *L. monocytogenes* were grown for 18 - 24 h in 50 mls TSB at 30°C with shaking (50 ± 10 rpm). Approximately 30 minutes before inoculation began, cultures were placed in ice-water baths to minimise changes in culture density during the inoculation procedure which required ~30 minutes to complete. The inoculum volume was adjusted in each block of experiments to achieve ~80% transmittance at the time of inoculation, and was typically 0.2 - 0.3 ml.

4.2.2.2 Growth rate estimation

Per cent transmittance (540nm) was monitored with a spectrophotometer (Spectronic 20). Measurement times were chosen to correspond to %T changes of 5 - 10% between consecutive measurements, and were continued at least until %T fell below 10%. Per cent transmittance readings were converted to change in %T (Δ%T) at 'time elapsed since inoculation' (Δt). Doubling times were calculated as described in 3.2.5. In early experiments the steepest tangent to lines of best fit drawn 'by eye' through growth curves were used. Subsequently, the steepest tangent was calculated from the parameters of a modified Gompertz function (Eqn. 3.13) fitted by nonlinear regression using a SAS[1] PROC NLIN routine written by Dr. G. McPherson, Mathematics Dept., University of Tasmania. All growth rate estimates used in the models developed for subsequent evaluation and validation (Chapter 5) were based on growth rate estimates derived from Eqn. 3.15 from fitted parameters of Eqn. 3.13. The basal medium for all *S. aureus* growth rate determinations was BHIB, and for *L. monocytogenes* was TSB.

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[1] SAS: Statistical Analysis System, SAS Institute Incorporated, SAS Circle, Cary, N. Carolina. USA.

4.2.2.3 Parameter estimation

General

Cultures were incubated in L-tubes containing 15 ml of growth medium on a shaking incubator (TGI, 40 ± 20 rpm) in a constant-temperature room. Linear regression analysis was performed using Cricket Graph 1.3.2[2] (Cricket Software, Malvern, PA, USA).

T_{min}

Cultures were incubated in a TGI as described above. When the %T of the culture had fallen below 10% the temperature in that tube was monitored (Fluke thermocouple) intermittently for several hours to establish the range of temperature experienced by that culture during incubation. The midpoint of that range was used as the nominal temperature for curve-fitting. Linear regression of the square root of rate, determined as per 4.2.2.2, as a function of incubation temperature in the sub-optimal range was performed. From the fitted equation:

$$\sqrt{rate} = b * Temperature + c$$

$$T_{min} = -c / b$$

$a_{w_{min}}$

Two methods for estimating $a_{w_{min}}$ were used. The first is the method of McMeekin *et al.* (1987) in which a series of broths of different water activities were incubated in a TGI. Growth rates, determined as per 4.2.2.2, as a function of temperature were fitted to Eqn. 1.1 (simple square root model) as described above. Linear regression of the square of the parameter b, as a function of medium a_w in the sub-optimal range, was performed. From the fitted equation:

$$b^2 = d * a_w + e$$

$$a_{w_{min}} = -e / d$$

In a simpler method, a series of broths of different a_w were prepared as follows. The a_w of culture media was adjusted by the inclusion of NaCl or sucrose to a level below that reported as the lower limit for growth of the species under investigation. Due to the volume changes upon addition of large amounts of solute, particular care was taken to ensure that final concentrations of the growth medium constituents in a_w adjusted media were the same as those achieved in normal preparations. An equivalent volume of the same medium, but without added solute, was also prepared.

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[2]Cricket Graph 1.3.2: Cricket Software, Malvern, PA, USA.

Both media preparations were autoclaved (105°C for 30 minutes to prevent turbidity of the broths due to formation of precipitates) simultaneously to avoid differences in absorbance of the medium which may result from different time-temperature combinations. Culture media, encompassing the a_w range of growth of the organism under study, were prepared by combining the two media in varying proportions in sterile L-tubes. In studies of the water activity and pH effect on growth rate of *L. monocytogenes,* Lactic acid (0.2M) was included as the acidulant, because of its significance in dairy products, which have been a vehicle of transmission in several food-borne listeriosis outbreaks. At each water activity level the growth rate was determined at pH (initial) 5.5 and 7.2. The L-tubes were incubated at constant temperature on a TGI. Growth rate at each water activity was determined as described in 4.2.2.2. Linear regression of the rate as a function of medium a_w in the sub-optimal range was performed. From the fitted equation:

$$rate = d * a_w + e$$

$$a_{w_{min}} = -e / d$$

The a_w of each medium at 25°C was calculated from the tables and equations of Robinson and Stokes (1949), Norrish (1966), Bromley (1973), Ross (1975), Chirife and Ferro Fontán (1980), Chirife and Resnik (1984) and (Chen, 1990) and measured at 25°C with a Novasina a_w meter. The effect of temperature on a_w for NaCl over the temperature range 15-50°C is negligible but increases slightly at lower temperatures (Resnik and Chirife, 1988). For sucrose, the water activity at 19.5°C is not different at the level of precision reported here, to the values calculated at 25°C (Ross, 1975). The values presented are considered to be accurate to about 0.003 - 0.004 a_w.

pH response

The pH of two preparations of over-strength TSB + 0.2 M lactate, simultaneously autoclaved, was adjusted by the addition of sterile NaOH or sterile HCl to pH ~4 and pH ~8 respectively. Broths were made up to final volume with sterile water. Culture media of varying pH were prepared by combining the two media in varying proportions in sterile L-tubes. The L-tubes were incubated at 19.5 ± 0.5°C in a TGI as described above. Growth rate at each pH was determined as described in 4.2.2.2. Aliquots (0.5 ml) were aseptically removed immediately after inoculation and at intervals during the incubation and the pH determined using a pH meter with surface probe.

4.2.2.4 Environmental history effects

Cultures of *Listeria monocytogenes* were incubated in TSB + 0.2M lactic acid, adjusted to a range of sub-optimal a_w (NaCl as humectant) and pH conditions, on a TGI with shaking at 19.5 ± 0.5°C. Growth rate was determined as described in 4.2.2.2. When the cultures had reached <10% transmittance, small aliquots were transferred to sterile TSB at the same temperature and the cultures incubated under the same conditions. Growth rate in this medium was determined as described in 4.2.2.2.

4.2.2.5 Model Generation

Experimental design

The *S. aureus* models were based on a data set (212 data) covering the entire suboptimal water-activity (NaCl)-temperature variables-space. The variables combinations tested are represented diagrammatically in Appendix 3, Fig. A1. From this large data set model parameters were estimated by the two methods described below. The results of that exercise led to the use of a minimal experimental design for the *L. monocytogenes* models, consistent with the hypotheses presented. Three single-variable experiments were undertaken from which to characterise well the response to each variable and from which to estimate the parameters T_{min}, a_{wmin} and pH_{min} individually. The variables combinations tested are represented diagrammatically in Appendix 3, Fig. A2 and are near to the minimum that could be used to derive models of the type of Eqn. 1.5 (*see* 2.2.9 re: 'quantity of data'). The same data set was fitted by nonlinear regression. The Murray B and Scott A data sets contain 54 and 72 data respectively. This experimental design was used in an attempt to 'test to destruction' the hypotheses presented, so that potential shortcomings of the strategy would be highlighted.

'Iterative' models

The parameters T_{min}, $a_{w_{min}}$ and pH_{min} of the square-root-type models were assumed to be characteristic features of the modelled organisms. Models were constructed by substitution of the parameter values, estimated by the methods described above, into Eqns. 1.3 and 1.5. The coefficient, b, was estimated algebraically by equating the model to the mean growth rate of replicated determinations under defined conditions. These replicated experiments were also used to estimate the variance in the response.

Nonlinear regression models

Models of the type of Eqns. 1.3, and 1.5 were fitted to data using SAS PROC NLIN routines written by Dr. D. Ratkowsky, University of Tasmania, Hobart, Australia.

The data sets used for generation of models by the two methods are shown in Appendices 3.1-3.

4.3 RESULTS

4.3.1 Parameter Estimation by 'Iterative' Methods

4.3.1.1 T_{min} estimates

Staphylococcus aureus

Table 4.1 summarises T_{min} determinations for *S. aureus* strains grown at a range of water activities (0.997 - 0.925). For each experiment estimates of T_{min} were determined from the generation time and from the time required for the %T of the culture to decrease by 30%.

It was noted that estimates of T_{min} for *S. aureus* were higher for data sets generated in low water activity media. To test a hypothesis, presented in detail in Chapter 7, that this may result from the range of data used in the fitting process, the lowest temperature at which growth was observed was compared to the T_{min} estimate obtained for individual data sets. The results are presented in Fig. 4.1.

Listeria monocytogenes

Table 4.2 presents estimates of T_{min} for two strains of *Listeria monocytogenes*. Estimates were derived for each data set based on generation time data, and the M parameter fitted to the data by Eqn. 3.11. Initial estimates were derived from a single experimental block for each strain. Other estimates were derived by augmentation of that data with complementary data from subsequent experiments (*see* Table 3.6).

4.3.1.2 $a_{w_{min}}$ estimates

Measured water activities were consistent with calculated values, within the degree of precision of the instrument (±2% RH). The instrument was unable to measure reliably water activities > 0.98, probably due to condensate formation on the sensor. Calibration of the instrument became increasingly difficult, and measurements more erratic, as the sensor aged. Higher precision was required and water activities > 0.98 were considered. Thus, all water activity values shown are calculated, rather than measured, values.

Staphylococcus aureus

The $a_{w_{min}}$ estimates for *Staphylococcus aureus* 3b (NaCl) by the method of McMeekin *et al.* (1987)was 0.855 (R^2 = 0.943). Estimation of $a_{w_{min}}$ by the second method described, for generation time data, is shown in Fig. 4.3, which also shows the temperature independence of the parameter $a_{w_{min}}$. Estimates of $a_{w_{min}}$ by that

Table 4.1 T_{min} estimates for strains of coagulase positive *S. aureus* .

Strain	T_{min} (°C)	±SD (n)
1a	7.29	0.40 (4)
1b	7.77	0.33 (4)
3a	6.98	0.79 (6)
BOV 2'b	8.69	0.87 (2)
BOV 4'	7.51	1.25 (2)
Cad. 010654	7.26	0.13 (2)
NCTC 6571	9.04	0.60 (3)
3b	7.27	0.78 (24)

Table 4.2 T_{min} estimates for two strains of *Listeria monocytogenes*

Strain	T_{min} (°C)	±SD	(n)
Initial experimental block			
Murray B	2.10	1.21	2
Scott A	2.98	1.22	2
Augmented data set			
Murray B	-0.26		1
Scott A	-0.29		1

method are 0.852 (34°C), 0.874 (30°C), and 0.851 (26°C). The R^2 values of the fitted lines are 0.974, 0.957 and 0.990 respectively. Estimates of $a_{w_{min}}$ for other strains of *S. aureus*, as a function of NaCl as humectant are shown in Table 4.3.

Using a method analogous to the latter method described in 4.2.2.3, cultures of *S. aureus* 3b in BHIB adjusted to a range of water activities by the addition of NaCl, were incubated (30°C) in a Bactometer[3] instrument. From preliminary experiments, linear regression of the reciprocal of impedance 'detection

[3] Bactometer: Bactomatic, A Division of MTC, Princeton, New Jersey, U.S.A.

Table 4.3 a_{wmin} estimates of coagulase positive *S. aureus* (NaCl as humectant).

Strain	a_{wmin}	r^2
Cad. 010654	0.880	0.947
	0.877	0.962
ATCC 25923	0.876	0.987
	0.882	0.920
NCTC 6571	0.871	0.985

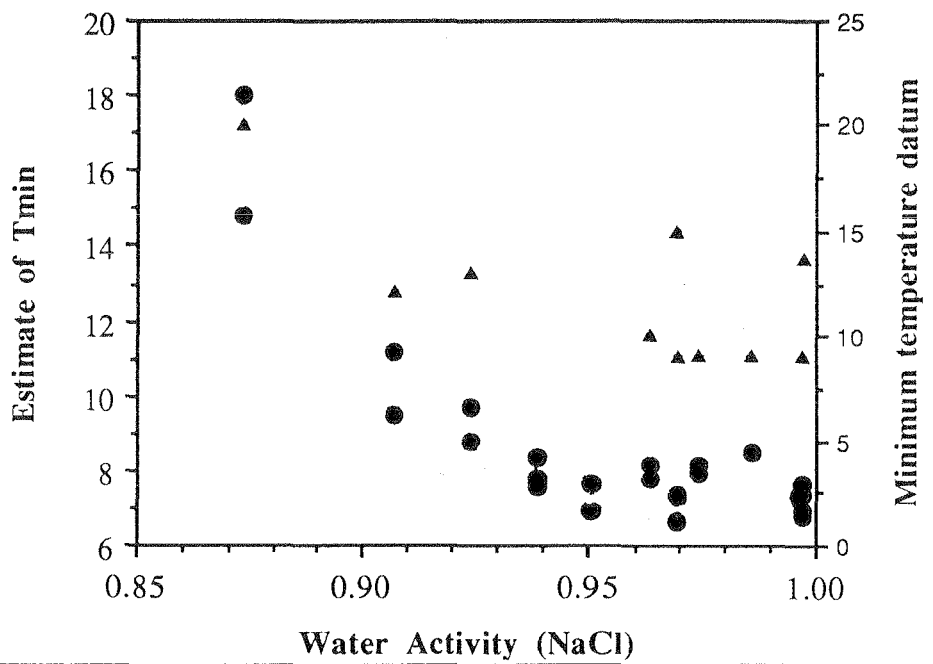


Fig. 4.1 Relationship between the estimate of T_{min} (•) and the lowest temperature (•) at which growth was recorded.

times' against water activity generated a_{wmin} estimates in the range 0.867-0.872 (results not shown).

The inhibitory effect of sucrose on growth rate of *S. aureus* is shown in Fig. 4.4 and details of fitted equations summarised in Table 4.4.

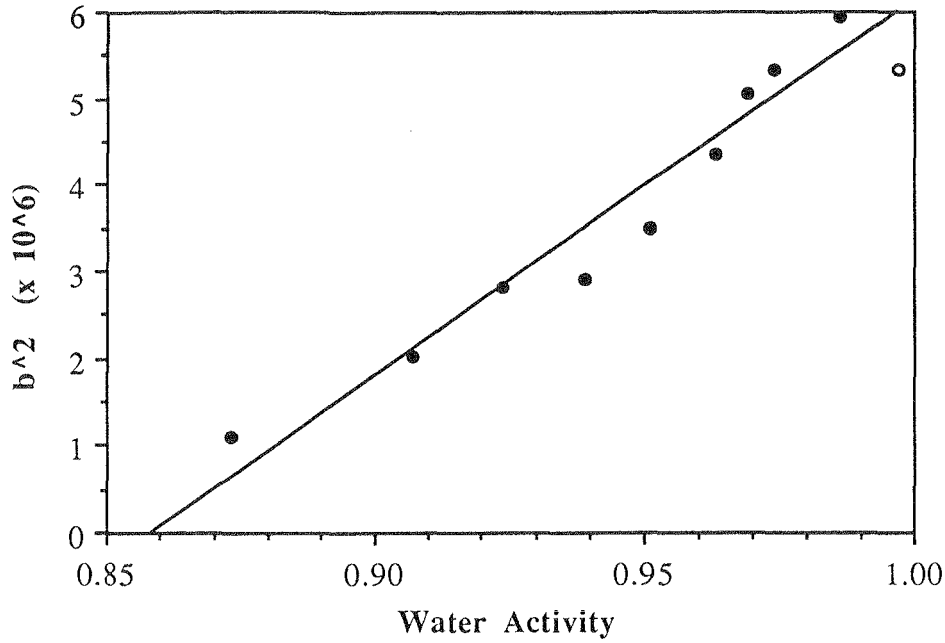


Fig. 4.2 Estimation of $a_{w_{min}}$ of *S. aureus* 3b by linear regression of the square of the parameter b of Eqn. 1.3 (NaCl as humectant).

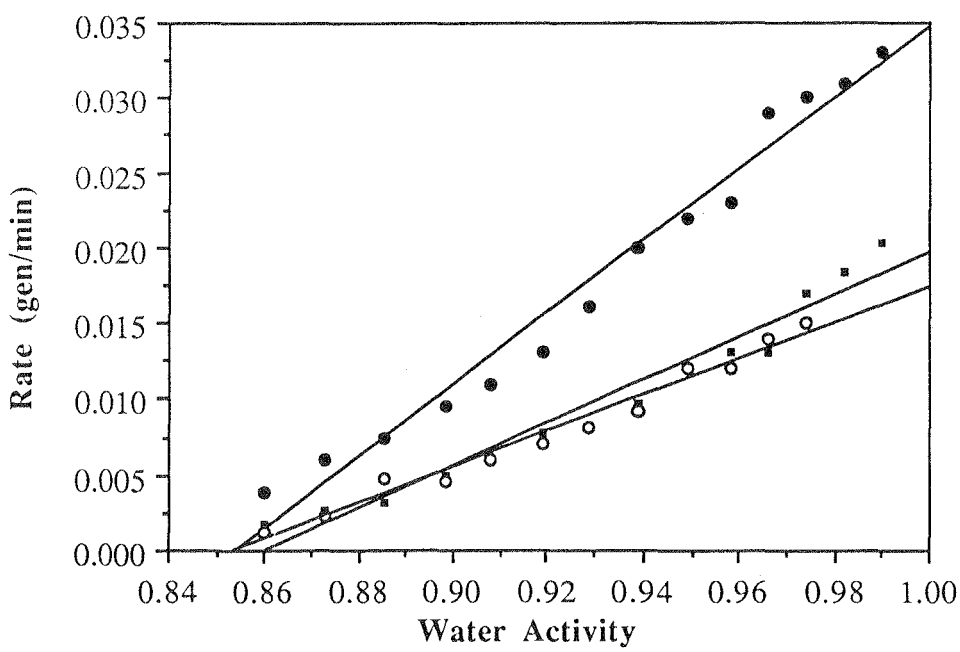


Fig. 4.3 Estimation of $a_{w_{min}}$ of *S. aureus* 3b by linear regression of the growth rate at constant temperature against water activity of the medium. ● : 34°C; ■ : 30°C; ○ : 26°C. (NaCl as humectant).

Table 4.4 $a_{w_{min}}$ estimates of coagulase positive *S. aureus* (sucrose as humectant).

Strain	$a_{w_{min}}$	R^2	Response
3b	0.924	0.978	generation time
	0.929	0.989	Time to $\Delta\%T_{30}$
NCTC 6571	0.908	0.872	generation time
	0.916	0.981	Time to $\Delta\%T_{30}$
	0.918	0.970	Lag Time

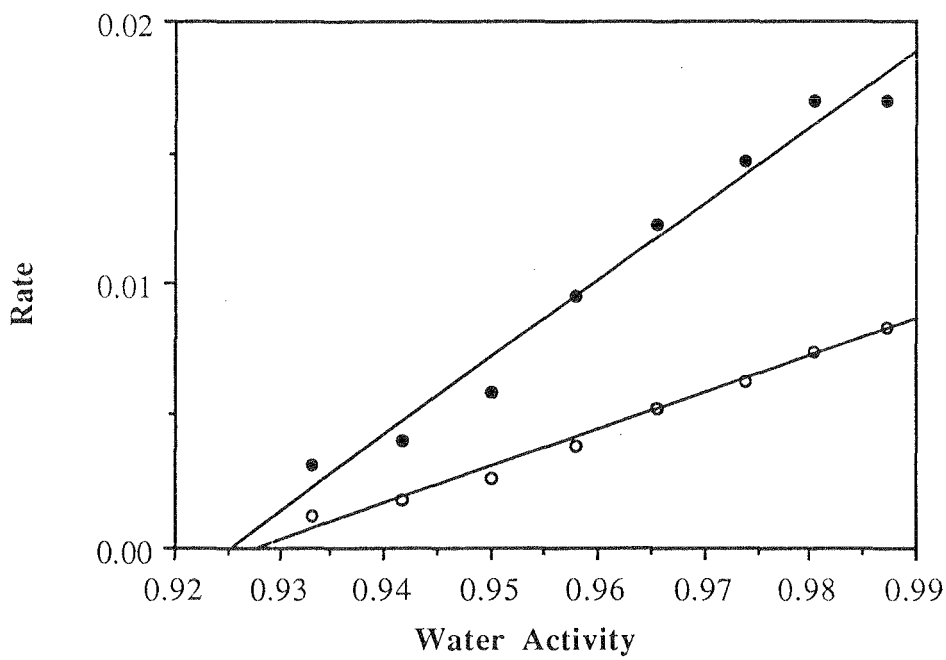


Fig. 4.4 Relationship between growth rate of *Staphylococcus aureus* 3b and water activity (sucrose as primary humectant). Rate determined from: ● : generation time (min); ○ : time (min) for 30% decrease in $\%T_{30}$.

Listeria monocytogenes

The effect of water activity on the growth rate of *L. monocytogenes* (NaCl as humectant) and estimation of a_{wmin} are shown in Fig 4.5. Estimates of a_{wmin}, based on generation time data, were 0.926 and 0.923 for *L. monocytogenes* strains Scott A and Murray B respectively. The R^2 values for the regression lines were 0.992 and 0.995 respectively. Neither strain grew in media at pH: 5.5 within 20 days.

4.3.2 pH Response

The relationship between the initial and final pH (pH_i and pH_f respectively) of the cultures is shown in Fig. 4.6a, and the dependence of that change upon population density shown in Fig. 4.6b for one culture. Fig. 4.6b is representative of the changes observed in all cultures, except those for which pH_i was less than 6.7. In these, the extent of growth and change in pH (ΔpH) was less than that for cultures with $pH_i > 6.8$. No cultures with $pH_i < 5.5$ showed growth and, in general, growth in all cultures ceased when the pH became less than 5.5. At this pH the lactate is 97.8% dissociated, i.e. under the experimental conditions the concentration of undissociated lactate is 4.3 mM. Cultures in which the transmittance had not fallen to <20% after ~90h were re-incubated at 30°C. After 24h the %T had not changed. These observations are consistent with the observations presented above for the growth of *Listeria monocytogenes* in media with reduced water activity. The effect of undissociated lactate, on the growth rate of both strains of *L. monocytogenes* tested, is shown in Fig. 4.7.

The change in pH closely paralleled the growth of the culture. The midpoint of the range pH_i to pH_f, designated pH_{mid}, was chosen as the response variable for assessment of the effect of pH on growth rate, as this pH was considered to be most representative of that at which the fastest growth rate was observed for each culture. Fig. 4.8 shows growth rate as function of pH_{mid}. Similar plots were obtained when the pH at the time 'M' of Eqn. 3.1, estimated from calibration curves, was used as the response variable. It was noted that for cultures in which pH_i was less than 6.7 (the eight lowest pH data points in Figs. 4.8) the final %T of the culture was not as low as for the other growth conditions. From Eqn. 3.4, reduced MPD would be expected to result in a lower observed growth rate. The initial %T, final %T and %T at time M obtained by fitting the data, were converted to equivalent CFU/ml by reference to a calibration chart derived from experiments described in 3.3. A correction factor for the expected decrease in rate, calculated at M, was derived from these data using Eqn. 3.4. It was found that the %T at M increased as pH_{mid} decreased, i.e. the maximum growth rate occurred at lower population density in those cultures with low pH_i and compensated the effect on growth rate of reduced

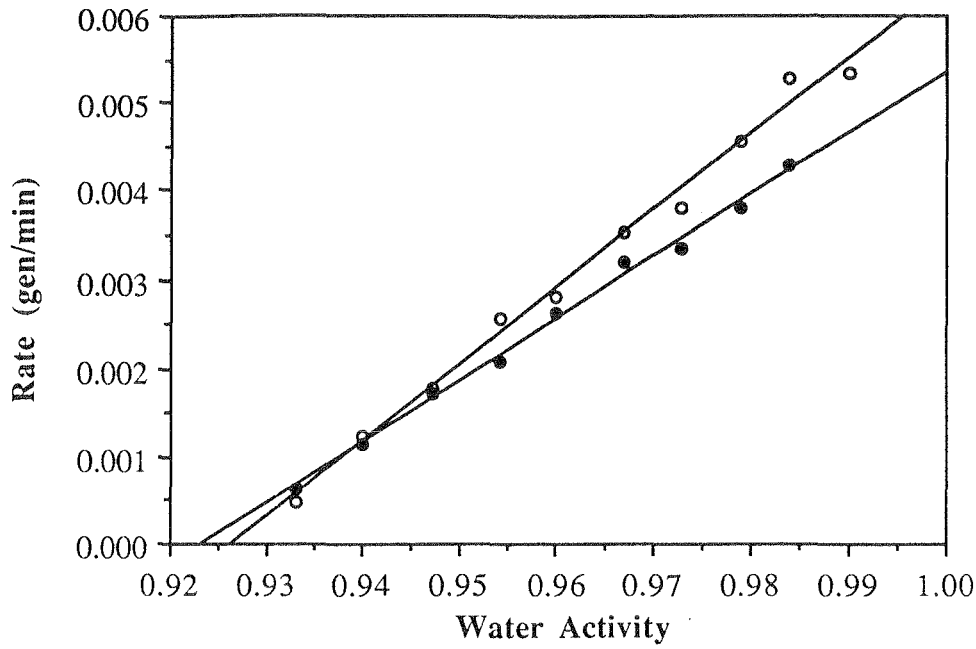


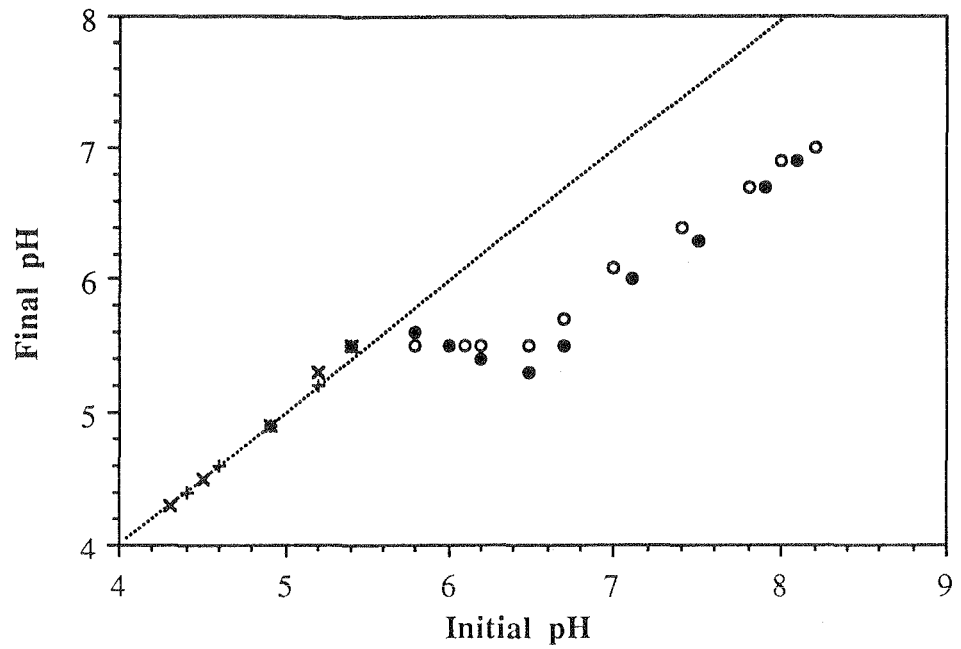
Fig. 4.5 The effect of water activity (NaCl as humectant) on the generation rate of *L. monocytogenes*. Scott A (●); Murray B (○).

MPD. The correction required was calculated to be of the order of 5 - 10% and, consequently, was not sufficient to explain the difference in estimated growth rates as pH decreased.

4.3.3 Environmental History Effects

The effect of growth of *Listeria monocytogenes* at sub-optimal water activity or pH, on the subsequent generation time of *L. monocytogenes* in non-limiting conditions is presented in Table 4.5. There was no consistent effect of preincubation conditions on the subsequent growth rate.

a.



b.

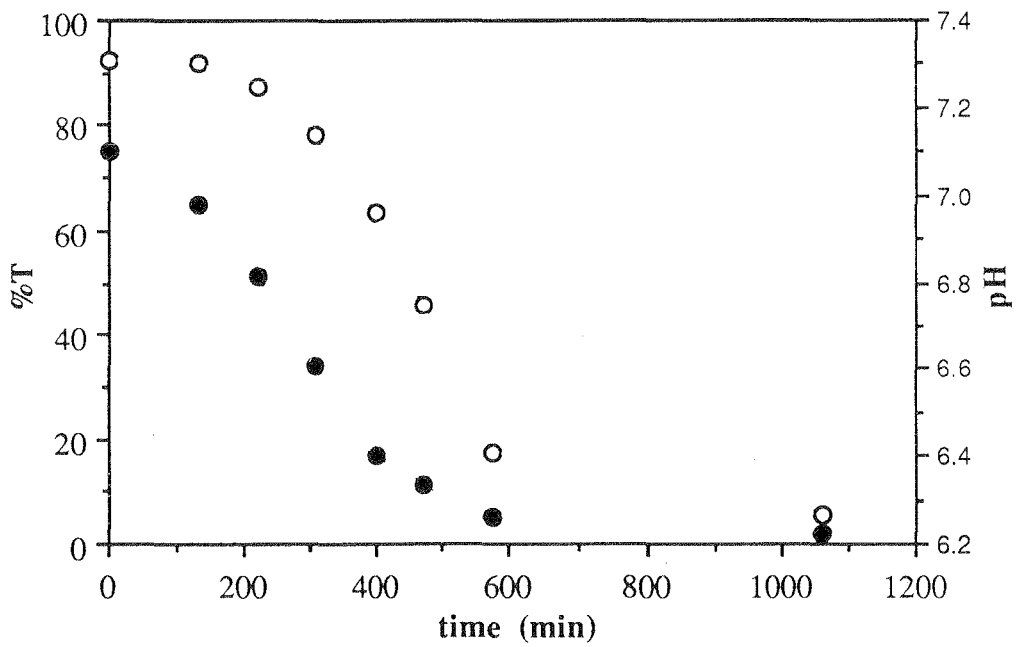


Fig. 4.6 a) Change in medium pH as a result of growth of *Listeria monocytogenes* at 19.5°C in TSB + 0.2M lactate. Strain Scott A (○), strain Murray B, (●). ×, +, represent cultures in which no growth occurred, strains Scott A and Murray B respectively.

b) Change in medium pH as a function of time and growth at 19.5°C of *L. monocytogenes* Scott A in TSB. pH of culture(○), %T of culture(●).

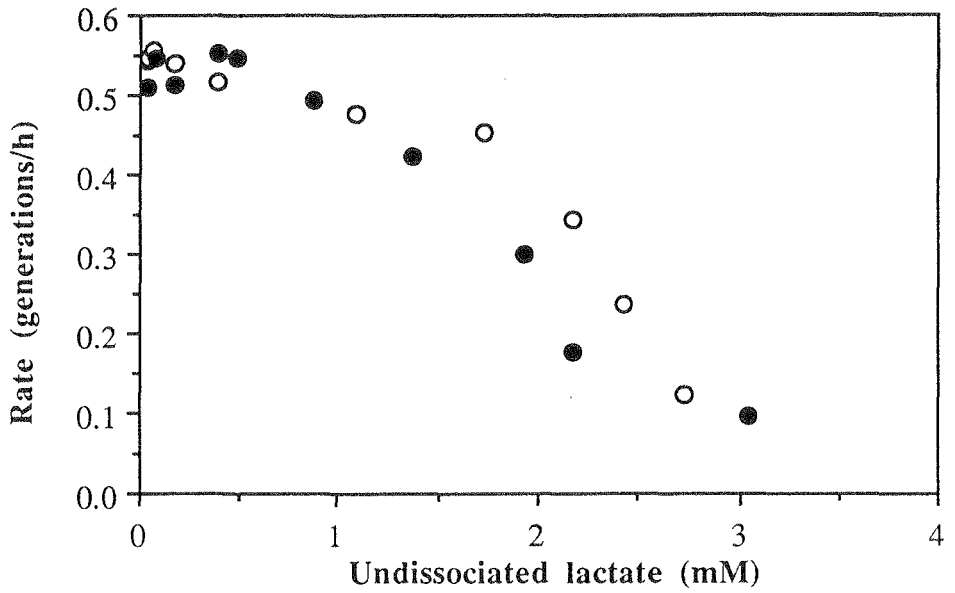


Fig. 4.7 Growth rate of *L. monocytogenes* strains Scott A (●) and Murray B (○) as a function of undissociated lactate concentration in the growth medium (TSB + 0.2M lactate at 19.5°C, $a_w = 0.990$).

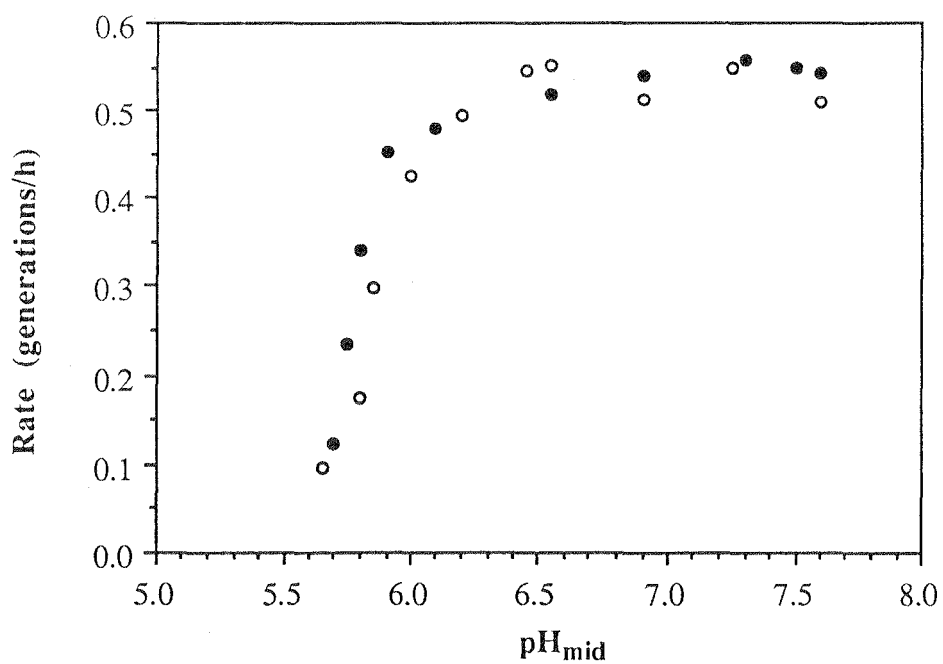


Fig. 4.8 Growth rate of *Listeria monocytogenes* strains Scott A (○) and Murray B(●) as a function of pH_{mid} in TSB + 0.2M lactate at 19.5°C.

Table 4.5 Effects of preincubation conditions on the subsequent generation time of *L. monocytogenes* in TSB at 19.5°C.

Strain	mls inoculum / mls medium	Pre-Incubation Conditions		Generation time (h)	
		water activity	pH	initial culture	subsequent culture
Scott A	0.1/15	0.990	6.5	2.82	1.76
	0.1/15	0.973	6.5	3.97	1.85
	0.1/15	0.954	6.5	5.90	2.38
	0.1/15	0.933	6.5	30.36	2.76
	0.4/15	0.990	7.6	1.96	1.70
	0.4/15	0.990	6.9	2.00	1.60
	0.4/15	0.990	6.0	2.41	1.65
Murray B	0.1/15	0.990	6.5	3.77	2.30
	0.1/15	0.973	6.5	4.49	1.90
	0.1/15	0.954	6.5	7.23	1.80
	0.4/15	0.990	7.6	1.89	1.62
	0.3/10	0.990	6.6	1.97	1.74
	0.4/15	0.990	5.8	2.99	1.74

4.3.4 Model construction

The data summarised above enable the construction of a number of models, but models were developed only for those organisms which had been most intensively studied, namely *S. aureus* 3b, and *L. monocytogenes* strains Scott A and Murray B.

4.3.4.1 Stochastic considerations

A clear dependence of $\mathrm{Var}(t_G)$ upon t_G was noted. Thus, other variance-homogenising transformations were considered. Variance in $\ln(t_G)$ and $1/\sqrt{t_G}$ for *L. monocytogenes* Scott A are presented in Fig. 4.9 as a function of mean generation time. The results do not clearly indicate which transformation of the response variable is more appropriate, but suggest that the square root transformation homogenises the variance at least as well as the logarithmic transformation. In the development of the models described below it is assumed that the variance is homogeneous in $\sqrt{\text{rate}}$.

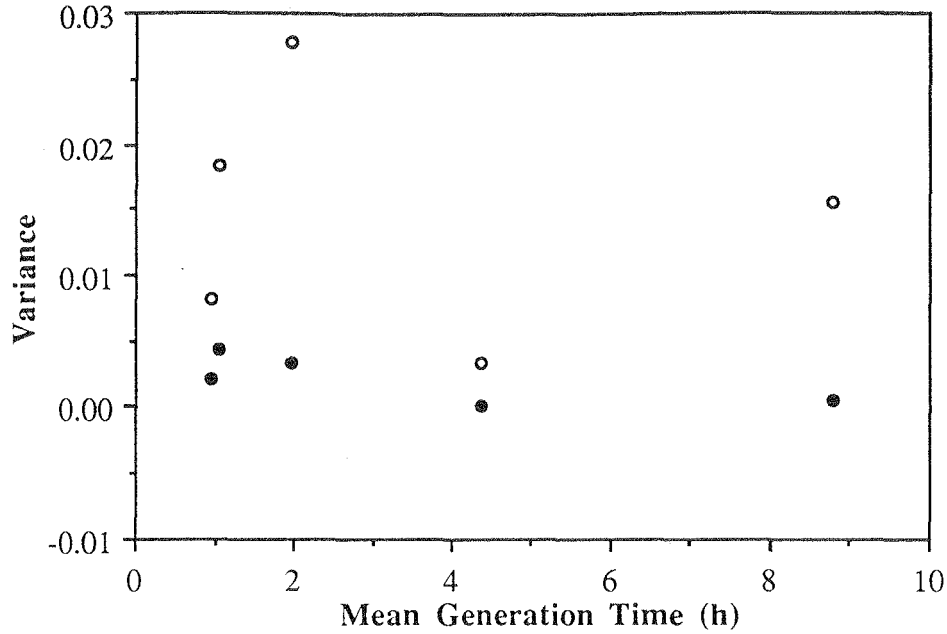


Fig. 4.9 Variance in transformations of generation time estimates of *Listeria monocytogenes* strains Scott A. Var(ln $(1/t_G)$): (○). Var$(1/t_G)$):(●)

McMeekin *et al.* (1993) reported that the error in the growth rate of *S. aureus* 3b was homogenous in √rate. Data obtained by the candidate (not presented) is consistent with this observation. From the data presented in Table 3.6 an estimate of the variance in √rate may be calculated and used to determine 95% confidence limits on the estimates of the models developed for *S. aureus* 3b. The confidence intervals for the *Listeria monocytogenes* models are based on replicate rate determinations at 19.5 °C, 0.997 a_w and pH 7.2.

4.3.4.2 *Staphylococcus aureus* 3b

In developing the iterative model the T_{min} of *S. aureus* 3b was estimated to be 8.02 ± 0.82 (SD) °C and $a_{w_{min}}$ estimated as 0.860 ± .0092 (SD) when NaCl is the humectant. To evaluate '*b*', these values were substituted into Eqn. 1.14 and equated to the mean of the replicated estimates of the generation time of *S. aureus* 3b at 27°C and a_w 0.997 (*see* Table 3.6). The model was subsequently corrected for the difference in GT estimates derived from Δ%T and VC data (*see* 3.3). Thus:

$$0.93\ (\text{h}) \quad = \quad 1/\left(b \times (T - 8.0) \times \sqrt{(a_w - 0.86)}\right)^2$$

$\therefore$ generation time$_{\Delta\%T}$ (h) = $1/(0.147 \times (T - 8.0) \times \sqrt{(a_w - 0.86)})^2$

$\therefore$ for $T \leq 36°C$, and $a_w \leq 0.997$

$$\text{generation time}_{VC}\ (\text{h}) = \frac{1}{\left(0.165 \times (T - 8.0) \times \sqrt{(a_w - 0.860)} \pm 0.12\right)^2} \tag{4.1a}$$

Parameter estimates (upper and lower 95% confidence limits in brackets) derived from nonlinear regression of the data to Eqn. 1.3 were '*b*' = 0.155 (0.1490, 0.1609), $a_{w_{min}}$ = 0.856 (0.852, 0.859), T_{min} = 7.5 °C (7.0, 8.0), and give rise to the following model:

$$\text{generation time}_{VC}\ (\text{h}) = \frac{1}{\left(0.155 \times (T - 7.5) \times \sqrt{(a_w - 0.856)} \pm 0.12\right)^2} \tag{4.1b}$$

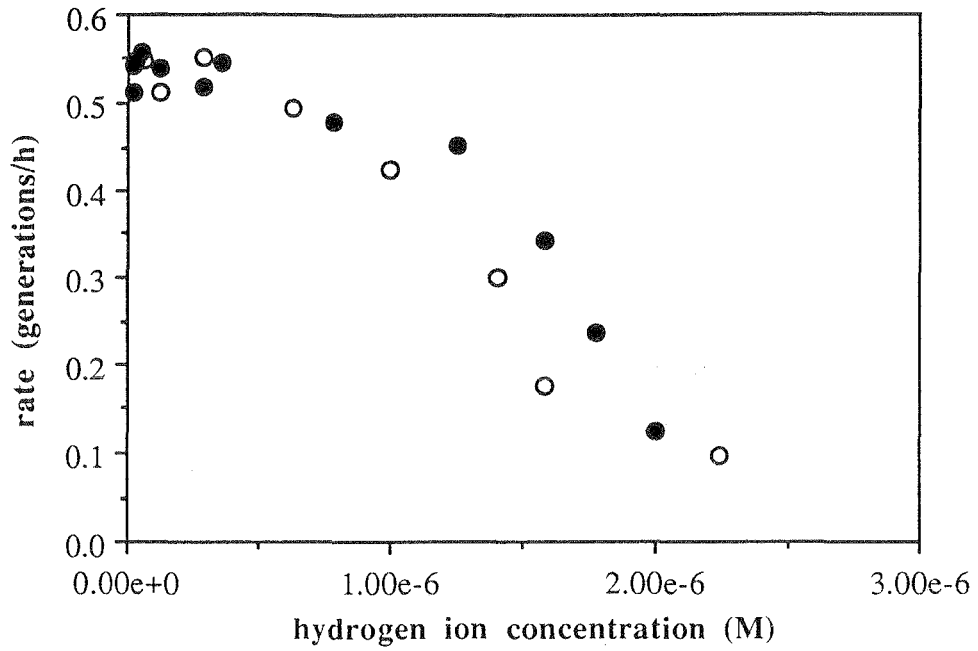
4.3.4.3 *Listeria monocytogenes*

The effect of pH (0.2M lactate) on growth rate (Fig. 4.7) indicates that there is little effect in the range pH 6.2 - 7.6, but that at pH < 6.2 the rate declines as a function of the pH and growth ceases at pH < 5.5. This response is not well described by the pH model presented by Adams *et al.* (1991). Cole *et al.* (1990) and Wijtzes *et al.* (1993) also presented models describing environmental effects, including pH, on growth rate. Cole *et al.* (1990) found a linear relationship between growth rate and hydrogen ion concentration. Fig. 4.10a shows that the decline in rate as a function of $[H^+]$ for both *Listeria* strains approximates a straight line only for $[H^+]$ concentrations greater than approximately 0.5 x 10^{-6}M (pH 6.3). A model of the type introduced, without explanation, by Wijtzes *et al.* (1993):

$$\sqrt{rate} = e \times (pH - pH_{\min}) \times \{1 - \exp[d \times (pH - pH_{\max})]\}$$

(similar to the pH model of Zwietering *et al.*, 1992a), was fitted to the *L. monocytogenes* Murray B pH-rate data. pH_{max} was set to 9.2, the literature value reported by Wijtzes *et al.* (1993). The fitted model and data are shown in Fig. 4.10b. Convergence was difficult to achieve, and it was noted that the estimates of the parameters *e* and *d* had extremely wide confidence intervals which were many times greater in magnitude than the estimate itself. These observations and the poor fit to the experimental data suggest that the model of Wijtzes *et al.* (1993) is not appropriate.

a.



b.

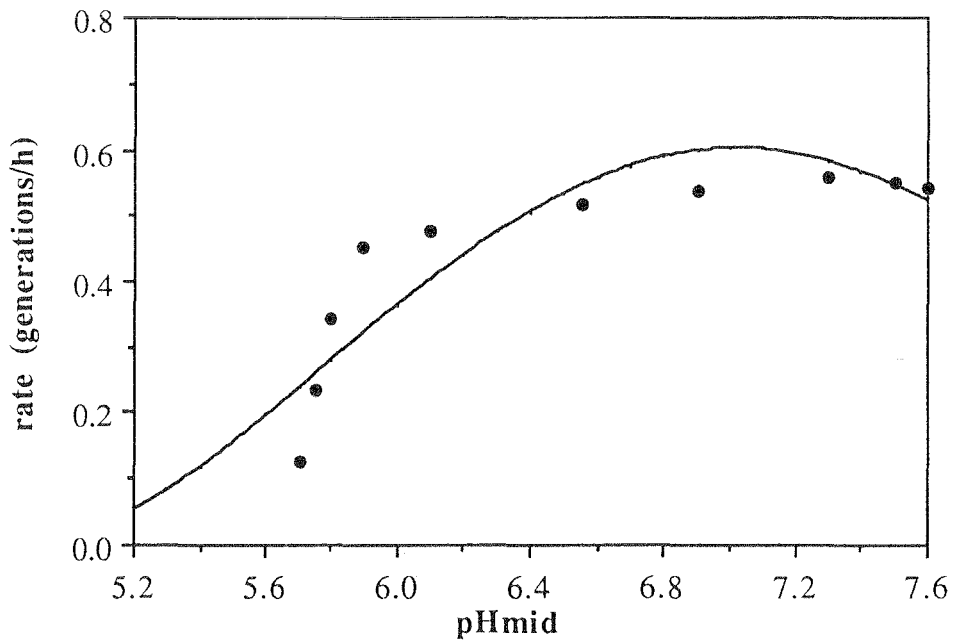


Fig. 4.10 a) Relationship between growth rate and [H^+] for *L. monocytogenes* strains Scott A (○) and Murray B (●)
b) The pH model of Wijtzes *et al.* (1993) fitted to data for the growth of *L. monocytogenes* Murray B. Data (●), fitted model (——).

Neither model adequately describes the response over the full data range. To simplify the 'iterative' model development, complementary models were developed. Below pH 6.2 the effect of pH is well described by a model of the type of Eqn. 1.5, but at higher pH a model of the type of Eqn 1.3 is sufficient. The following 'iterative' model for the generation time of *Listeria monocytogenes* Scott A, calibrated to viable count methods was derived:

For $T \leq 37°C$, $pH \geq 6.2$ and $a_w \leq 0.997$:

$$\text{generation time}_{VC}\ (\text{h}) = \frac{1}{\left(0.165 \times (T + 0.3) \times \sqrt{(a_w - 0.926)} \pm 0.10\right)^2}$$

and for $T \leq 37°C$, aw ≤ 0.997 and $pH \leq 6.2$ in the presence of 0.2M lactate:

$$\text{generation time}_{VC}\ (\text{h}) = \frac{1}{\left(0.197 \times (T + 0.3) \times \sqrt{(a_w - 0.926)} \times \sqrt{(\text{pH} - 5.5)} \pm 0.10\right)^2} \quad (4.2a)$$

Similarly, for *Listeria monocytogenes* Murray B:

For $T < 37°C$, $pH > 6.2$ and $a_w < 0.997$:

$$\text{generation time}_{VC}\ (\text{h}) = \frac{1}{\left(0.159 \times (T + 0.3) \times \sqrt{(a_w - 0.923)} \pm 0.12\right)^2}$$

and for $T < 37°C$, $a_w < 0.997$ and $pH < 6.2$ in the presence of 0.2M lactate:

$$\text{generation time}_{VC}\ (\text{h}) = \frac{1}{\left(0.191 \times (T + 0.3) \times \sqrt{(a_w - 0.923)} \times \sqrt{(\text{pH} - 5.5)} \pm 0.12\right)^2} \quad (4.3a)$$

To overcome the need to model the temperature/pH/water activity response surface by multiple equations, a function to describe the pH response which could be integrated with the temperature and water activity terms, was sought. The required function should have values near one over much of its range, decline to zero at the lower limiting value, and have some degree of interpretability. The function

$$rate \propto 1 - \frac{pH_{\min}}{pH}$$

fulfils some of these requirements but will always display the same rate of decline as a function of pH. Accordingly, the n^{th} root of the function was taken, which also takes values between 1 and 0, but the rate of decline can be varied to better fit the data. Thus, the term:

$$rate = b \times \sqrt[n]{1 - \frac{pH_{\min}}{pH}} \tag{4.4}$$

was devised. The function is nonlinear.

A new temperature-pH-water activity model of the form:

$$\sqrt{\text{rate}} = b \times (T - T_{\min}) \times \sqrt{(aw - aw_{\min})} \times \sqrt[n]{1 - \frac{pH_{\min}}{pH}} \tag{4.5}$$

was fitted to the data in Appendices 3.2 and 3.3. The parameter estimates of the models for *L. monocytogenes* strains Scott A and Murray B are presented in Table 4.6. The models corresponding to those parameter estimates for strains Scott A and Murray B will be referred to as Eqns. 4.2b and 4.3b respectively. The goodness of fit of the various iterative and nonlinear regression model is compared in Table 4.7.

4.4 DISCUSSION

Before discussing the results it is useful to reiterate that the parameters T_{min}, a_{wmin} and pH_{min} are *theoretical* lower limiting values of temperature, a_w and pH respectively. These values cannot be experimentally determined: by definition they are conditions at which the generation time is infinite. Consequently, they must be estimated by extrapolation. There is continued confusion in the literature concerning the meaning of these parameters with some authors (e.g. Neidhardt *et al.*, 1990; Wijtzes *et al.*, 1993), equating them with the lowest conditions at which growth is *observed.* The determination of the lowest conditions at which growth is observed is itself problematic. Some reports fail to distinguish between growth being possible, as opposed to simply not being detected within the time frame of the experiments, and many reports are based on detection systems which require many generations of growth before a positive result (i.e. growth) is recorded. Both lag and generation times are increasingly prolonged as conditions for growth become less favourable, thus investigators must be cautious in declaring that growth is not possible under a particular set of conditions. Using the predictive models developed in this chapter, several published experimental methods to determine minimum conditions for

Table 4.6 Parameter estimates for *L. monocytogenes* strains Scott A and Murray B fitted to Eqn. 4.5 (Δ%T data).

Parameter	Scott A (Eqn.4.2b)	95% confidence interval	Murray B (Eqn. 4.3b)	95% confidence interval
b	0.176	0.157 to 0.195	0.156	0.141 to 0.171
T_{min} (°C)	-0.1	-1.2 to +1.0	+0.1	-1.5 to +1.7
a_{wmin}	0.930	0.927 to 0.934	0.930	0.926 to 0.934
pH_{min}	5.65	5.63 to 5.66	5.70	5.70 to 5.70
n	0.131	0.071 to 0.192	0.063	0.018 to 0.107

Table 4.7 Mean Square Error (√rate) of Models Developed in this Chapter for *S. aureus* 3b, and *L. monocytogenes* strains Scott A and Murray B

Organism	**Mean Squared Error** (√rate) "Iterative" Method	Eqn.	Nonlinear Regression	Eqn.
S. aureus 3b	0.006143	4.1a	0.005231	4.1b
L. monocytogenes				
Scott A	0.01572	4.2a	0.003767	4.2b
Murray B	0.02382	4.3a	0.005187	4.3b

growth were analysed. For some (e.g. Tapia de Daza *et al.*, 1991; Farber, *et al.*, 1992) the duration of the experiments would have precluded sufficient generations (even ignoring lag phase duration) for growth to be recorded by the criterion used (e.g. time to turbidity, time for 1000-fold increase). Thus, conclusions regarding combinations of factors which preclude growth must be interpreted with caution.

4.4.1 Responses to Individual Constraints

4.4.1.1 Temperature and water activity

Troller (1986) points out that the minimum values of temperature and water activities which permit the growth of *Staphylococcus aureus* can be specific to the test conditions. Many reports, for example, indicate limiting a_w values in the region 0.90 to 0.86. From several of the compilations of published values of limiting temperatures and water activities for *S. aureus* growth (Jay, 1986; Beuchat, 1987; Troller, 1986; Ewald and Notermans, 1988), a temperature range for growth of from 7 to 45°C, and a lower water activity limit of 0.86 are the most frequently observed. A lower temperature limit for growth of 6.5 °C for many strains was reported by Schmidt *et al.*, (1990). Troller (1976) observed growth of *S. aureus* at water activities as low as 0.83. Jay (1986) concludes, however, that 0.86 a_w and 7°C are the generally recognised minimum water activity and temperature for growth.

Minimum growth temperatures reported for *Listeria monocytogenes* are typically near zero. Walker *et al.* (1990) reported values for three strains in the range -0.4 to -0.1°C; Junttila *et al.* (1988) reported values in the range 0.5° to 3.0°C for 77 pathogenic strains. Other workers observed growth of *L. monocytogenes* on commercial meat products at 0.1°C (Grau and Vanderlinde, 1992) and at 0°C in a model meat system (Grau and Vanderlinde, 1993). Estimates of T_{min} range from -2.55 to -1.75°C (Wijtzes *et al.*, 1993), -2.2 to -2.4°C (Grau and Vanderlinde, 1993), and values in the range -4.5 to -1.5°C may be calculated from the data presented in Walker *et al.* (1990). The latter data set covers the range 0 to 9.5 °C only.

The minimum water activity for the growth of *L. monocytogenes* was found to be 0.92, for NaCl as humectant, by several investigators (Miller, 1991; Tapia de Daza *et al.*, 1991; Farber *et al.*, 1992). Wijtzes *et al.* (1993) estimated the $a_{w_{min}}$ for *L. monocytogenes* to be 0.912 - 0.916.

The minimum pH for *L. monocytogenes* growth was reported to be 4.39 (George *et al.*, 1989). Wijtzes *et al.* (1993) estimated pH_{min} (HCl as acidulant) as 4.15 from pH data in the range pH ≤ 6.3, and 4.03 from pH data in the range pH ≤ 6.7. In phosphate buffered TSB containing 0.1M citrate, Cole *et al.* (1990) found that the lowest pH which permitted growth (~10^4 increase in numbers at 30°C within 60 days) was 4.66.

4.4.1.2 Derived estimates

The parameter values derived from the data presented in this chapter are generally consistent with literature values, and estimates obtained by either method of model development are very similar. To simplify discussion, the organisms will be considered separately.

Staphylococcus aureus

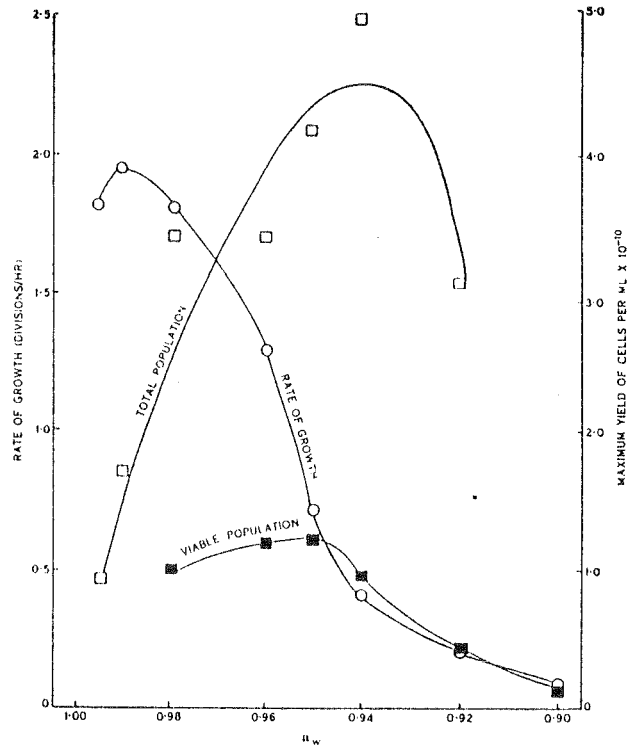
The estimate of a_{wmin} (NaCl) for *S. aureus 3b* is very consistent with previously reported values for the lower water activity limit. Some strain to strain variation was noted (Table 4.3). Scott (1953) reported no difference in the minimum water activity for growth of *S. aureus* when either sucrose or NaCl were the primary humectants for many strains. Similarly, from the results of Broughall *et al.* (1983) using glucose as humectant, an a_{wmin} of ~0.86 is estimated. Conversely, Nunheimer and Fabian (1940) found that sucrose prevented growth of *S. aureus* at a_w 0.95 - 0.93. Li and Torres (1993b) estimated lower limits for growth using various humectants by extrapolation of a straight line fitted to rate *vs* a_w. Their estimates of a_{wmin} for *S. aureus* ATCC 13566, derived from data in the range 0.980 - 0.947 were close to 0.93 for both NaCl and sucrose as the primary humectants. A partial explanation for the divergence of reported values may be found by considering the actual growth rate response to water activity as distinct from the modelled response. Fig. 4.11a is reproduced from Scott (1953). Fig. 4.11b is derived from Broughall *et al.* (1983) using the method of McMeekin *et al.* (1987). These figures, and Figs. 4.2 - 4.4, display curvature in the water activity-rate response. A similar trend is apparent in analogous data for *Staphylococcus xylosus* (McMeekin *et al.*, 1987). Li and Torres (1993a) alluded to the same effect.

A consequence of fitting a straight line to such data is that the water activity value at which the fitted line corresponds to zero rate will depend upon the range of data. Data collected at high water activities will lead to higher estimates of a_{wmin}, whilst data predominantly at the lower extreme of the water activity range will give rise to lower estimates of a_{wmin}. This emphasises the importance of gathering data over as full a range of the response variable as possible (McMeekin *et al.*, 1993, Chapter 2), and also the risks of extrapolation beyond the range of data used to generate models. In practical terms, a straight line is a good approximation to the water activity response within a given range, but would be expected to result in overprediction of the rate in the middle of the range being modelled, and underestimation of rate at the upper and lower extremes of the range modelled. This pattern of residuals was found upon examination of the residuals of the fitted values of Eqn. 4.1a and b. Nonetheless, the observed values may be well approximated within the range of the data.

Thus, if the experiments on sucrose had been continued for a longer time period, or conducted at temperatures nearer the optimum for growth, a response similar to that observed by Scott (1953), i.e. Fig. 4.11a, may have been observed.

A slight curvature was also noted in many square root plots ($\sqrt{}$rate *vs.* temperature), and has the same predicted consequence, i.e. if less data is collected at the lower end of the temperature range, a higher estimate of T_{min} would be expected.

a.



b

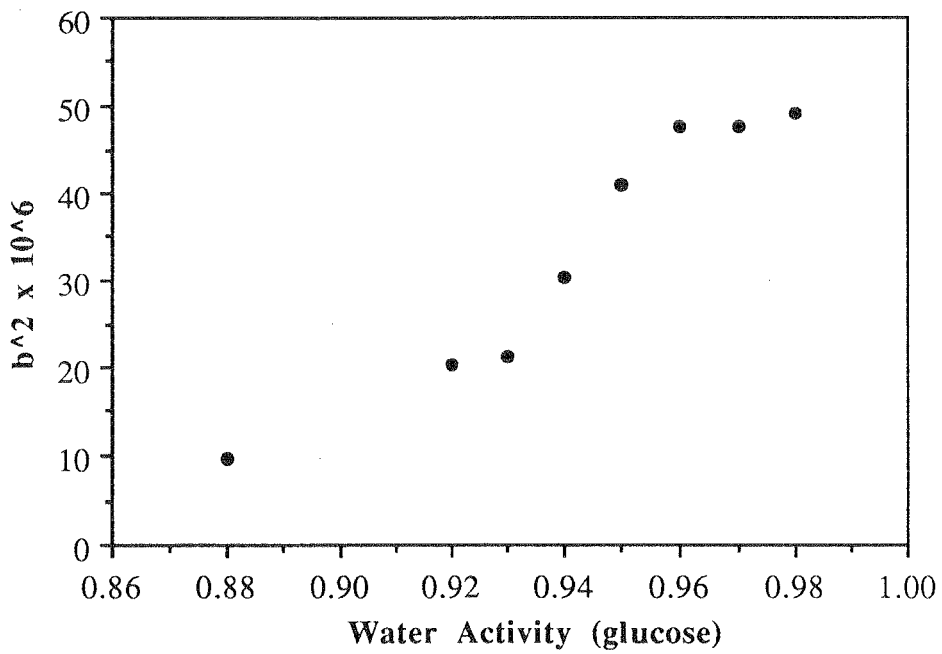


Fig. 4.11 Relationship between water activity and growth rate of *S. aureus*. a) Reproduced from Scott (1953) for strain 49/1974 growing in BHIB with modified water content. b). Data of Broughall *et al.* (1983) for strain NCTC 10655 in glucose modified UHT milk, analysed by the method of McMeekin *et al.* (1987) .

This was demonstrated in Fig. 4.1. The same analysis was applied to the results of Broughall *et al.* (1983) and is presented in Fig. 4.12. The similarity of these responses is consistent with suggestions (M. Cole, pers. comm.) that the relationship between temperature and microbial growth rate may consistently deviate from that described by a 'square root response'. This possibility is more fully discussed in Chapter 7. Once again, in practice the deviation may be small, and still give acceptable predictions within the range of the data used to generate the model. These possibilities will be evaluated in Chapter 5.

Listeria monocytogenes

The estimates of a_{wmin} determined here are generally consistent with literature values, but the estimates obtained by either method of model development were consistently different and were explored more closely. Linear regression of the data presented in Fig. 4.5, which were obtained from a single experimental block, yielded a_{wmin} estimates of 0.923 and 0.926 for strains Scott A and Murray B respectively. Other experimental blocks contained data at 19.5°C and a_w 0.990. When these were combined with the data in Fig. 4.5, and a_{wmin} estimated from the combined data the a_{wmin} estimates were both slightly higher because all the additional data had faster rate values at 19.5°C and, in consequence, 'skewed' the fitted line. The same effect would have occurred when the entire data set was fitted to Eqn. 1.5 by nonlinear regression. The data presented in Fig 4.5, from a single experimental block, is self consistent both within and between strains. Rate data from other experiments is not consistent. The reasons for the differences are unclear, but suggest a lack of control of some variable between experiments. It is possible that the rate of rocking of the TGI, which was not well controlled between experiments may be responsible, e.g. due to the differences in the rate of aeration of the cultures, oxygen depletion may be affecting observed growth rates at the high population levels being measured. Pearson and Marth (1990a,b) reported shorter lag times (4.41h *cf.* 8.15h), shorter generation times and higher MPDs (9.18 *cf.* 8.39 $\log_{10}$CFU/ml) for *L. monocytogenes* cultures which were agitated rather than quiescent. Whilst this inconsistency may lead to biased estimates of the parameters when fitting the data by nonlinear regression, the manual method of modelling will be insensitive to such differences and may, from this perspective, be preferable.

The estimates of T_{min} derived for both organisms from the original experimental block were higher than published estimates, and even when the data set used for T_{min} estimation was supplemented with data from other experimental blocks, the results were not consistent with reports cited in 4.4.1.1. The reason for the relatively high estimates is unknown, but does accentuate the need for replication. The problem will be manifest when applying the model to independent

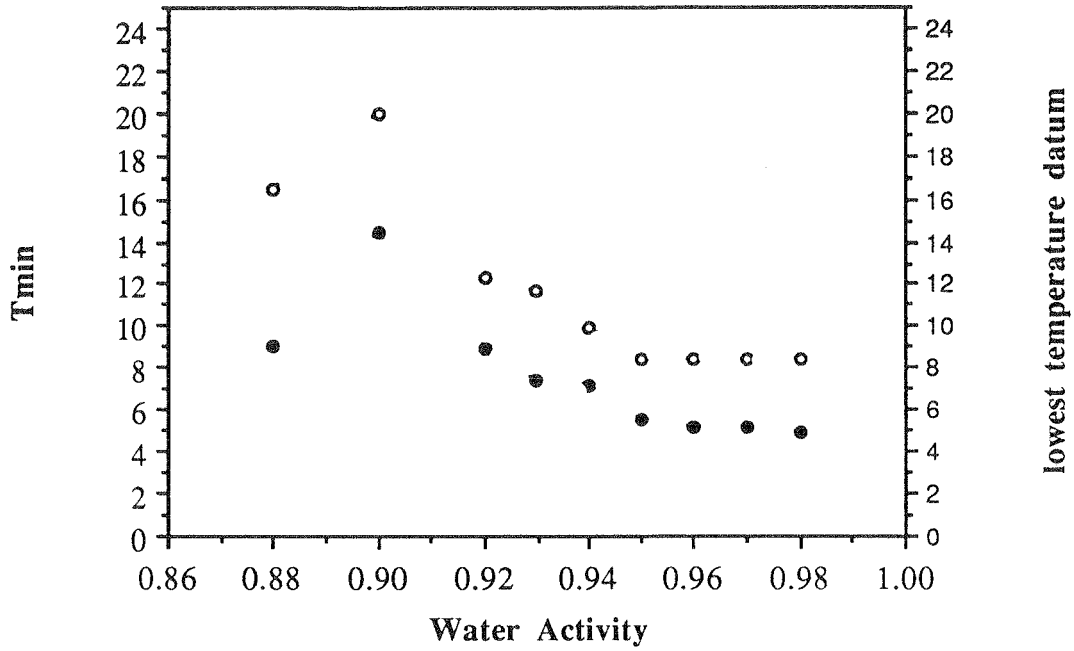


Fig. 4.12 Relationship between the estimate of T_{min} (●) and the lowest temperature (○) at which growth was recorded for the data of Broughall *et al.* (1983).

data for the growth of these organisms, and would be expected to lead to overestimation of the generation times at temperatures a few degrees above 0°C. Despite the known discrepancy in the estimates of T_{min}, these data sets were selected for analysis to provide a rigorous evaluation of the hypotheses summarised at 4.4.4.

4.4.1.3 pH response

The pH response noted here is inconsistent with others previously described in the predictive microbiology literature (Adams *et al.*, 1991; Cole *et al.*, 1990, Wijtzes *et al.*, 1993) but is consistent with the more general response described by Neidhardt *et al.* (1990), i.e. a range of one or two pH units in which growth rate is largely unaffected by pH, beyond which growth rate declines rapidly as a function of pH. From the results presented in this chapter, inhibition due to pH appears to become significant only below pH ~6.2. Pearson and Marth (1990b) reported that in milk at pH 6.28 the growth of *L. monocytogenes* was not significantly different to that in laboratory broth (pH 7.15), and also noted that *L. monocytogenes* decreased the pH of a modified Tryptose Phosphate Broth by ~0.5 pH unit. George *et al.* (1988) and

Petran and Zottola (1989) recorded similar decreases in pH in broth media containing sugars as a result of *Listeria* growth. The decline in growth rate below pH~6.2 is approximately proportional to the decline in pH, as observed by Adams *et al.* (1991) but also appears to be proportional to the decline in $[H^+]$ as reported by Cole *et al.* (1990). Extrapolation of the 'straight line portion' of the response for pH < 6.2, for either independent variable, results in a lower limiting value corresponding to pH ~ 5.5.

The data of Adams *et al.* (1991) for *Yersinia enterocolitica* are restricted to the range pH < 6.4 and, thus, may represent suboptimal pHs only. For the data of Wijtzes *et al.* (1993) the estimate of pH_{min} most consistent with published data was obtained from data in the range pH < 6.3 only. Extending the range to pH< 6.7 resulted in a lower estimate of pH_{min} which would be expected if the rate above pH ~6.2 did not change as a function of pH, i.e. the fitted line would be less steep and have a lower pH axis intercept. The pH range tested by Cole *et al.* (1990) was pH < 7.0. Thus, the apparent qualitative differences may again be a reflection of the data range used, and the use of a model inadequate to fully describe the response.

The inability of *L. monocytogenes* to grow at pH 5.5 in the presence of lactate has been noted before. Irvin (1968) reported complete growth inhibition in silage with pH slightly below pH 5.5, but observed growth just above 5.5. Ryser *et al.* (1985) found a similar response in cottage cheese. In silage at pH < 5.5, viable counts of cells of *L. monocytogenes* inoculated into the silage declined (Irvin, 1968). A similar limiting pH was found in cultured and uncultured whey (Ryser and Marth, 1988). The results of Buchanan *et al.* (1993b) for the rate of inactivation of *L. monocytogenes* due to lactic acid/pH also seem consistent with these observations. The latter authors inferred that while growth was possible in the presence of low lactate concentrations at some pH's, growth was not possible below pH 5 in the presence of ≥ 0.1 M lactate. They also concluded the rate of inactivation was in part due to the concentration of undissociated lactate. At pH 5.5 approximately 97.9% of the lactate is in the dissociated form. In the presence of 0.2M lactate this corresponds to 4.3 mM of the undissociated acid. If the inhibition were solely due to the undissociated form, in the presence of 0.1M lactate growth would not be expected to occur at pH < 5.2.

By sequentially removing the lowest pH datum in the data set it was noted that the estimate of pH_{min} fitted to Eqn. 4.5 was always very close to the lowest pH at which data were available. Attempts were made to fit the pH data of the *L. monocytogenes* Scott A and Murray B (*see* Fig. 4.8) to Eqn. 4.4 but convergence was not achieved. Even using the fitted parameter estimates (pH_{min}, n) shown in Table 4.6, the fit to the data at pH values below pH 6.2 was poor. This was confirmed by comparing the residuals of the 'iterative' and the nonlinear regression models. In the majority of conditions the nonlinear regression model estimate was

closer to the observed value, but for those data at pH < 6.2, the two component 'iterative' models produced estimates which were orders of magnitude closer to the observed value. More promising results were obtained when $[H^+]$ was used as the independent variable in Eqn. 4.4. This work is continuing.

4.4.2 Environmental History Effects

The effect of preincubation conditions on subsequent growth rate of *L. monocytogenes* Scott A presented in Table 4.5 suggest that there may be a water activity history effect. The analogous results for strain Murray B, however, contradict this conclusion. Scott (1953) alluded to a lack of water activity history effect also: "...cells grown at 0.96 a_w had no greater capacity to form colonies at low a_w s than cells grown at 0.999". There is no evidence of a pH history effect from the, albeit limited, data presented.

4.4.3 Strain to Strain Variability

The similarity between estimates of the parameters T_{min}, $a_{w_{min}}$, and pH_{min} for two strains of *Listeria monocytogenes* considered in this work suggests that a single model may be sufficient for this species for a given acidulant and humectant. Literature values for growth limiting NaCl concentrations for other strains are also consistent with the results presented here, and literature values for T_{min} are also very similar between strains. Similarly, the lower water activity limit for *S. aureus* growth is reasonably consistent between strains, as remarked upon by Scott (1953). The greater variation reported in this chapter may arise, in part, from the difference between a_{wmin} an the limiting a_w for growth and their respective methods of estimation. With regard to temperature, Schmidt *et al.* (1990) found that for 77 strains of *S. aureus* the minimum temperature for growth was found to range between 6.5 and 12.5°C. This does not of necessity mean that the T_{min} range is as wide. When one considers the sources of errors referred to earlier, Table 4.1 may indicate that T_{min} may be more consistent than the observed lowest growth temperature. Nonetheless, the T_{min} estimates reported in this chapter appear to be higher than would be expected from the literature. If it can be shown that one or more of the parameters of the square root type models are constant between strains, the work involved in the generation of models would be further reduced. The significance of this possibility justifies further study.

4.4.4 Evaluation of Hypotheses

The hypotheses in 4.1 can be summarised as follows:

i) that the effects of temperature, water activity and pH on microbial growth are independent and, in consequence, that the parameters T_{min}, a_{wmin} and pH_{min} are independent

ii) that this knowledge allows the unambiguous definition of the response surface by the independent estimation of the values of these parameters and their sequential incorporation into models of increasing complexity without the need for nonlinear regression modelling expertise

iii) that these requirements may be met by the use of an economical experimental design in which the response to each variable is well defined, by intensive one-variable experiments, which obviates the need for complex response surface designs.

4.4.4.1 Independence of effects

The independence of the effects of temperature and water activity on microbial growth rate demonstrated in this study agree with previous reports (Davey, 1989a, Adams *et al.*, 1991; Chandler and McMeekin, 1989b, c; Buchanan *et al.*, 1993a). No experimental results were obtained to assess potential interactions between pH and temperature, or pH and water activity, but there were no significant correlations between the parameters T_{min}, a_{wmin}, and pH_{min} fitted by nonlinear regression to any data set. This conclusion was reinforced by sequentially fitting parameters by nonlinear regression: the same parameter estimates were generated irrespective of the starting values, or realistic values assigned to other parameters. Fitted T_{min} and a_{wmin} values for both *Listeria* data sets after deletion of data for pH < 6.2 were within 0.1°C and 0.0002 a_w of the estimates from entire data sets. Estimates of the parameter values also appear to be insensitive to the response used to generate them (i.e. $1/t_G$, $1/\Delta\%T30$, 1/impedance detection time, 1/M etc.), but may be dependent upon the range of the data used to estimate them.

4.4.4.2 Iterative approach

The similarity of the parameter estimates obtained by the two modelling approaches support, in general, the hypothesis that square-root-type models can be constructed by sequential experiments to determine the values of parameters, and the variability in the response and the scaling constant. The models developed for *S. aureus* 3b best illustrate this point. As suggested above, slight differences in the a_{wmin} estimates for the complementary *L. monocytogenes* models by the two methods may be due to, as yet, unexplained inconsistencies in the data from separate experimental blocks. The pH_{min} estimates are not truly comparable because of the different functions fitted.

The MSEs of models generated by the two methods show that nonlinear regression produces models that better describe the data. The MSE of the nonlinear regression model to the *S. aureus* 3b data set is only slightly better than that by the 'iterative' method. The difference is much greater for the two *L. monocytogenes* data sets. Consideration of the data used to generate the model explains much of the

Table 4.8 Parameter estimates of Eqn. 1.3 for the *L. monocytogenes* data in Appendices 3.2 and 3.3, after removal of low pH data.

Parameter	Scott A	95% confidence interval	Murray B	95% confidence interval
b	0.161	0.152 to 0.169	0.166	0.147 to 0.170
T_{min} (°C)	-0.1	-1.1 to +0.9	+0.2	-2.0 to +1.7
$a_{w_{min}}$	0.930	0.927 to 0.933	0.930	0.926 to 0.934

difference. It was pointed out above that the water activity data set had anomalously low rate values. In attempting to produce the best fit to the data set it is probable that these values will lead to larger residuals. This was confirmed by examination of the residuals. A distinction must be made, however, regarding the ability of a model to describe well a specific data set and its ability to model the general response. This can be assessed only by comparing predictions to novel data, as will be considered in the Chapter 5.

4.4.4.3 Experimental design

The full data set for *S. aureus* 3b covers a large proportion of the temperature-water activity response surface, but an almost identical model could be derived by considering the temperature response at a single value of water activity, and the water activity response at a single value of temperature. This observation was applied in the development of the *L. monocytogenes* models. The use of a minimal experimental design for the generation of *L. monocytogenes* models did reveal potential pitfalls in the design, as intended. In using such a minimal design, the relative weight of an anomalous result will be greater. Furthermore, while it is easy to recognise inconsistencies within the results of a single experimental block, it may be difficult to perceive inconsistencies between data from separate experimental blocks, particularly if the data gathered only intersect at one point in the variables space as is the case with the experimental design proposed (*see* Appendix 3, Fig. A2). Thus, unless the source(s) of these inconsistencies can be found and controlled, a more conservative design may be required which measures the full range of each variable at two or more values of each other variable. This is also borne out in the

T_{min} estimates for both *L. monocytogenes* data sets. The experiment from which the T_{min}s were to be estimated yielded a value inconsistent with literature reports, and was re-estimated after additional data became available. In the absence of literature values, however, the initial estimate would have been accepted. Had T_{min} been estimated from a separate but analogous data set, the inconsistency would have been detected and investigated.

Nonetheless, the methodology proposed appears to satisfy almost all of the desirable features of a response surface design (*see* Table 2.1), with the exception perhaps of point 1: the distribution of information throughout a region of interest. In the case of the development of temperature-water activity-pH kinetic models it appears that extrapolation of the qualitative response to one factor to all values of the other factors, is valid. This is possible because of the independence of the effects of pH, temperature and water activity on microbial growth rate. If the results presented in this chapter are widely representative of bacteria, it should be possible to construct efficiently models for the combined effects of these three parameters, using the proposed methodology, but subject to the limitations discussed above.

4.4.5 Summary

The results presented in this chapter provide support for the hypotheses presented in 4.1. The practical utility of the models generated has not yet been considered. Comparison of the predictions of models developed in this chapter to independent data may reveal important differences in the two methods of model generation and will be assessed in Chapter 5.

A number of important considerations in model development are, however, highlighted by the results presented in this chapter.

i) It is important to measure responses over as wide a range as possible to obtain consistent estimates of the parameters of square-root type models.

ii) there may be systematic deviations from the microbial growth rate responses to temperature, water activity and pH predicted by Eqn. 1.5

Points i) and ii) may not be significant in practice, provided that predictions are made only by interpolation within the data range used to generate a model.

iii) the experimental design proposed in this chapter may be too simplistic and may fail to reveal anomalous or aberrant results

iv) anomalous, but self-consistent data, from single experimental blocks suggest that there are important, but uncontrolled, variables in the experimental protocol used to generate the data in this chapter.

v) it is important to compare observed and predicted values individually, e.g. simultaneous plotting of predicted and observed responses, and examination of the residuals for patterns of deviation, to fully assess the appropriateness of models.

5 MODEL EVALUATION

5.1 INTRODUCTION

In 2.4 it was stated that models must be shown to predict accurately the behaviour of microorganisms in foods in commercial processing and distribution before they can be used in practice. Demonstration of this ability, a process generally termed *validation*, remains an ill defined aspect of predictive microbiology. Even though some models are claimed to have been validated, a standard method or set of criteria has not been published to date, by which a predictive model for the growth of microorganisms in foods can be said to be validated.

Wijtzes *et al.* (1993) plotted literature values for the generation time of *L. monocytogenes* against the corresponding predictions of a model generated in TSB. From this plot, predictions which would be unsafe in practice could be readily visualised, and the overall reliability of the model assessed. Duh and Schaffner (1993) developed a model for *Listeria* growth based on rates measured in BHIB. Complementary literature values for the growth of the organism in food were then added to the data set and the model fitted to the supplemented data. The close similarity in MSE and r^2 values of the equations fitted to either data set were taken as an indication of the reliability of the models when applied to foods. Another measure of the accuracy of models was introduced by McClure *et al.* (1993). Those authors compared their models on the basis of the sum of the squares of the differences of the natural logarithm of observed and predicted values:

$$\sum\left(\ln(GT_{published}) - \ln(GT_{predicted})\right)^2 \qquad (5.1)$$

A smaller value indicates a model which, on average, better predicts the observed response.

Two important aspects of the predictive ability of a model are its precision, and freedom from systematic error, or bias. In this chapter, indices of bias and precision of models are developed and used to assess the goodness-of-fit of published models to the data used to generate them. Values of these indices are used as a baseline from which to assess the goodness-of-fit of models to independent data. Similarly, predictions from models developed in Chapter 4 are compared to novel and literature data broadly relevant to the range of conditions for which the models were developed. Bias and precision indices are compared to evaluate the effect of the method of model generation on the precision and accuracy of Eqns. 4.1 - 4.3. Despite that Eqns. 4.2a, b and 4.3a, b are based on apparently anomalous estimates of T_{min}, for the purposes of this thesis further T_{min} determinations were not undertaken. Instead, the practical consequences of points i) - iii) in 4.4.5 were investigated by assessing the models without alteration.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Full details of reagents and equipment used are presented in Appendix 2.

5.2.2 Data Sources and Bases of Comparison

5.2.2.1 Data sources

Data for the growth of the modelled microorganisms, independent of that used to generate the models, were obtained from three sources: i) by determination of the rates of growth of *S. aureus* 3b inoculated on to foods of varying water activity (described in 5.2.3) and stored at various temperatures; ii) Within a collaborative project[1], Dr. F. Grau and Mr. P. Vanderlinde of the CSIRO Meat Research Laboratory, Brisbane, Australia modelled the growth of *L. monocytogenes* Murray B in a range of meat products. Comparisons are based on results presented in Tables 1 and 2 of Grau and Vanderlinde (1993) and a third unpublished data set. iii) By reference to other published data for the growth of the modelled organisms.

Some of the literature values were manually calculated (*see* Fig. 3.1) from growth curves presented in publications and must be considered as approximate values. Relevant information (i.e. pH, temperature, a_w) upon which to base predictions was not documented in all publications. In some cases, representative values appropriate to the product were used. Alternatively, values cited in other publications (Buchanan and Phillips, 1990; Buchanan *et al.*, 1993a; Wijtzes *et al.*, 1993) in which literature values were compared to model predictions, were adopted to facilitate comparison between published models and those developed in Chapter 4. For data in which the temperature exceeded the range of the modelled data, the predicted rate at the highest temperature in the modelled data set was used.

For publications in which a range of generation times were recorded for one or more strains, the shortest recorded generation time was chosen as the basis for comparison so that the ability of the models to make 'safe' predictions was assessed. Where the models predicted growth rates ≤ 0 (i.e. infinite generation times), predictions could not be included in calculation of the precision and bias estimates and were omitted. Similarly, if growth was predicted but not observed, the logarithm of the ratio could not be calculated. These data were also omitted from calculation of the indices.

To assess further the reliability of the square-root type models developed, and in contravention of sound modelling practice, predictions were made by extrapolation beyond the range of the temperature data used to generate the model, in the suboptimal range only (i.e. predictions were made in the region between T_{min} and 6°C, the lowest temperature at which growth was recorded). Bias and precision

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[1] Jointly funded by the following Australian agencies: Rural Industries Research and Development Corporation; Meat Research Corporation; Dairy Research and Development Corporation and Pig Research and Development Corporation.

indices were calculated both with and excluding extrapolated predictions.

5.2.2.2 Indices of bias and precision

Generation time predictions from Eqns. 4.1 - 3 were assessed for their deviation from the observed rates. A number of indices were considered, but a measure similar to Eqn. 5.1 was considered most appropriate. To permit the derivation of an 'average' deviation, the *ratio* of the predicted and observed generation times was chosen to standardise the deviation because the data varied greatly in magnitude. The ratio alone, however, may be misleading because a 'factor of 10' underprediction of generation time (observed/predicted = 10) will have more weight than a 'factor of 10' overprediction (observed/predicted =0.1) in the calculation of a mean. Thus, the logarithm of the ratio was chosen so that over- and underprediction were given equal weight in determining the average deviation, i.e. the geometric mean of the standardised deviation was calculated. The antilogarithm of this value was then calculated which may be interpreted as the average ratio of the predicted and observed generation times.

$$\text{BIAS factor} = 10^{\frac{\sum \log(GT_{observed}/GT_{predicted})}{n}}$$

A value less than one indicates that the predicted generation time is, on average, greater than the observed generation time and is thus 'fail dangerous'. Conversely, a value greater than one indicates that the model is 'fail safe'. Under- and over-prediction will tend to 'cancel out' in this measure which, consequently, provides no indication of the average precision of estimates. The average of the sum of the *absolute* values of the logarithm of the ratio (similar to Eqn. 5.1 in which the *square* of the ratio is used to make all values positive) was thus calculated. The antilogarithm of this value will always be greater than or equal to one.

$$\text{PRECISION factor} = 10^{\frac{\sum \left|\log(GT_{observed}/GT_{predicted})\right|}{n}}$$

The larger the value, the less precise is the average estimate. Thus, a precision factor of two indicates that the prediction is, on average, a factor of two different from the observed value i.e. either half as large or twice as large, while a value of one indicates that there is perfect agreement between all predicted and observed values.

5.2.3 Growth Rate Determinations in Foods

5.2.3.1 Preparation of inoculum

S. aureus 3b was grown in BHIB for 18 - 20 h at 37 ± 2 °C with shaking (25 ± 5

rpm). The cell suspension was centrifuged (10,000g x 20 min) at 4 ± 1 °C. The supernatant was decanted and the pellet resuspended in chilled (4 ± 1 °C) 0.9% saline. The centrifugation and resuspension steps were repeated twice. In the final resuspension, sufficient chilled 0.9% saline was used to produce a just visibly turbid suspension. This suspension was diluted 100-fold in chilled 0.9% saline, and then stored in an ice-water bath.

5.2.3.2 Product inoculation

One hundred millilitres of a washed cell preparation, containing approximately 1 x 10^4 CFU *S. aureus* 3b per ml of chilled (4 ± 1°C) 0.9% saline was prepared and placed in a sterile 150 ml beaker, resting in an ice water bath. Food portions were weighed and dipped in this suspension for 5 s, removed and placed onto a stainless steel mesh to drain off excess liquid, and reweighed. After ~20 inoculations the suspension became fouled with food particles and a new suspension was prepared in an identical manner. All samples were kept on ice before and after inoculation.

5.2.3.3 Incubation of inoculated product

Weighed portions of inoculated product were placed in sterile 180 x 350 mm plastic bags (Disposable Products). Excess air was gently squeezed out by hand, and the bags firmly folded in half several times, and secured with an elastic band. The weight before and after inoculation was recorded on the bag. Batches of 16 - 20 samples were then placed in water-tight plastic bags in water baths (Lauda) at various temperatures (± 0.1°C). The bags were weighted to ensure that the water level was above all samples. Bags were used to improve temperature equilibration after removal of samples, i.e. the bags collapsed under the pressure of the water and thus minimised the insulating air space between the heating/cooling menstruum and the samples.

5.2.3.4 Assessment of growth

At 7 - 10 intervals, duplicate samples were withdrawn from the bags. Chilled diluent (0.1% peptone, 4 ± 1°C) was added in the ratio 9 mls per gram of product (pre-inoculation weight). In some samples pH was determined in this dilution. The sample was then homogenised (Colworth Stomacher) for 1 min. From this homogenate serial tenfold dilutions were prepared in 0.1% peptone (ambient temperature). Spread plates of three dilutions on Baird-Parker Agar (BPA), in duplicate, and Plate Count Agar (PCA, not replicated) were prepared. BPA plates were incubated for 36 - 48 h at 37°C, and PCA plates incubated at room temperature for 96 ± 10 h. In all blocks of experiments three or four samples were withheld at the commencement of incubations. These samples were immediately processed as described above to provide estimates of 'zero time' counts for incubations at all

temperatures in that block. Numbers of *Staphylococcus aureus* 3b were determined from the number of typical colonies on BPA. Viable counts were determined from the number of colonies on PCA. Colony counting procedures used are described in Appendix 2.

5.2.3.5 Growth rate estimation

Growth rates, estimated from colony counts, were calculated from the fitted parameters of Eqn. 3.1 using Eqn. 3.8.

5.2.3.6 Growth on prawns

Green prawns (*Penaeus monodon* (Fabricius, 1798)), were obtained commercially. They were manually (sterile gloves) de-shelled and de-veined in a laminar flow cabinet on a surface-sterile cutting board lying on a bed of ice. Prior to and after deveining the prawns were kept on ice in sterile plastic bags. Some prawns were placed into boiling 2% brine for two minutes. Following overnight (16-18h) storage at 2 ± 1°C, the prawns were divided into 10 ± 1g portions. Prawns were inoculated and growth rates determined as per 5.2.3.2 - 5.2.3.5. Cooked prawns were incubated at 20 and 30 °C. Uncooked prawns were incubated at 12.5, 17.5, 20, 25, 30, and 32.5°C. These experiments were undertaken in two stages. Raw and cooked prawns at 20 and 30°C were studied in the first block and all other experiments on prawns in a second block.

5.2.3.7 Growth on smoked Atlantic salmon

A side (~2 kg) of commercially smoked, sliced (~3mm thickness), Atlantic salmon was obtained from a local producer. Variation in water activity within the product was assessed by measuring the water activity of samples (~5g) taken from various sites using a water activity meter (Novasina). From the sites with the most consistent a_w, 16 cm^2 portions were prepared. The samples were inoculated as per 5.2.3.2 with the following modifications. To obviate the possibility that residual moisture from the inoculation process would increase the water activity, the washed cell suspension was prepared in 7% saline to match the a_w of the product. Earlier experiments (results not shown) indicated that no loss of viability of cells resulted from this suspending medium over the time course of the inoculation procedure. Water activities of product were determined as described in 5.2.3.10 immediately after inoculation (n = 5) and at the end of incubations. Incubation temperatures were 12.5, 17.5, 22.5, 25.0, 27.5, 32.5 and 35.0°C, undertaken in three blocks. Growth rates were determined as described in 5.2.3.3 - 5.2.3.5.

5.2.3.8 Growth on re-brined smoked salmon

A commercial fisheries product of low a_w ,.e. ≤ 0.93, could not be readily obtained. Consequently, the smoked salmon product described above was rebrined to achieve the desired a_w. After initial experiments to determine brining times and concentrations, samples of smoked salmon were immersed in a sterile 2.2M ($a_w \approx 0.92$) NaCl solution for 18 h at 2 ± 1°C. Before inoculation the samples were allowed to drain for 24 h at 2 ± 1°C on sterile tissue paper in sterile petri dishes to remove excess brine. The inoculation procedure was as described in 5.2.3.3 except that the suspending medium was 2.2M NaCl. Four inoculated samples were retained for water activity determination as described in 5.2.3.10. Incubations were performed as described in 5.2.3.3 at 12.6, 17.5, 22.5, 27.5, and 32.5°C in a single experimental block. Growth rates were determined as per 5.2.3.3 - 5.2.3.5.

5.2.3.9 Growth in milk

Homogenised and pasteurised whole (~3.5% [w/v] milk fat) milk was obtained at retail on the day of packaging. 'Full cream' (≤ 3.9% [w/w]) UHT milk with >60 days shelf life remaining was also obtained at retail. Duplicate 50 ml samples of whole milk, and unreplicated 50 ml samples of UHT milk, were aseptically transferred to sterile 250 ml Erlenmeyer flasks. The flasks were suspended by elasticised tape in water baths at various temperatures (± 0.1°C). All flasks were inoculated with 0.4 ml of a washed cell suspension of *S. aureus* 3b, prepared in 0.9% NaCl as described in 5.2.3.1, and containing ~2 x 10^4 CFU/ml. Water circulation in the bath provided gentle agitation throughout the incubation. In addition, flasks were vigorously shaken for 3 - 5 s at all sampling times to aerate the sample. At intervals, 1 ml aliquots were withdrawn from the flasks and *S. aureus* 3b and viable aerobic organisms enumerated as per 5.2.3.3 - 5.2.3.4, and growth rates determined as per 5.2.3.5.

5.2.3.10 Water activity determinations

Water activities in smoked salmon samples were determined by measurement with a Novasina a_w meter, calibrated against saturated salt solutions. The a_w of milk and prawns are literature-based estimates. The reported a_w of solutions is based on published tables (*see* 4.2.2.3).

5.3 RESULTS

Table 5.1a compares generation times predicted by Eqns. 4.1a and b to those determined by inoculation of *S. aureus* 3b into a range of foods at various temperatures. Table 5.1b compares the generation time predictions of Eqns. 4.1a and b, to literature reports for the growth of various strains of *S. aureus*, both in

laboratory media and in foods. The references cited in that table are

1: Li and Torres (1993b)

2: Buchanan *et al.* (1993a)

3: Broughall *et al.* (1983).

Tables 5.2 a-c are comparisons of the predictions of Eqns. 4.3a and b for the generation times of *L. monocytogenes* Murray B to the generation times for that organism determined by Grau and Vanderlinde (*see* 5.2.2) in meat products.

Tables 5.3a-c present comparisons of the predictions of Eqns. 4.2a and b, developed for *L. monocytogenes* Scott A. Table 5.3a compares those equations to rates determined in laboratory media. Table 5.3b compares the equation predictions to generation times determined in foods. To assess the generality of models, Eqns. 4.2a and b were used to make predictions of the generation time for given conditions and were compared to reported generation times of other strains of *L. monocytogenes*. As wide a range of literature values as possible was collated so that any deficiencies of the model could be better appreciated.

Each table details whether the observed generation time falls within the 95% confidence limits of the model, and includes the value of the bias and precision index for the data set, exclusive of the extrapolated values which are denoted by italics. Bias and precision estimates, based on all predictions are presented separately in Table 5.4. In addition, to assess whether the modelled pH response (i.e. in 0.2M lactate) was appropriate to the data of Grau and Vanderlinde (Table 5.2b), the indices were again determined after deletion of data representing growth rates at pH < 6. The revised values are also shown in Table 5.4.

Table 5.5 presents bias and precision estimates for a number of published models, both to the data used to generate the model, and to literature values presented in those papers, by the authors, by which to assess their models.

In all tables, 'NG' denotes that no growth (i.e infinite generation time) was predicted by the model.

Table 5.1a Evaluation of Eqns. 4.1 a and b for *S. aureus* 3b Growth by Comparison to Novel Data

Food Type	Parameters		Observed	Eqn. 4.1a		Eqn. 4.1b	
	temperature (°C)	water activity	GT (h)	predicted GT (h)	In 95% limits	predicted GT (h)	In 95% limits
Milk (Whole)	12.50	0.995	16.50	13.53	yes	11.80	yes
"	12.50	0.995	7.27	13.53	yes	11.80	yes
" (UHT)	12.50	0.995	7.51	13.53	yes	11.80	yes
Milk (Whole)	17.50	0.995	1.57	3.02	no	2.97	no
"	17.50	0.995	8.31	3.02	no	2.97	no
" (UHT)	17.50	0.995	2.69	3.02	yes	2.97	yes
Milk (Whole)	22.50	0.995	1.51	1.30	yes	1.32	yes
"	22.50	0.995	1.25	1.30	yes	1.32	yes
" (UHT)	22.50	0.995	1.29	1.30	yes	1.32	yes
Milk (Whole)	27.50	0.995	0.89	0.72	no	0.74	yes
"	27.50	0.995	0.80	0.72	yes	0.74	yes
" (UHT)	27.50	0.995	0.93	0.72	no	0.74	no
Milk (Whole)	32.50	0.995	0.44	0.45	yes	0.48	yes
"	32.50	0.995	0.54	0.45	no	0.48	yes
" (UHT)	32.50	0.995	0.51	0.45	yes	0.48	yes
Milk (Whole)	37.50	0.995	0.37	0.31	no	0.33	yes
"	37.50	0.995	0.40	0.31	no	0.33	no
" (UHT)	37.50	0.995	0.51	0.31	no	0.33	no
Prawns	32.50	0.995	0.40	0.45	yes	0.48	no
" (Cooked)	30.00	0.995	0.57	0.56	yes	0.59	yes
"	30.00	0.995	0.47	0.56	no	0.59	no
"	25.00	0.995	0.79	0.94	yes	0.97	yes
" (Cooked)	20.00	0.995	1.80	1.89	yes	1.90	yes
"	20.00	0.995	1.64	1.89	yes	1.90	yes
"	17.50	0.995	2.48	3.02	yes	2.97	yes
"	12.50	0.995	6.12	13.53	no	11.80	yes
Smoked salmon	12.50	0.965	11.47	17.40	yes	15.05	yes
"	17.50	0.965	4.05	3.89	yes	3.79	yes
"	22.50	0.975	1.65	1.52	yes	1.54	yes
"	25.00	0.955	1.90	1.34	no	1.36	no
"	27.50	0.975	0.73	0.84	yes	0.87	yes
"	32.50	0.965	0.57	0.58	yes	0.61	yes
"	35.00	0.955	0.50	0.53	yes	0.55	yes
Smoked salmon	12.60	0.920	43.31	29.13	yes	24.65	yes
"	17.50	0.920	7.93	6.80	yes	6.45	yes
"	22.50	0.920	3.78	2.91	yes	2.87	yes
"	27.50	0.920	1.58	1.61	yes	1.62	yes
"	32.50	0.920	0.99	1.02	yes	1.04	yes
		Bias factor		0.994		0.998	
		Precision factor		1.253		1.245	

Table 5.1b Evaluation of Eqns. 4.1 a, b for *S. aureus* Growth by Comparison to Published Data

Medium (Strain)	Parameters temperature (°C)	water activity	Observed GT (h)	Eqn. 4.1a prediction (h)	In 95% limits	Eqn. 4.1b prediction (h)	In 95% limits	Ref.
BHIB + NaCl	26.00	0.970	1.28	1.03	yes	1.06	yes	1
(ATCC 13566)	20.00	0.968	2.22	2.37	yes	2.36	yes	1
	16.00	0.967	4.17	5.38	yes	5.14	yes	1
	12.00	0.968	8.33	21.43	no	18.06	yes	1
	26.00	0.950	1.82	1.26	no	1.29	no	1
	20.00	0.950	7.14	2.84	no	2.81	no	1
	16.00	0.950	10.31	6.40	yes	6.07	yes	1
	12.00	0.950	25.00	25.72	yes	21.52	yes	1
BHIB + NaCl	37.00	0.997	0.30	0.34	yes	0.36	no	2
(196E)	28.00	0.997	0.60	0.67	yes	0.70	yes	2
	19.00	0.997	1.95	2.22	yes	2.21	yes	2
	19.00	0.973	3.20	2.69	yes	2.67	yes	2
	19.00	0.946	5.70	3.54	yes	3.47	no	2
	19.00	0.997	3.50	2.22	no	2.21	no	2
	12.00	0.997	8.70	16.89	yes	14.35	yes	2
BHIB	37.00	0.990	0.50	0.36	no	0.38	no	2
Determinations in laboratory media								
Bias factor				1.073		1.103		
Precision factor				1.432		1.416		
UHT Milk	23.2	0.98	1.12	1.33	yes	1.35	yes	3
+ glucose	20	0.98	1.24	2.13	no	2.13	no	3
(C-246-3A)	16.4	0.98	3.04	4.35	yes	4.20	yes	3
	12.3	0.98	6.41	16.68	no	14.35	no	3
	20.00	0.96	1.62	2.55	no	2.54	no	3
	16.40	0.96	3.31	5.22	yes	5.01	yes	3
	12.30	0.96	8.61	20.01	no	17.11	yes	3
	26.20	0.90	2.77	2.77	yes	2.69	yes	3
	20.00	0.90	12.20	6.39	yes	6.01	no	3
	20.00	0.88	15.30	12.77	yes	11.02	yes	3
	16.40	0.88	34.40	26.11	yes	21.69	yes	3
	20.00	0.93	4.95	3.65	yes	3.57	yes	3
	16.40	0.93	9.45	7.46	yes	7.04	yes	3
	12.30	0.93	34.40	28.59	yes	24.05	yes	3
Raw pastry	25.00	0.91	1.60	2.54	no	2.50	no	2
	30.00	0.91	1.20	1.52	yes	1.51	yes	2
	37.00	0.91	0.70	0.92	no	0.93	no	2
Steak and Kidney	13.00	0.98	8.70	12.32	yes	10.95	yes	2
Pie Mix	21.00	0.98	2.30	1.81	yes	1.83	yes	2
	30.00	0.98	0.60	0.63	yes	0.66	yes	2
	42.00	0.98	0.40	0.38	yes	0.41	yes	2
Determinations in Foods								
Bias factor				0.865		0.910		
Precision factor				1.413		1.426		
Combined Results								
Bias factor				0.949		0.989		
Precision factor				1.421		1.422		

Table 5.2a Evaluation of Eqns. 4.3a and b for the Growth of *L. monocytogenes* Murray B. Beef (fat) Data of Grau and Vanderlinde (1993).

Temperature (°C)	Water activity	pH	Observed GT (h)	Predicted Generation Times (h) Eqn. 4.3a	In 95% Limit?	Eqn. 4.3 b	In 95% Limit?
0.0	0.993	7	62.89	*6685.43*	yes	*NG*	no
0.0	0.993	7	58.48	*6685.43*	yes	*NG*	no
2.5	0.993	7	58.14	*72.23*	yes	*112.00*	yes
4.7	0.993	7	26.46	*22.58*	yes	*30.49*	yes
4.8	0.993	7	31.94	*21.70*	yes	*29.20*	yes
7.5	0.993	7	10.67	9.27	yes	11.78	yes
10.1	0.993	7	5.00	5.21	yes	6.45	yes
10.1	0.993	7	2.32	5.21	no	6.45	no
14.9	0.993	7	2.22	2.44	yes	2.95	yes
15.0	0.993	7	1.87	2.40	yes	2.91	no
19.8	0.993	7	1.83	1.39	yes	1.66	yes
19.9	0.993	7	1.68	1.38	yes	1.65	yes
22.0	0.993	7	1.52	1.13	no	1.35	yes
24.8	0.993	7	1.56	0.89	no	1.06	no
25.0	0.993	7	1.29	0.88	no	1.04	yes
27.4	0.993	7	1.3	0.73	no	0.87	no
30.6	0.993	7	4.33	0.59	no	0.69	no
30.6	0.993	7	0.81	0.59	no	0.69	yes
	Bias factor			1.314		1.091	
	Precision factor			1.578		1.506	

Table 5.2b Evaluation of Eqns. 4.3a and b for the Growth of *L. monocytogenes* Murray B. Data (lean beef) of Grau and Vanderlinde (1993).

Temperature (°C)	Water activity	pH	Observed GT (h)	Predicted Generation Times (h) Eqn. 4.3a	In 95% Limit?	Eqn. 4.3 b	In 95% Limit?
0.0	0.993	5.61	NG	*42552.33*	yes	NG	yes
2.4	0.993	5.60	58.48	*544.01*	yes	NG	no
2.5	0.993	5.55	58.14	*1011.42*	yes	NG	no
5.1	0.993	5.56	26.46	*225.83*	no	NG	no
5.5	0.993	5.51	31.94	*1174.23*	no	NG	no
10.1	0.993	5.61	10.67	33.15	no	NG	no
15.5	0.993	5.55	5.00	31.58	no	NG	no
22.3	0.993	5.56	2.32	12.86	no	NG	no
22.6	0.993	5.59	2.22	8.35	no	NG	no
24.9	0.993	5.55	1.87	12.41	no	NG	no
25.0	0.993	5.59	1.83	6.84	no	NG	no
25.4	0.993	5.60	1.68	5.96	no	NG	no
27.3	0.993	5.56	1.52	8.62	no	NG	no
27.4	0.993	5.59	1.56	5.70	no	NG	no
29.8	0.993	5.57	1.29	6.21	no	NG	no
29.8	0.993	5.60	1.30	4.35	no	NG	no
14.8	0.993	5.73	4.33	7.52	yes	4.68	yes
35.0	0.993	5.73	0.81	1.37	no	0.83	yes
0.0	0.993	6.06	81.30	*8358.49*	yes	NG	yes
2.4	0.993	6.09	34.12	*92.21*	yes	*139.45*	yes
4.8	0.993	6.09	18.41	*25.75*	yes	*33.40*	yes
10.0	0.993	6.09	6.71	6.30	yes	7.53	yes
14.4	0.993	6.10	3.60	3.04	yes	3.60	yes
14.9	0.993	6.11	3.42	2.80	yes	3.35	yes
15.7	0.993	6.08	3.11	2.65	yes	3.04	yes
19.8	0.993	6.11	1.94	1.60	yes	1.89	yes
19.9	0.993	6.11	1.90	1.58	yes	1.87	yes
25.1	0.993	6.08	1.22	1.05	yes	1.18	yes
25.4	0.993	6.11	1.12	0.98	yes	1.15	yes
30.0	0.993	6.08	0.85	0.74	yes	0.83	yes
30.1	0.993	6.11	0.79	0.70	yes	0.81	yes
35.0	0.993	6.08	0.67	0.54	no	0.61	yes
5.0	0.993	6.34	24.63	*20.09*	yes	*29.01*	yes
15.5	0.993	6.32	3.17	2.26	yes	2.95	yes
25.1	0.993	6.34	1.03	0.87	yes	1.11	yes
0.0	0.993	6.68	66.67	*6685.43*	yes	NG	no
2.4	0.993	6.66	28.57	*77.70*	yes	*125.90*	yes
4.9	0.993	6.7	15.85	*20.87*	yes	*28.78*	yes
5.2	0.993	6.98	13.55	*18.65*	yes	*24.84*	yes
10.0	0.993	6.71	5.88	5.31	yes	6.76	yes
14.8	0.993	6.68	3.00	2.47	yes	3.08	yes
15.0	0.993	6.98	2.79	2.40	yes	2.91	yes
20.0	0.993	6.71	1.79	1.37	yes	1.67	yes
26.0	0.993	6.68	0.96	0.81	yes	0.99	yes
	Bias factor			0.631		0.990	
	Precision factor			1.937		1.050	

Temperature (°C)	Water activity	pH	Observed GT (h)	Predicted Generation Times (h) Eqn. 4.3a	In 95% Limit?	Eqn. 4.3 b	In 95% Limit?
5	0.993	6.89	14.58	*20.09*	yes	*27.11*	yes
5	0.993	6.79	15.16	*20.09*	yes	*27.37*	yes
5	0.993	6.72	15.32	*20.09*	yes	*27.56*	yes
5	0.993	6.54	14.79	*20.09*	yes	*28.15*	yes
5	0.993	6.46	15.95	*20.09*	yes	*28.46*	yes
5	0.993	6.41	17.37	*20.09*	yes	*28.68*	yes
5	0.993	6.26	18.10	*20.09*	yes	*29.46*	yes
5	0.993	6.23	16.75	*20.09*	yes	*29.65*	yes
5	0.993	6.23	15.51	*20.09*	yes	*29.65*	yes
5	0.993	6.2	16.13	*20.10*	yes	*29.85*	yes
5	0.993	6.17	17.37	*21.00*	yes	*30.06*	yes
5	0.993	6.15	18.34	*21.64*	yes	*30.21*	yes
5	0.993	6.12	17.16	*22.69*	yes	*30.46*	yes
5	0.993	6.08	18.87	*24.25*	yes	*30.82*	yes
5	0.993	5.96	17.18	*30.58*	yes	*32.25*	yes
5	0.993	5.87	18.71	*38.02*	yes	*33.96*	yes
5	0.980	6.74	15.01	*24.67*	yes	*34.65*	yes
5	0.980	6.72	15.85	*24.67*	yes	*34.73*	yes
5	0.980	6.64	16.37	*24.67*	yes	*35.03*	yes
5	0.980	6.43	16.58	*24.67*	yes	*36.02*	yes
5	0.980	6.26	19.98	*24.67*	yes	*37.12*	yes
5	0.980	6.11	21.04	*28.32*	yes	*38.49*	yes
5	0.980	6.1	21.43	*28.79*	yes	*38.60*	yes
5	0.980	6.09	19.09	*29.28*	yes	*38.71*	yes
5	0.980	5.93	18.38	*40.17*	yes	*41.24*	yes
5	0.968	6.82	16.61	*31.25*	yes	*45.24*	yes
5	0.968	6.82	16.37	*31.25*	yes	*45.24*	yes
5	0.968	6.72	18.09	*31.25*	yes	*45.69*	yes
5	0.968	6.71	20.48	*31.25*	yes	*45.74*	yes
5	0.968	6.61	19.54	*31.25*	yes	*46.26*	yes
5	0.968	6.50	18.78	*31.25*	yes	*46.91*	yes
5	0.968	6.49	20.70	*31.25*	yes	*46.98*	yes
5	0.968	6.40	21.31	*31.25*	yes	*47.62*	yes
5	0.968	6.36	20.54	*31.25*	yes	*47.93*	yes
5	0.968	6.33	27.38	*31.25*	yes	*48.19*	yes
5	0.968	6.22	21.92	*31.25*	yes	*49.26*	yes
5	0.968	6.13	29.13	*34.73*	yes	*50.36*	yes
5	0.968	6.13	26.02	*34.73*	yes	*50.36*	yes
5	0.968	6.13	26.57	*34.73*	yes	*50.36*	yes
5	0.968	6.10	29.50	*36.47*	yes	*50.79*	yes
5	0.968	6.09	31.11	*37.09*	yes	*50.94*	yes
5	0.968	6.05	26.65	*39.79*	yes	*51.60*	yes
5	0.960	6.71	20.34	*38.01*	yes	*57.94*	yes
5	0.960	6.69	23.30	*38.01*	yes	*58.06*	yes
5	0.960	6.63	24.35	*38.01*	yes	*58.45*	yes
5	0.960	6.40	24.67	*38.01*	yes	*60.31*	yes
5	0.960	6.28	28.29	*38.01*	yes	*61.61*	yes
5	0.960	6.16	27.60	*40.32*	yes	*63.29*	yes
5	0.960	6.14	37.75	*41.58*	yes	*63.62*	yes
5	0.960	6.12	27.31	*42.92*	yes	*63.96*	yes
5	0.960	6.10	34.71	*44.36*	yes	*64.33*	yes
5	0.960	5.94	38.54	*60.48*	yes	*68.38*	yes
	Bias factor			0.699		0.495	
	Precision factor			1.430		2.016	

Table 5.3a Comparison of Predictions of Eqns. 4.2a and b to Published Generation Times of *L. monocytogenes* in Laboratory Broth Media

Reference (Medium) Strain	Temperature (°C)	Water activity	pH	Observed GT (h)	Predicted Generation Times (h) Eqn. 4.3a	In 95% Limit?	Eqn. 4.3 b	In 95% Limit?
Petran and Zottola (1989)	4	0.995	7	33.50	*29.37*	yes	*36.30*	yes
Tryptic Soy Broth	13	0.995	7	4.80	3.03	yes	3.56	yes
Scott A	35	0.995	7	0.70	0.43	no	0.50	no
Buchanan *et al.* (1989)	37	0.995	6	0.60	0.54	yes	0.61	yes
Tryptose Phosphate Broth	37	0.973	6	0.40	0.79	no	0.92	no
Scott A	37	0.995	7.5	0.60	0.38	no	0.42	no
	37	0.973	7.5	0.70	0.56	no	0.63	yes
	28	0.995	6	0.50	0.93	no	1.06	no
	28	0.973	6	0.60	1.37	no	1.60	no
	28	0.995	7.5	0.60	0.67	yes	0.72	no
	28	0.973	7.5	0.90	0.98	yes	1.10	yes
	19	0.995	6	0.90	2.01	no	2.29	no
	19	0.973	6	2.50	2.95	yes	3.46	yes
	19	0.995	7.5	1.20	1.44	yes	1.57	no
	19	0.973	7.5	2.20	2.11	yes	2.37	yes
	5	0.995	6	14.40	*26.97*	yes	*32.09*	yes
	5	0.973	6	22.20	*39.59*	yes	*48.52*	yes
	5	0.995	7.5	13.30	*19.26*	yes	*22.00*	yes
	5	0.973	7.5	14.10	*28.28*	yes	*33.25*	yes

Table 5.3a (cont.) Comparison of Predictions of Eqns. 4.2a and b to Published Generation Times of *L. monocytogenes* in Laboratory Broth Media

Reference (Medium) Strain	Temperature (°C)	Water activity	pH	Observed GT (h)	Predicted Generation Times (h) Eqn. 4.3a	In 95% Limit?	Eqn. 4.3 b	In 95% Limit?
Walker *et al.* (1990)	0	0.995	7	NG	*7884.30*	yes	*61025.40*	yes
Chicken broth	2.5	0.995	7	30	*69.97*	yes	*90.27*	yes
433	5	0.995	7	25	*19.26*	yes	*23.46*	yes
	7.5	0.995	7	13	8.85	yes	10.57	yes
	9.3	0.995	7	5	5.83	yes	6.91	yes
Walker *et al.* (1990)	0	0.995	7	131	*7884.30*	yes	*61025.40*	yes
Chicken broth	2.5	0.995	7	45	*69.97*	yes	*90.27*	yes
433	5	0.995	7	19	*19.26*	yes	*23.46*	yes
	7.5	0.995	7	9	8.85	yes	10.57	yes
	9.3	0.995	7	7	5.83	yes	6.91	yes
Wilkins *et al.* (1972)	5	0.997	7.2	23.25	*18.72*	yes	*22.12*	yes
1%tryptone, 1% yeast	10	0.997	7.2	6.93	4.92	yes	5.64	yes
extract, 0.3%K_2HPO_4	25	0.997	7.2	1.58	0.81	no	0.91	no
0.1% glucose	30	0.995	7.2	0.91	0.58	no	0.83	yes
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		Bias factor			1.008		0.873	
		Precision factor			1.425		1.411	

Table 5.3b Comparison of Predictions of Eqns. 4.2a and b to Published Generation Times of *L. monocytogenes* Scott A in Foods

Reference	Product	Temperature (°C)	a_w	pH	Observed GT (h)	Predicted Generation Times (h) Eqn. 4.3a	In 95% Limit?	Eqn. 4.3 b	In 95% Limit?
Ferguson and Shelef (1990)	Soymilk	22	0.995	6.5	1.26	1.08	yes	1.38	yes
		5	0.995	6.5	9.04	19.26	no	25.97	no
Connor *et al.* (1986)	Clarified cabbage juice	30	0.995	5.6	1.90	4.07	no	NG	no
		30	0.989	5.6	1.90	4.46	no	NG	no
	Clarified cabbage juice	30	0.995	6.1	1.70	0.68	no	0.87	no
		30	0.989	6.1	2.20	0.74	no	0.95	no
Marshall and Schmidt (1988)	whole milk	10	0.995	6.4	10.00	5.06	no	6.81	yes
		10	0.995	6.4	7.00	5.06	yes	6.81	yes
Berrang *et al.* (1989)	asparagus	4	0.98	5.9	46.00	*65.67*	yes	*70.18*	yes
		15	0.98	5.9	7.23	5.12	yes	5.17	yes
	broccoli	4	0.98	6.5	79.90	*37.53*	yes	*52.24*	yes
		15	0.98	6.5	10.50	2.92	no	3.85	no
Ryser and Marth (1987)	Camembert cheese	6	0.986	6.1	50.70	18.25	yes	24.48	yes

Table 5.3b (cont.) Comparison of Predictions of Eqns. 4.2a and b to Published Generation Times of *L. monocytogenes* Scott A in Foods

Reference	Product	Temperature (°C)	a_w	pH	Observed GT (h)	Predicted Generation Times (h) Eqn. 4.3a	In 95% Limit?	Eqn. 4.3 b	In 95% Limit?
Ryser and Marth (1988)	Uncultured whey	6	0.995	5.6	28.90	95.20	yes	NG	no
		6	0.995	6.2	21.10	13.60	yes	20.10	yes
		6	0.995	6.8	18.00	13.60	yes	16.97	yes
	Cultured whey	6	0.995	5.6	19.40	95.20	no	NG	no
		6	0.995	6.2	10.30	13.60	yes	20.10	yes
		6	0.995	6.8	9.50	13.60	yes	16.97	yes
Rosenow and Marth (1987)	Whole milk	4	0.995	6.5	30.50	*29.37*	yes	*40.18*	yes
		8	0.995	6.5	13.00	7.81	yes	10.30	yes
		13	0.995	6.5	5.90	3.03	no	3.94	yes
	Chocolate milk	4	0.986	6.5	30.00	*33.77*	yes	*46.64*	yes
		8	0.986	6.5	10.80	8.98	yes	11.95	yes
		Bias Factor				1.162		1.245	
		Precision Factor				1.974		1.509	

Table 5.3c Comparison of Predictions of Eqns. 4.2a and b to Published Generation Times of Various *L. monocytogenes* Strains in Foods

Reference	Product	Strain	Temperature (°C)	a_w	pH	Observed GT (h)	Predicted Generation Times (h) Eqn. 4.3a	In 95% Limit?	Eqn. 4.3 b	In 95% Limit?
Rosenow and Marth (1987)	Chocolate milk	V7	13	0.986	6.4	4.50	3.49	yes	4.70	yes
		V7	8	0.986	6.4	8.60	8.98	yes	12.30	yes
		V37CE	4	0.986	6.4	29.50	*33.77*	yes	*48.01*	yes
		V7	21	0.986	6.4	1.60	1.36	yes	1.81	yes
		V7	35	0.986	6.4	0.68	0.49	no	0.66	yes
	Whole milk	CA	4	0.995	6.4	30.00	*29.37*	yes	*41.36*	yes
		V7	8	0.995	6.4	10.80	7.81	yes	10.60	yes
		V7	13	0.995	6.4	5.00	3.03	no	4.05	yes
		V7	21	0.995	6.4	1.90	1.18	no	1.56	yes
		V7	37	0.995	6.4	0.65	0.38	no	0.51	no
Ryser and Marth (1988)	Uncultured whey	OH	6	0.995	5.6	25.20	95.20	yes	NG	no
		V7	6	0.995	6.2	14.80	13.60	yes	20.10	yes
		V7	6	0.995	6.8	14.00	13.60	yes	16.97	yes
	Cultured Whey	OH	6	0.995	5.6	16.50	95.20	no	NG	no
		Scott A	6	0.995	6.2	10.30	13.60	yes	20.10	yes
		OH	6	0.995	6.8	7.30	13.60	yes	16.97	no
Donnelly and Briggs (1986)	Whole milk	F5069	35	0.995	6.4	0.43	0.43	yes	0.56	no
		F5069	21	0.995	6.4	1.08	1.18	yes	1.56	no
		F5069	10	0.995	6.4	3.71	5.06	yes	6.82	no
		F5069	4	0.995	6.4	12.10	*29.37*	no	*41.36*	no

Table 5.3c (cont.) Comparison of Predictions of Eqns. 4.2a and b to Published Generation Times of Various *L. monocytogenes* Strains in Foods

Reference	Product	Strain	Temperature (°C)	a_w	pH	Observed GT (h)	Predicted Generation Times (h) Eqn. 4.3a	In 95% Limit?	Eqn. 4.3 b	In 95% Limit?
Ryser and Marth (1987)	Camembert	OH	6	0.986	6.1	21.69	18.25	yes	24.49	yes
Pearson and Marth (1990b))	Skim Milk	V 7	30	0.995	6.5	0.87	0.58	no	0.75	yes
			35	0.995	5.6	0.80	3.00	no	NG	no
Connor *et al.* (1986)	Clarified cabbage juice	LCDC 81-861	30	0.995	5.6	1.42	4.07	no	NG	no
			30	0.989	5.6	2.12	4.46	no	NG	no
Hudson and Mott (1993b)	Smoked salmon	cocktail	10	0.983	6.1	6.70	7.15	yes	9.44	yes
			5	0.983	6.1	21.20	*27.20*	yes	*37.01*	yes
Hudson and Mott (1993a)	Pate	NCTC 7973	4	0.995	6.1	68.70	*34.26*	yes	*46.70*	yes
		L70	4	0.995	6.1	69.00	*34.26*	yes	*46.70*	yes
		NCTC 7973	10	0.995	6.1	14.30	5.91	no	7.70	yes
		L70	10	0.995	6.1	14.40	5.91	no	7.70	yes

Table 5.3c (cont.) Comparison of Predictions of Eqns. 4.2a and b to Published Generation Times of Various *L. monocytogenes* Strains in Foods

Reference	Product	Strain	Temperature (°C)	a_w	pH	Observed GT (h)	Predicted Generation Times (h) Eqn. 4.3a	In 95% Limit?	Eqn. 4.3 b	In 95% Limit?
Walker *et al.* (1990)	UHT Milk	433	0	0.997	7.2	77.00	*7662.20*	no	NG	no
		433	2.5	0.997	7.2	33.00	*68.00*	yes	*85.09*	yes
		433	5	0.997	7.2	19.00	*18.72*	yes	*22.12*	yes
		433	7.5	0.997	7.2	9.50	8.60	yes	9.96	yes
		433	9.3	0.997	7.2	9.00	5.67	yes	6.51	yes
Grant *et al.* (1993)	Roast beef and gravy	CRA 198	5	0.99	6	18.86	*29.07*	yes	*34.77*	yes
		CRA 198	10	0.99	6	9.43	7.64	yes	8.87	yes
		CRA 433	5	0.99	6	17.24	*29.07*	yes	*34.77*	yes
		CRA 433	10	0.99	6	8.37	7.64	yes	8.87	yes
Ferguson and Shelef (1990)	Soymilk	DA 3	5	0.995	6.5	18.07	*19.26*	yes	*25.98*	yes
		Brie 1	5	0.995	6.5	13.46	*19.26*	yes	*25.98*	yes
Hart *et al.* (1991)	Chicken breast	NCTC 11994	6	0.993	5.8	16.90	32.68	yes	28.64	yes
		NCTC 11994	15	0.993	5.8	4.52	5.50	yes	4.67	yes
				Bias factor			0.920		0.911	
				Precision factor			1.589		1.330	

Table 5.4 Summary of Bias and Precision Indices for Eqns 4.1-4.3 and the Effect of Including Extrapolated Predictions

Table No.	Eqn. No.	BIAS factor		PRECISION Factor	
		Inc. Extrapolation	Within Range	Inc. Extrapolation	Within Range
5.1a	4.1a		0.994		1.253
	4.1b		0.998		1.245
5.1b	4.1a		0.949		1.421
	4.1b		0.989		1.422
5.2a	4.3a	0.735	1.314	2.446	1.578
	4.3b	1.027	1.091	1.475	1.506
5.2b	4.3a	0.422	0.631	2.762	1.937
	4.3b	0.823	0.990	1.251	1.050
5.2b	4.3a		1.188		1.188
(pH>6)	4.3b		0.994		1.049
5.2c	4.3a	0.699		1.430	
	4.3b	0.495		2.106	
5.3a	4.2a	0.806	1.008	1.634	1.425
	4.2b	0.648	0.873	1.784	1.411
5.3b	4.2a	1.106	1.162	1.864	1.974
	4.2b	1.079	1.245	1.552	1.509
5.3c	4.2a	0.828	0.920	1.604	1.589
	4.2b	0.809	0.911	1.462	1.330

5.4 DISCUSSION

There are limitations in comparing the bias and precision of models on the basis of literature values for response times. It is not always possible to obtain all the relevant information from published reports to enable an appropriate prediction from the models. The degree of control over important variables in experimental determinations is also often unknown, e.g. how well was the temperature controlled, did the pH measured reflect that throughout the incubation? etc. Further, it was not possible to obtain published values for the full range of a_w modelled for *L. monocytogenes*. For these reasons the most objective comparisons in this chapter relate to the data in Table 5.1.

Table 5.5 Bias and Precision Indices for Published Models

Organism (Reference)	Data Type	Bias Factor	Precision Factor
S. aureus 196E	literature	0.548	2.393
Buchanan *et al.*(1993a)	originating	0.998	1.353
L. monocytogenes Scott A	literature	2.004	2.004
Buchanan and Phillips (1990)			
L. monocytogenes NCTC 9863	literature	1.770	1.770
Wijtzes *et al.*(1993)			
B. thermosphacta MR 165	literature	1.413	1.824
McClure *et al.*(1993)			
Salmonella (cocktail of 3)	literature	1.225	1.408
Gibson *et al.*. (1988)			
L. monocytogenes Murray B	originating (fat)	0.993	1.07
Grau and Vanderlinde (1993)	originating (lean)	0.999	1.07

5.4.1 Model for *S. aureus* 3b

The results in Table 5.1 strongly support the hypotheses proposed in 4.1. Models developed by the 'iterative' method perform at least as well as models developed by nonlinear regression of a full data set. There is virtually no bias in either model. The precision of the models is ± ~25% which compares very favourably with other published models (Table 5.5).

When Eqns. 4.1 a and b are compared to published values for the growth of *S. aureus* (Table 5.1b) the models are seen to over-predict generation times in foods, i.e. they are 'fail dangerous', but are very similar to published generation times for the growth of *S. aureus* in laboratory broth media. The bias involved is quite small for both Eqns. 4.1a and b, and the accuracy is as good as other published models. It is also noteworthy that all the generation times determined in foods reported in Table 5.1b were generated at the Unilever Research Laboratories, Bedford, UK. It was not possible from the reports to determine whether the same strain was used for all experiments, but it is possible that all those determinations are based on a single, fast, growing strain.

The number of values which fell outside the 95% confidence intervals of both models is higher than would be expected, with the nonlinear model performing slightly better. The number of observations outside of the predicted 95% range suggests that the variance in growth rates determined in laboratory media may be narrower than those observed in foods. Foods are more heterogeneous than laboratory broths and, for the data presented in Table 5.1a, included normal spoilage biota. These uncontrolled factors and potential interactions may be expected to lead to increased variability in responses determined in foods, and may need to be considered more fully in the setting of confidence intervals on model predictions.

5.4.2 Models for *L. monocytogenes* Murray B

Comparisons of models on the basis of the generation times of *L. monocytogenes* Murray B in foods (Tables 5.2a-c), which were determined under well controlled conditions, would also be expected to be an objective basis for the evaluation of the hypotheses and validation of the models. Table 5.2a indicates that Eqn. 4.3b outperforms its 'iterative' counterpart. From Table 5.2a, Eqn. 4.3b is seen to display very little bias and to have about the same degree of precision as other good models. Similarly, in Table 5.2b the performance indices of Eqns. 4.3a appear poor. Table 5.4 shows that when data relating to pH < 6 were deleted, however, the performance indices of Eqn. 4.3a improved, while the performance indices of Eqn 4.3b were almost unchanged. Eqn. 4.3b predicted 'no growth' for all but two data in the range pH < 6. 'No Growth' predictions were not included in the calculation of the indices. The comparison of indices are somewhat misleading in this case. The improved performance of Eqn. 4.3a after removal of pH < 6 data suggests that the pH response in the presence of 0.2M lactate does not reflect the behaviour of the organisms in the meat products tested, in which the lactate concentration was ≤ 0.1M (F. Grau, pers. comm). Also, the pH values reported were the average values of the pH recorded at each sampling time which may differ from the pH_{mid}. The performance of Eqn. 4.3b was generally better than that of the iterative model, Eqn. 4.3a, for the data sets presented in Tables 5.2a - b.

Table 5.2c includes predictions entirely based on extrapolation of the model to 5°C. In this case Eqn. 4.3a, which has a slightly lower T_{min} than Eqn. 4.3b, consistently outperforms the latter equation. Both models overpredicted all generation times observed. As noted in 5.1, the T_{min} estimates for all the *L. monocytogenes* models derived in Chapter 4 were anomalously high. A consequence of this is that as the incubation temperature approaches T_{min}, predictions from the model will be expected to become increasingly erroneous. This is demonstrated in Table 5.6 in which the relative generation times at various temperatures are compared for models based on a T_{min} of -0.3°C (Eqn. 4.3a) and T_{min} of -2.2°C (representative literature value). From Table 5.6 a bias factor of ~0.5

Table 5.6 Relative Generation Times Predicted by Square-root-type Models With Different T_{min} Values

Temperature (°C)	$\frac{GT\ (T_{min} = -0.3°C)}{GT(T_{min} = -2.2°C)}$
6	1.69
5	1.85
4	2.08
3	2.48
2	3.33
1	6.06
0	53.78

would be expected on the basis of the anomalous T_{min}, and is similar to that observed for both models. At higher temperatures the difference in estimates as a result of the anomalous T_{min}, will be less pronounced. Thus, the anomalously high T_{min} estimates explain the poor performance of Eqns. 4.3a and b at chill temperatures. Despite the poor performance indices, use of the shortest generation times within the 95% confidence intervals would have produced fail safe estimates in all but one case in samples with pH >5.8. As indicated above, the pH_{min} estimate is likely to be specific to the experimental conditions employed.

5.4.3 Models for *L. monocytogenes* Scott A

The effect of the anomalous T_{min} estimates can also be perceived in the results presented in Table 5.3a for Eqns. 4.2a and b which favour the use of Eqn. 4.2a (iterative). Again, under well controlled conditions, there is good agreement between the predictions of models and independent observations, and the average precision is within ± ~40%. The results in Table 5.3b are encouraging from the perspective of application of predictive models. Both Eqns. 4.2 a and b underestimate observed generation times by approximately 10% and thus provide 'fail safe' predictions.

Table 5.3c presents an assessment of the ability of a model developed for one strain of bacterium to predict the growth rate of another strain of the same species. The results indicate that the 'iterative' model outperforms the complementary model developed by nonlinear regression. The performance indices indicate slight underprediction of growth rates. Remembering that the fastest growth

rates were selected where ranges of rates and strains were presented in publications, it is perhaps not surprising that Eqns. 4.2a and b slightly over-predict the reported generation times. This observation, and the comments in 5.4.2 relating to the *S. aureus* data in Table 5.1b, supports the proposal of McMeekin *et al.*(1993), discussed in 2.2.6, that the fastest growing strains of a species should be selected for model development.

The performance indices of both Eqns. 4.2a and b, developed for *L. monocytogenes* Scott A, when applied to other strains of *Listeria monocytogenes* are as good as any of the models assessed in Table 5.5, suggesting that a model developed for a relevant strain of one species may have general applicability to other strains of the same species. To test this hypothesis an iterative-type temperature and water activity model for *Listeria monocytogenes* was developed based on published T_{min} estimates and the minimum water activities permitting growth (*see* 4.4.1.1). The scaling factor, b, was determined by equating the model to the average generation time of *L. monocytogenes* Scott A at 19.5°C, a_w =.997 (Table 3.6). No pH term was included. The model generated is:

$$\text{Generation time(h)} = \left(\frac{1}{0.122 \times \sqrt{(a_w - 0.92)} \times (T + 2.2)} \right)^2 \qquad (5.2)$$

and was compared to the literature data in Table 5.3c for pH > 6. The variance determined for *L. monocytogenes* Scott A data was used to estimate the 95% confidence intervals for this model. The derivation and assessment of Eqn. 5.2 also test again the hypothesis of Chandler and McMeekin (1989b) regarding the development of temperature and water activity models.

The results, shown in Table 5.7, show that the model performs at least as well as the equations previously developed and supports the hypothesis, presented above, that a large component of the lack of fit of Eqns. 4.2 - 4.3 stems from the anomalous estimates of T_{min}. The results also strongly support the hypotheses regarding model generation presented in 4.1, and the inter-strain applicability of models for *L. monocytogenes*.

5.4.4 Extrapolation

Table 5.4 demonstrates the effect of extrapolation on the bias and precision of models to independent data. In most cases poorer performance was indicated when extrapolated predictions were made. In some cases, however, the performance increased, which indicates that the results are subject to some data set specific effects. The data sets collated are relatively small, so that the contribution of individual values may be noticeable in the overall results.

Table 5.7 Evaluation of Eqn. 5.2- a Theoretical Model for *L. monocytogenes*

Reference	Strain	Temperature (°C)	a_w	pH	Observed GT (h)	Predicted GT Eqn. 5.2	In 95% Limit?
Rosenow and Marth	V7	13	0.986	6.4	4.50	4.44	yes
(1987)	V7	8	0.986	6.4	8.60	9.87	yes
	V37CE	4	0.986	6.4	29.50	26.70	yes
	V7	21	0.986	6.4	1.60	1.91	yes
	V7	35	0.986	6.4	0.68	0.74	yes
	CA	4	0.995	6.4	30.00	23.50	yes
	V7	8	0.995	6.4	10.80	8.68	yes
	V7	13	0.995	6.4	5.00	3.91	yes
	V7	21	0.995	6.4	1.90	1.68	yes
	V7	37	0.995	6.4	0.65	0.59	yes
Ryser and Marth	V7	6	0.995	6.2	14.80	13.43	yes
(1988)	V7	6	0.995	6.8	14.00	13.43	yes
	Scott A	6	0.995	6.2	10.30	13.43	yes
	OH	6	0.995	6.8	7.30	13.43	yes
Donnelly and Briggs	F5069	35	0.995	6.4	0.43	0.65	no
(1986)	F5069	21	0.995	6.4	1.08	1.68	no
	F5069	10	0.995	6.4	3.71	6.07	no
	F5069	4	0.995	6.4	12.10	23.50	yes
Hudson and Mott	cocktail	10	0.983	6.1	6.70	7.22	yes
(1993b)		5	0.983	6.1	21.20	20.74	yes
Ryser and Marth (1987)	OH	6	0.986	6.1	21.69	15.27	yes
Pearson and Marth (1990)	V7	30	0.995	6.5	0.87	0.87	yes
Hudson and Mott	NCTC 7973	4	0.995	6.1	68.70	23.50	yes
(1993a)	L70	4	0.995	6.1	69.00	23.50	yes
	NCTC 7973	10	0.995	6.1	14.30	6.07	no
	L70	10	0.995	6.1	14.40	6.07	no
Walker *et al.*	433	0	0.997	7.2	77.00	181.77	yes
(1990)	433	2.5	0.997	7.2	33.00	39.83	yes
	433	5	0.997	7.2	19.00	16.97	yes
	433	7.5	0.997	7.2	9.50	9.35	yes
	433	9.3	0.997	7.2	9.00	6.65	yes
Grant *et al.*	CRA 198	5	0.99	6	18.86	18.67	yes
(1993)	CRA 198	10	0.99	6	9.43	6.50	yes
	CRA 433	5	0.99	6	17.24	18.67	yes
	CRA 433	10	0.99	6	8.37	6.50	yes
Ferguson and Shelef	DA 3	5	0.995	6.5	18.07	17.42	yes
(1990)	Brie 1	5	0.995	6.5	13.46	17.42	yes
			Bias factor			1.049	
			Precision factor			1.336	

5.4.5 Conclusions

The overall performance of the models developed in Chapter 4 is at least as good as other published models in terms of bias and precision. Published models were assessed only on the basis of the data presented by their authors and may have yielded different index values if calculated on the basis of the data collated and presented in this chapter.

Table 5.4 summarises the performance indices of all 'iterative' and nonlinear regression fitted models to all relevant data sets and shows that neither method of model generation consistently provides lower bias. Nor does either method of model generation lead to consistent over- or underprediction. The nonlinear regression models generally appear to show better precision, but this may be misleading because of the large number of 'no growth' predictions by the nonlinear regression models. The exclusion of 'no growth' predictions from the index calculations tends to improve the precision index of the nonlinear models compared to the iterative models. For those data sets where both models gave the same number of growth predictions the precision was virtually identical.

Thus, the hypothesis of Chandler and McMeekin (1989b) for the development of temperature and water activity models is supported by the results of this work, and in particular the development and evaluation of Eqn. 5.2. The performance of the models evaluated in this chapter also support the general modelling philosophy developed in Chapter 2, and confirm the reliability of turbidimetric methods of growth rate estimation developed in Chapter 3.

The hypothesis of McMeekin *et al.* (1992) in relation to the generation of temperature-water activity-pH models cannot be fully assessed on the basis of this work due to the difficulty of finding a suitable function to model the pH response, and also the uncertain relationship between the experimental system (0.2M lactate) and the conditions under which published generation times were obtained. Adams *et al.* (1991) demonstrated the acidulant specificity of pH_{min} in their models, and the effects of organic acids are believed to be not solely due to pH effects (Eklund, 1989). Should an appropriate function to describe the pH response over a reasonable range be found, the results in this chapter and the documented independence of temperature, water activity and pH effects on microbial growth rate would be expected to permit the derivation of reliable models by the proposed approach.

Potential problems in the proposed methodology have again been highlighted in this chapter, in particular the consequences of anomalous parameter estimates combined with extrapolation beyond the range of the data. It is noteworthy, however, that the T_{min} estimates of Wijtzes *et al.* (1993) for *L. monocytogenes* were from -1.75 to -2.55°C, and have an average which is close to the value used in Eqn. 5.2. The T_{min} estimates of Wijtzes *et al.* (1993) were

derived, however, from experiments in the temperature range 5 - 35 °C. This suggests that the poorer performance of Eqns. 4.2 - 4.3 when extrapolated predictions were made may have its basis in the anomalous T_{min} estimate used, rather than extrapolation of the square-root-type models beyond the bounds of the data *per se*.

Returning to the points raised in 4.4.5, this chapter has again highlighted the need for an experimental design which generates reliable estimates of the parameters of square-root-type models. Secondly, if there are systematic deviations in the responses predicted by the square-root-type temperature-water activity models, they have not been shown to reduce their performance compared to other model types.

The results of model evaluations suggest that there may a limit to the precision of model predictions to independent data. The highest degree of precision of any of the models developed when applied to a literature data set was ~ ± 35%. Even for the *S. aureus* 3b models predictions are only within ± ~25% of the generation times determined for the same strain in foods under well controlled laboratory conditions. These observations are in contrast to that of Monod (1949), and may result from the difficulty in determining the growth rates of microorganisms in heterogeneous and ill-defined environments such as food. Together with the use of the bias index developed in this chapter, the apparent lower limiting value of the precision index provides a basis for a rational set of criteria whereby a model can be said to have been validated.

6 TERTIARY MODELS: TEMPERATURE FUNCTION INTEGRATION

6.1 INTRODUCTION

Predictive modelling offers a rapid, non-invasive, and objective means of assessing the effect on product quality and safety of processing, storage and distribution conditions without recourse to traditional (culture-based) microbiological skills and facilities. One major area of application is the prediction of product shelf life and safety under controlled and constant conditions, e.g. evaluation of the effects of changed product formulation. Another is the assessment of elapsed or remaining shelf life of foods on the basis of conditions actually experienced during processing and distribution. It is easier and faster in almost all situations to monitor environmental parameters over time and relate these to bacterial growth than to measure that growth directly. Such 'environmental histories', moreover, can be analysed by many models specific for groups or species of interest to predict the extent of their growth. Using existing methodology, groups or species of microorganisms of interest must be isolated and/or enumerated separately.

Technology which predicts the growth of populations of microorganisms under static and defined conditions is well developed. The PMP (Buchanan, 1991b), distributed free-of-charge, includes models for the growth of eight pathogens as functions of temperature, salt concentration, pH, nitrite concentration and aerobic/anaerobic atmosphere, and is contained on a single computer disc. Food Micromodel offers essentially the same information but is also able to make predictions for the effect of fluctuating conditions. Currently, access to the model is available only via a trained operator and at considerable expense. The Delphi logger and software (Gill *et al.*, 1988a,b) enable interpretation of temperature histories but for one or two organisms only. To improve the access of non-expert users to the benefits of predictive microbiology for interpretation of environmental histories simpler 'user friendly' systems are also required.

Using temperature-based models as examples, this chapter describes some simple devices and general strategies for the interpretation of environmental histories in terms of potential growth of microorganisms.

6.2 INTEGRATION

Predictive microbiology relies on the prediction of rates of growth of microorganisms. The rate is expressed in generations per unit time (e.g. gen h^{-1}). Ultimately it is of interest to know how this relates to actual numbers of microorganisms on the product and the consequences for product safety and remaining shelf life, i.e. it is necessary to know how many generations of growth have been possible in the period monitored. If it is assumed that, under the

conditions of interest, the time taken for growth rate to 'equilibrate' to new conditions is negligible, the answer is given by the product of rate and time:

Growth rate (gen h^{-1}) x Time (h)
= Number of generations

The number of generations corresponds to the number of population doublings, thus three and a half generations represents a greater than tenfold increase in microbial numbers, and six and a half generations correspond to nearly a one hundred-fold increase.

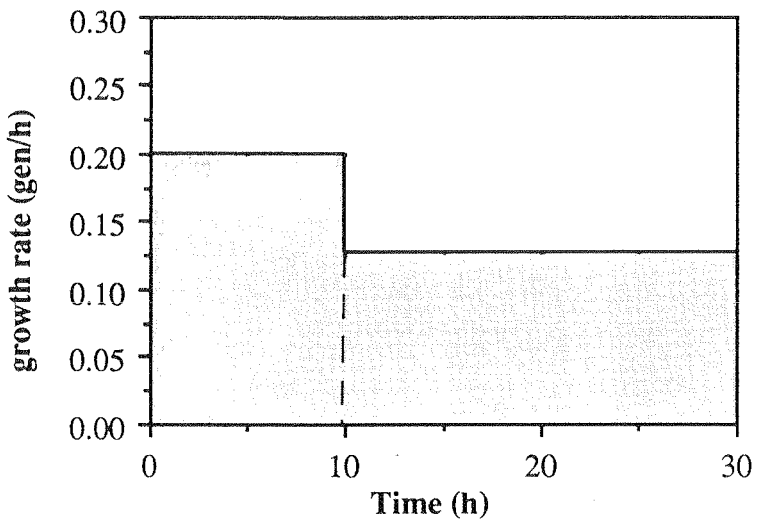
Geometrically, integration of rate can be visualised as the area under a graph of rate *versus* time. Provided the growth rate is constant the above calculation is a simple one, as is its geometrical interpretation (Fig. 6.1a). Where rate fluctuates the same mathematics hold; they are simply more difficult to apply. A primitive method is to plot the data on graph paper and count the squares under the curve, or even to cut out the area under the curve and calculate the area from the weight of paper. A more accurate method is to divide the graph into many small time intervals and determine the number of generations in each, using as an approximation the average rate value in that interval. The growth calculated for each of these intervals is summed to give the total number of generations over the entire time (Fig. 6.1b). The smaller the time interval chosen the more accurate will be the estimate. If the relationship between time and rate can be described by a mathematical equation, the time interval can be chosen to be vanishingly small and an exact value for the area under the graph calculated. This is the mathematical process of integration.

6.1.2 Temperature Function Integration and Food

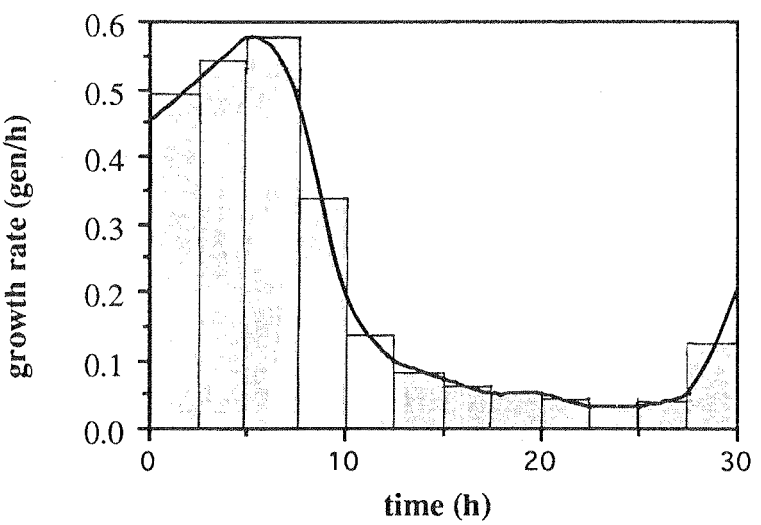
The concept of temperature function integration for predicting bacterial growth was envisaged by Scott (1936, 1937), and was again considered in the 1970's by Nixon (1971) who presented, and subsequently patented, working designs for predictive devices for use with fisheries products. Nixon based his work on results showing that the relative spoilage rate of fish over the temperature range 0 - 10°C, and above a certain limiting value (analogous to T_{min}), was directly proportional to temperature. Thus he was able to integrate temperature values directly and multiply the result by a constant value to obtain the extent of spoilage relative to that known at some reference temperature.

The simple relationship between temperature and relative rate of spoilage used by Nixon (1971) is inadequate to describe rate of growth or spoilage over a wider temperature range and for other systems. More complex mathematical

a.


0.30
0.25
0.20
0.15
0.10
0.05
0.00
growth rate (gen/h)
0
10
20
30
Time (h)

b.


0.6
0.5
0.4
0.3
0.2
0.1
0.0
growth rate (gen/h)
0
10
20
30
time (h)

functions are required to model the relationship between temperature, other environmental factors, and microbial growth rate. Predictive models define those relationships.

6.2.2 Temperature Function Integration Devices

A first step in an environmental history integration is to use the predictive model to convert the values of the measured environmental parameters at each time interval into a predicted growth rate. The environmental factors governing microbial growth rate in food are not usually a simple function of time, unless the product is stored under very well controlled conditions. In most situations it will not be possible to express the environmental history as a mathematical function to be integrated implicitly. Instead one must revert to first principles, a tedious, repetitive process requiring the determination of a mean value of rate over the time interval, its multiplication by the time interval, and the summation of all the separate intervals. Alternatively, one could take a mean of all the rate values calculated and multiply this by the total time.

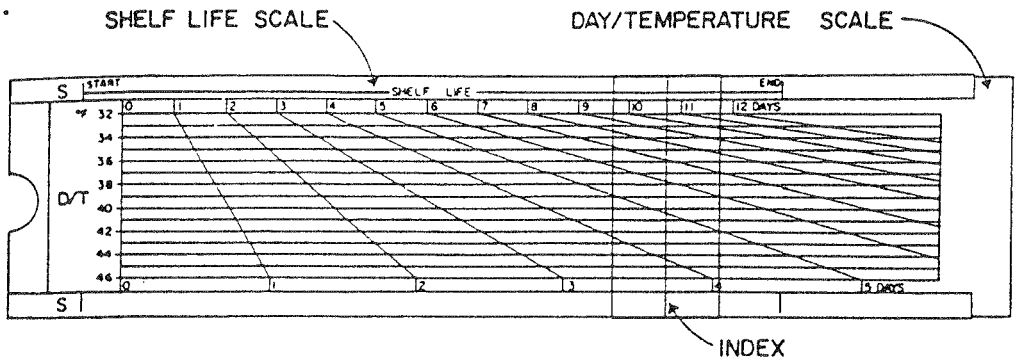
6.2.3 A Manual Tertiary Model

An ingenious device to simplify the process was devised by Ronsivalli and Charm (1975) and is shown diagrammatically in Fig. 6.2a. This mechanical integrator, named the 'shelf life prediction slide rule' or 'predictor', consists of three parts:

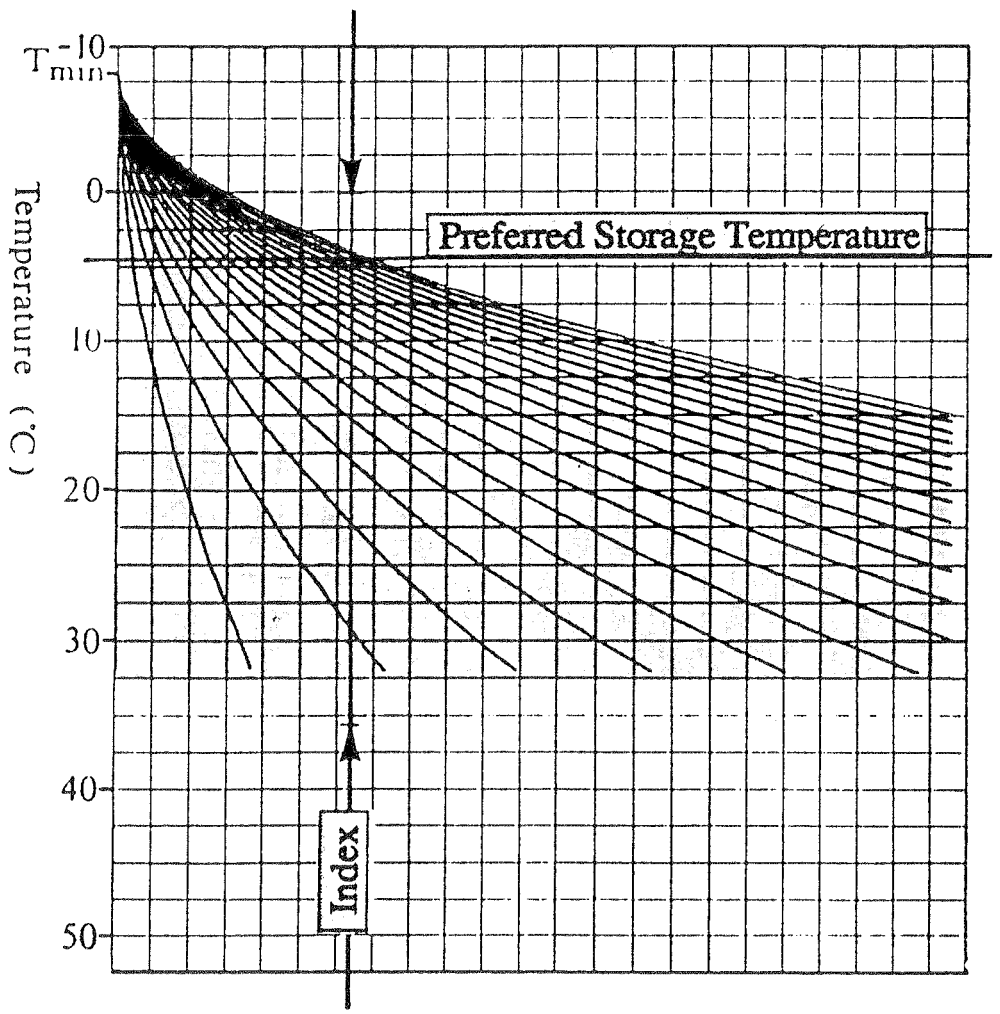
i) an index (sliding cursor),
ii) a shelf life scale, and
iii) a temperature scale.

The user selects the observed temperature on the temperature scale, and moves the cursor along that line for the appropriate amount of time that the product has been at that temperature. Starting with the cursor at this new point, the next time-temperature combination is added. This process is repeated for each time-temperature combination the product is known to have experienced (for example, by reference to a chart record), so that the cursor always moves further to the right. When all parts of the temperature history have been integrated, the overall effect on storage life is determined by reading the final position of the cursor against the shelf life index, which represents the remaining shelf life of the product at some ideal reference temperature. One of the claimed benefits of the device is that users can see the economic benefits which can be realised by keeping the product at as low a temperature as possible. At the time of invention the relationship between temperature and spoilage rate was poorly defined and the device was only reliable in

a.


SHELF LIFE SCALE
DAY/TEMPERATURE SCALE
START
SHELF LIFE
END
12 DAYS
D/T
5 DAYS
INDEX

b.


T_{min}
-10
0
10
20
30
40
50
Temperature (°C)
Preferred Storage Temperature
Index

the range 0-7°C. The integrating principal of the device remains sound, however, and by incorporating a more appropriate rate-temperature relationship a simple, inexpensive, and reliable predictive device could still be manufactured to enable interpretation of the effects of temperature history.

Figure 6.2b depicts a device which incorporates the square root model into the predictor. Relative rate values predicted by the square root relationship are shown on the shaded scale, for equal time intervals. The relative rate scale slides along the temperature axis, however, so that the predictor can be used for any organism or spoilage process which the square root model describes. The zero rate point, marked 'T_{min}' is aligned with the temperature corresponding to the T_{min} of the organism or spoilage process being integrated. The cursor is used as in the original version; i.e the growth at each time interval is integrated by moving the cursor the appropriate time measure to the right, using the relative rate scale, along the line corresponding to the temperature observed over that time interval. In addition, rather than using a fixed reference temperature, an adjustable 'reference temperature cursor' could be used. This would enable calculation of the elapsed shelf life relative to that at *any* preferred storage temperature. These changes would make its operation general for a wide range of organism/food systems.

6.2.4 A Spreadsheet-based Tertiary Model

Unless the time-temperature relationship can be defined mathematically the process of integration of time-temperature histories is tedious and repetitive, even with an aid like the predictor. Repetitive tasks, however, are something to which microprocessors are perfectly suited. Microprocessors, together with transducers to supply the data in a form that the microprocessor can manipulate, hold one solution to the routine application of predictive microbiology. An alternative to the construction of dedicated integrators is to use data loggers, which are widely available, and to develop applications software to interpret the environmental histories collected. The potential of this technology was reviewed by the candidate in McMeekin *et al.* (1993, Chapter 9).

Applications software written to interpret environmental histories is now described, initially for the simple case of temperature as the only fluctuating variable, and is exemplified by reference to prototype Excel[1]-based software for the growth of *E. coli*. To simplify the description, it is considered in terms of data input, processing and output aspects of the application.

The software is based upon the analysis of a file containing time and corresponding temperatures, however, the logger output may have to be processed first to achieve the required format. For example, the data may be in the form of

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[1]Microsoft Excel Version 4.0 © 1985 -1992. Microsoft Corporation, 1 Microsoft Way, Redmond, WA, USA.

temperature records only, together with the duration of the recording interval and the time of the initial temperature log. Data of the required format can readily be derived from this type of information using available spreadsheet functions.

6.2.4.1 Input

The fundamental user input is the time-temperature data file. Further inputs are necessary to generate growth curves representative of the growth of the organisms of concern in foods although, as described in 1.1.5, useful predictions of relative increases can be made without this information. Other desirable inputs are

i) the initial inoculum level
ii) the known lag time of the organisms on the product at a given temperature
iii) the level of concern, e.g. the population density at which spoilage occurs, a specification which must not be exceeded, etc.
iv) the maximum population density (MPD) achieved by the organism on the product

The initial bacterial load is required only for quantitative determinations of $N_{(t)}$. If this information is unavailable, the increase in bacterial numbers relative to that at $t = 0$ can be calculated using a default value of 0 Log(CFU/unit food), i.e. 1 CFU/unit food. An alternative strategy was used by Gill *et al.* (1988a) who requested the user to input the 'assured storage life' of red meats at some reference temperature. Assuming that spoilage occurred at 10^7CFU-spoilers/cm^2, they derived an initial bacterial load estimate from the rate of growth of the spoilage organisms at that temperature. This approach assumes some knowledge of the lag phase duration also.

The treatment of the lag phase inputs are discussed fully below. Briefly they are used to calculate an equivalent number of generation times to that required for lag resolution. In the current application, if details of the lag period are unknown a default value of zero will yield a conservative, i.e. 'fail safe', estimate.

The MPD input is used to model the decrease in growth rate as a function of population density and is also discussed more fully below. If the information is unavailable a default value of 10^9CFU/ml or g or cm^2, representative of the MPD in many products in which temperature only is growth-limiting, may be used. Other MPD values may be more appropriate to other product types.

6.2.4.2 Processing

The increase in bacterial numbers is determined by integration. The number of generations of growth occurring in each interval between logs is calculated and summed to give the number or increase in number of bacteria at the end of each time interval. This approach was also used by Broughall *et al.* (1983) and Gill and

Harrison (1985), but a number of refinements are presented here to generate a full growth curve.

The core of the application is a model which relates the temperature to the rate of growth. Let us call this model $f(T)$. Over each interval the rate predicted by $f(T)$ is calculated from the average of the temperature at the beginning and end of that interval. The interval can be calculated from the difference between any of the time records, whether entered as absolute or relative time. For the initial interval there is no initial temperature datum available and a 'dummy' initial value is used, equal to the temperature at the end of the interval.

The rate estimated by this method can only be considered as a potential growth rate because it is dependent upon the population density. In many situations of interest to food microbiologists the decline in growth rate as the population size increases is insignificant until after spoilage or toxigenic levels are exceeded. It is, nonetheless, desirable to model the entire growth curve including lag, and stationary phases of growth. The dependence of growth rate on population density was modelled mechanistically by Jason (1983), who showed that the rate of microbial growth depends on the initial density as well as the size of the population relative to the maximum population density. Jason modelled the decline in rate, as the population density approaches the maximum population density (MPD) by Eqn. 3.4:

$$\mu_{(t)} = \mu_{\max} \frac{MPD - N_{(t)}}{MPD - N_{(0)}}$$

Thus, the rate over an interval, $t_{(i)}$is given by:

$$rate_{obs} = f(T) \frac{MPD - N_{(t_{i-1})}}{MPD - N_{(0)}} \qquad (6.1)$$

To avoid circular references within the spreadsheet the value for N estimated at the end of the previous interval (i - 1) is used in this calculation. If the amount of growth calculated within an interval is large it is possible that the above calculations lead to a value for $N_{(t)}$ that is greater than MPD. To circumvent this a further conditional statement is used so that, if the predicted $N_{(t)}$ exceeds MPD, the value of MPD is returned. MPD may be a function of the environment which the product presents to the organisms (Jason, 1983; Buchanan *et al.*, 1993a). Consequently, given the appropriate information, a more sophisticated version of the software could include routines to calculate the MPD from product formulation information.

There are finite temperature limits for each organism beyond which growth is not normally possible. None of the kinetic models in contemporary use (see 1.1.4) define these limits. Square-root-type models do predict 'zero and negative' growth

rates beyond finite limits, but the lower limits may be inappropriate because growth typically ceases several degrees above T_{min} (Chandler and McMeekin, 1989b; Neidhardt *et al.*, 1990). Other situations exist for which the temperature range of concern is narrower than the growth range, e.g. due to reduced water activity, growth is greatly reduced at temperatures at which the products become frozen; *S. aureus* toxin production occurs over a narrower temperature range than growth (Smith *et al.*, 1983; Schmitt *et al.*, 1990; *see* 4.1). Such limits should be built into interpretive models to prevent overprediction of risk, and can be readily incorporated into the spreadsheet, using conditional statements. A further refinement would be to incorporate models which relate the temperature limits for growth to other environmental factors, and to use these to control the conditional values. Currently, such models have not been developed.

In the example presented here, for temperatures above T_{max} and below 7.8°C, the temperature at which some strains of *E. coli* cease to grow (Ross, unpublished; Friedmann *et al.*, 1969), $rate_{obs}$ is equated to zero. The calculation of $rate_{obs}$, including the conditional values, may be undertaken in a single spreadsheet function. With the advent of more sophisticated models, negative growth rates (e.g. thermal death, loss of viability) may be modelled and, thus, extend the temperature range of applicability of the model.

The *potential* number of generations over the interval is calculated by multiplication of the modified rate value and the time interval. The realised growth will depend, however, on the time required to resolve the lag phase. In the absence of details of the lag phase duration, a worst case scenario is adopted and the predicted growth is equivalent to the potential growth. If, however, the user has details of the lag phase duration in the product at a known temperature the lag phase at other temperatures can be predicted also.

The specified lag time is converted into an equivalent number of generations as the product of lag time and the generation rate at that temperature, i.e.

$$\text{lag generations} = \text{lag time} \times f(T) \times \left(\frac{MPD - N_{(t-1)}}{MPD - N_{(0)}} \right) \qquad (6.2)$$

This strategy was used by Broughall *et al.* (1983), and Labuza and Riboh (1982), and is routinely employed by workers at the University of Tasmania for planning experimental sampling times. It is based upon the observation that the temperature-lag time relationship is qualitatively the same as the temperature-generation time relationship (McMeekin *et al.*, 1993, pp. 211-212), although this realtionship has not been rigorously tested under all conditions and variables combinations. Cell density at t may now be calculated from the difference between the potential number of generations and lag generations, using conditional functions. If the potential number

of generations exceeds the lag generations, the population density is calculated, based on the Monod equation:

$$\log N_{(t)} = \log N_{(0)} 2^{(\text{potential generations} - \text{lag generations})} \qquad (6.3)$$

If the lag generations exceed the potential generations, the lag phase has not yet been resolved, and $N_{(t)} = N_{(0)}$. It should be noted that all operations described above are on absolute numbers of cells, rather than log numbers. As it is normal practice to plot the logarithm of cell numbers, the absolute cell density predicted is converted to the logarithm at this stage. As stated above, if the user is unable to supply a value for $N_{(0)}$ the processing steps are unchanged, $\log N_{(0)}$ is set to one, i.e. $N_{(0)} = 1$, and the results represent numbers *relative* to those at $t = 0$.

All of the operations described above can be applied to each temperature datum using 'fill' and 'last cell' commands under the control of command macro's to generate the predicted density of cells at the end of each time interval.

6.2.4.3 Output

The principal output is a graph showing the increase in numbers of the microorganisms of interest as a product of temperature and time. An 'overlay' chart of temperature and $\log N_{(t)}$ offers the advantage of showing the effect of temperature on rate of population increase, and the use of absolute time on the display enables times of product temperature abuse to be readily determined. Hence, the location of the product, or the agent responsible for the product, at the time of temperature abuse may also be identified.

The remaining storage life at a given temperature may also be determined from the following expression

$$\text{Storage Life Remaining} = \frac{[Tolerance - \log N_{(final)}] \times (1/\log_{10} 2)}{f(T)} \qquad (6.4)$$

where $\log N_{(final)}$ is the calculated value of $\log N_{(t)}$ at the end of the temperature history and *Tolerance* is the level of concern, expressed as log(CFU/unit food). The calculation is subjected to a conditional statement which yields the calculated value of Eqn 6.4 for positive values, and a statement such as "PRODUCT IS ALREADY SPOILED" for negative values. The model used for this calculation, $f(T)$, should also have conditional statements for the temperature range, as discussed above. For storage conditions in which no growth is predicted, the statement 'INDEFINITE' is returned for positive values of Eqn. 6.4. The application includes responses for nonsense inputs, e.g. if the input value $N_{(0)}$ is set greater than MPD.

6.2.4.4 User interface

Ideally, the operations described above must be presented to the user in an easy to use format, which is foolproof, and will not generate nonsense predictions. A prototype application incorporating the above approach was developed in Microsoft Excel 4.0, which was chosen because it includes many built in functions which permit the development of applications software by relatively unsophisticated users. The ability to create dialog boxes, command macro's, protect cells and spreadsheets, and to link documents enable the basic operations described above to be automated.

An overview of the prototype application is shown in Fig. 6.3. It is based on spreadsheet files, most of which could be replaced by dialog boxes for greater security and user robustness. The data file is pasted in to a template file in which the data processing occurs. The data file/worksheet and the 'macrosheet', which has instructions for management of the files/dialog boxes and processing instructions, are hidden from the user. If spreadsheets are used as the interface 'windows', they can be opened and closed automatically by command macro's linked to radio buttons on the spreadsheets so that as the user moves through the various stages of the analysis only one window is open at a time. The user is also given options to return to earlier windows (e.g. to re-read instructions), bypass them, or to quit the application from any window. A 'users view' of the application is shown in Figs. 6.4a-d.

Thus, the user is presented with a number of information screens, enters the requested information on the input screens, and selects the temperature history file. Processing occurs in the 'background' and the user is presented only with the output data, and the option of further analysis, i.e. remaining shelf life estimation or to return to earlier steps. The application also has potential as an educational tool, and an interactive spreadsheet combining the input and output screens is depicted in Fig. 6.4e. A demonstration version of this application is available.

6.2.4.5 Further developments

The potential of applications based on this approach is great. More sophisticated applications can be built up as information is obtained, or the need arises. For example, the inclusion of the effects of other limiting environmental factors may be accommodated by the use of additional data entry boxes, and reference to that entered value in the model used for rate calculation. The effects of a second fluctuating variable can be accommodated by the addition of another data column in the environmental history file and an appropriate model.

In 1.1.5 it was reported that the interactions between microorganisms in foods may, in many cases, be insignificant until the population densities are near to MPD. To extend this application to model the growth of mixed cultures requires only the relatively simple inclusion of models appropriate to the organisms present.

This would be useful for predicting the differential effect of environmental conditions on the growth of pathogens *cf.* spoilage organisms, and the consequences for shelf life *cf.* safety of foods under various formulations and storage conditions. It may be necessary, however, to provide some synthesis of the growth of mixed populations in a single environment. The synthesis required may be quite simple. An example is based on the results from inoculation of *S. aureus* 3b on to foods (see 5.2 - 5.3). *S. aureus* 3b was observed to enter stationary phase, irrespective of the *S. aureus* density, if the spoilage microbiota reached 'their' MPD first (results not shown). The results of Herten *et al.* (1989) show a similar effect. In this case the interaction may be modelled by using the cumulative *combined* population density to control the reduction in growth rates as the total population density increases.

6.3 CONCLUSIONS

Theoretically any electronic data loggers may be combined with predictive microbiology computer programs to yield estimates of the extent of growth of any microorganism of interest. Software can be written to analyse an environmental history or any part of it and, combined with a kinetic model, predict the specific increase in the number of bacteria of interest, whether spoilage organisms or pathogens. Relationships of any complexity could be incorporated into a predictive device, in principal, by recourse to 'look up' tables of experimentally determined rate values. Consequently, it is not strictly necessary to model mathematically the environment-growth rate relationship.

A single software package could include models for the growth of different bacteria. Using such information it is theoretically possible to assess objectively the effect of any given period of the environmental history on the overall shelf life and safety of a product. This information would be invaluable for Hazard Analysis, appraisal of problem areas in processing and distribution chains, for allocation of responsibility for loss of product quality through mishandling and for evaluating cost *versus* benefit of changed processing protocols. The complexity of the modelling undertaken is governed only by computer technology, and the availability of appropriate information upon which to base the models.

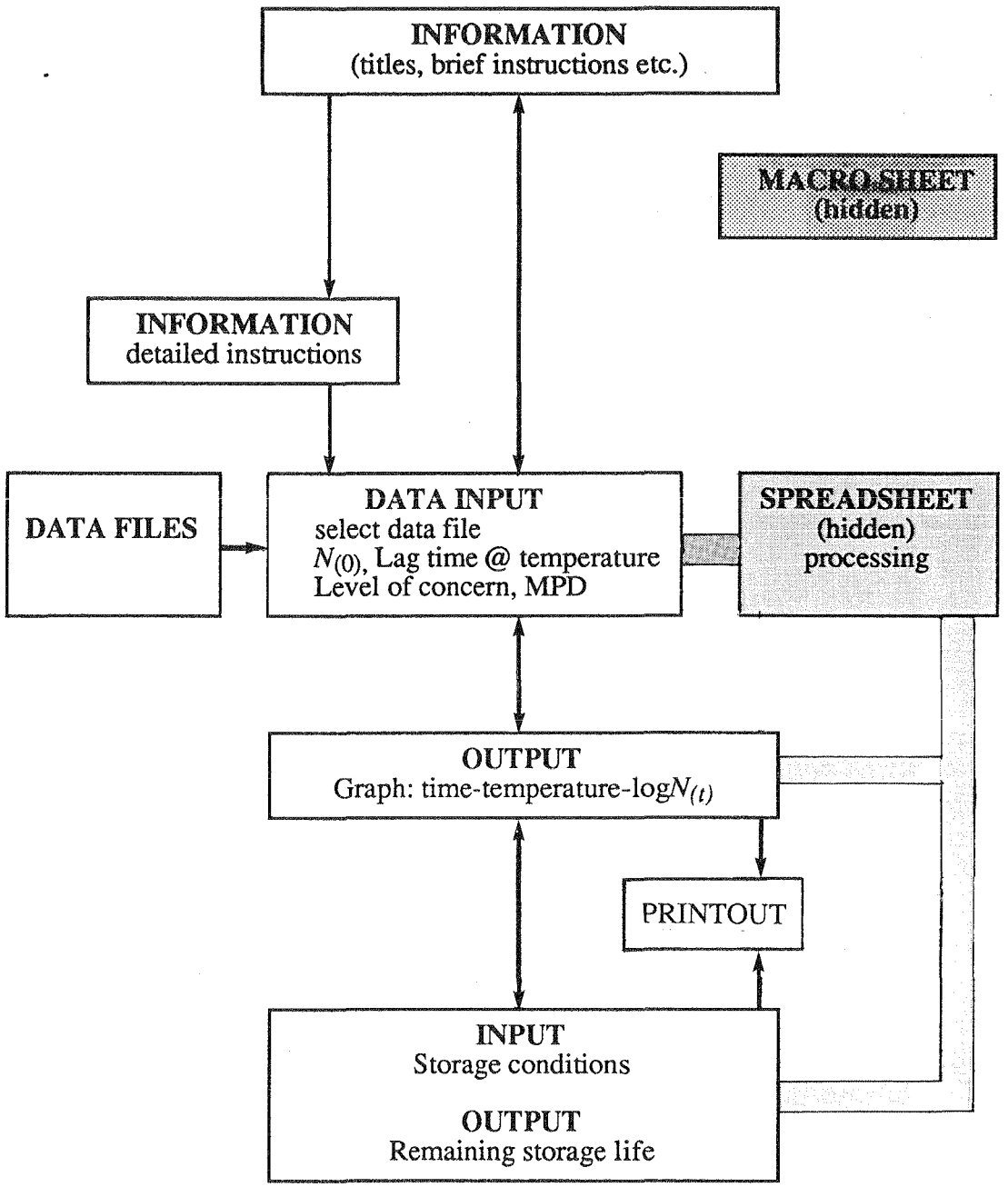


Fig. 6.3 Schematic diagram of a tertiary model for the interpretation of temperature histories as potential bacterial growth and remaining shelf life of products. (Wide shaded lines denote linked spreadsheets. 'Inputing' a new value in any of the linked files will lead to automatic recalculation of values, and amendment of output such as graphs or remaining shelf life).

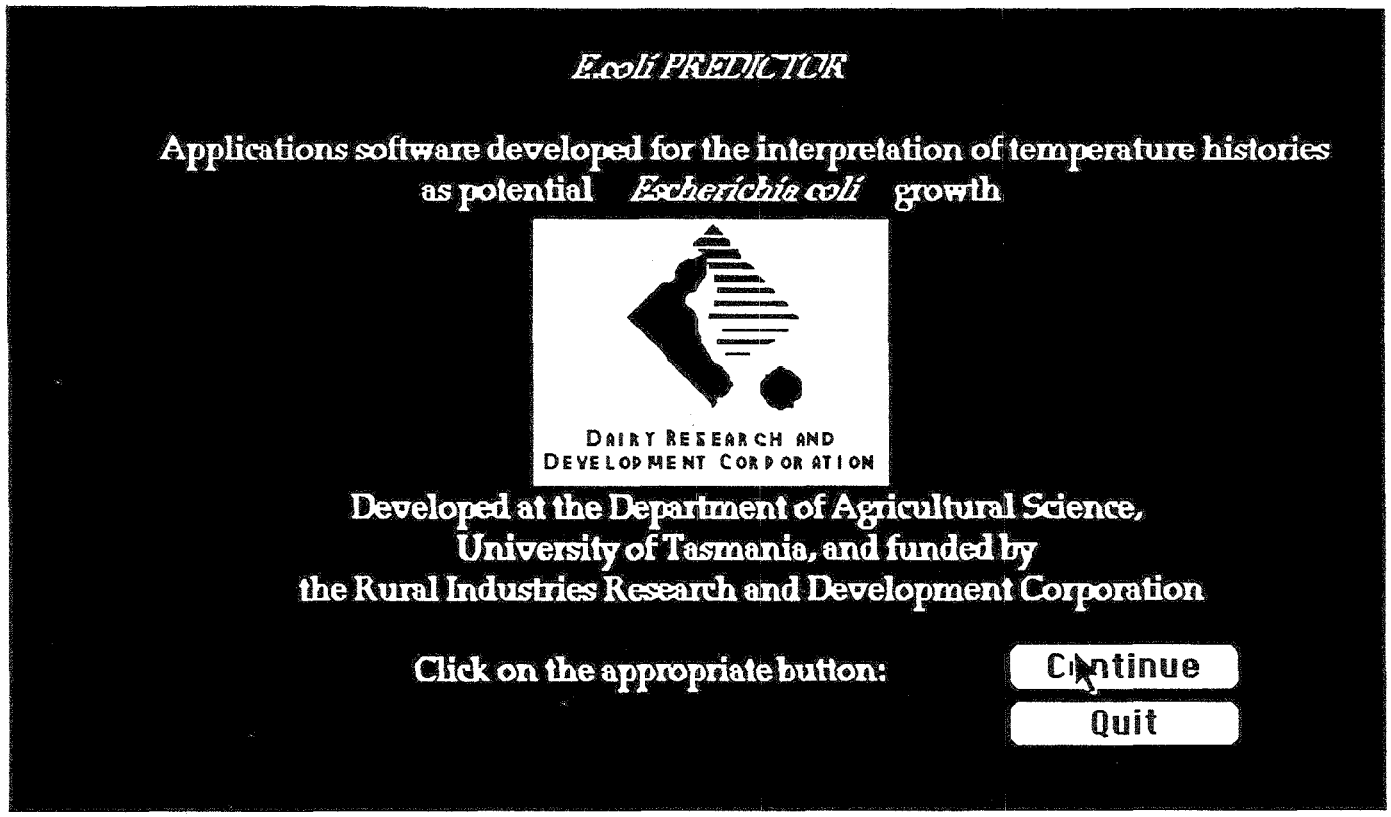


Fig. 6.4a Introductory window of a prototype tertiary model for bacterial growth and shelf life prediction.

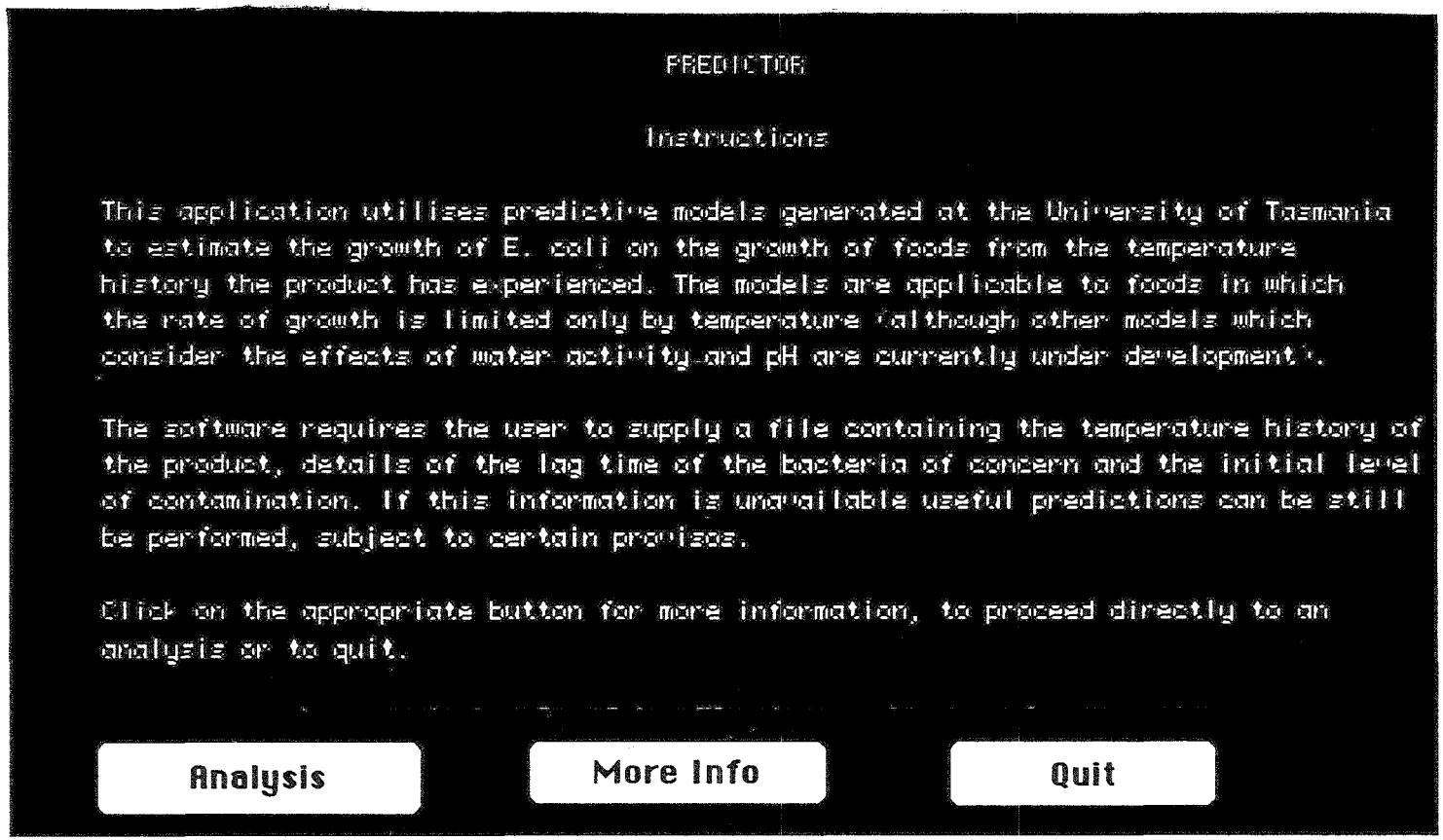


Fig. 6.4b An example of an 'instructions for use' window for a prototype tertiary model for bacterial growth and shelf life prediction.

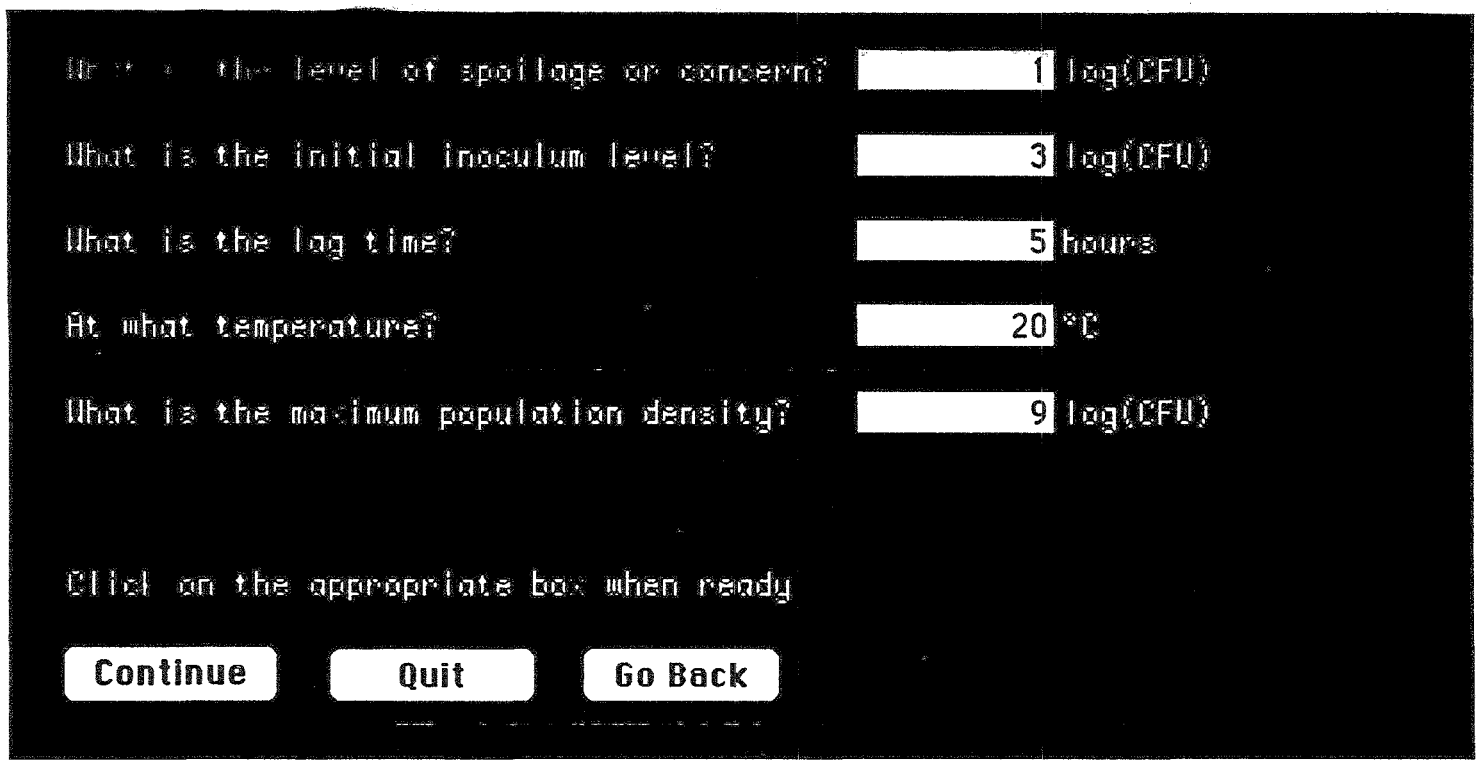


Fig. 6.4c An example of a 'data input 'window for a prototype tertiary model for bacterial growth and shelf life prediction.

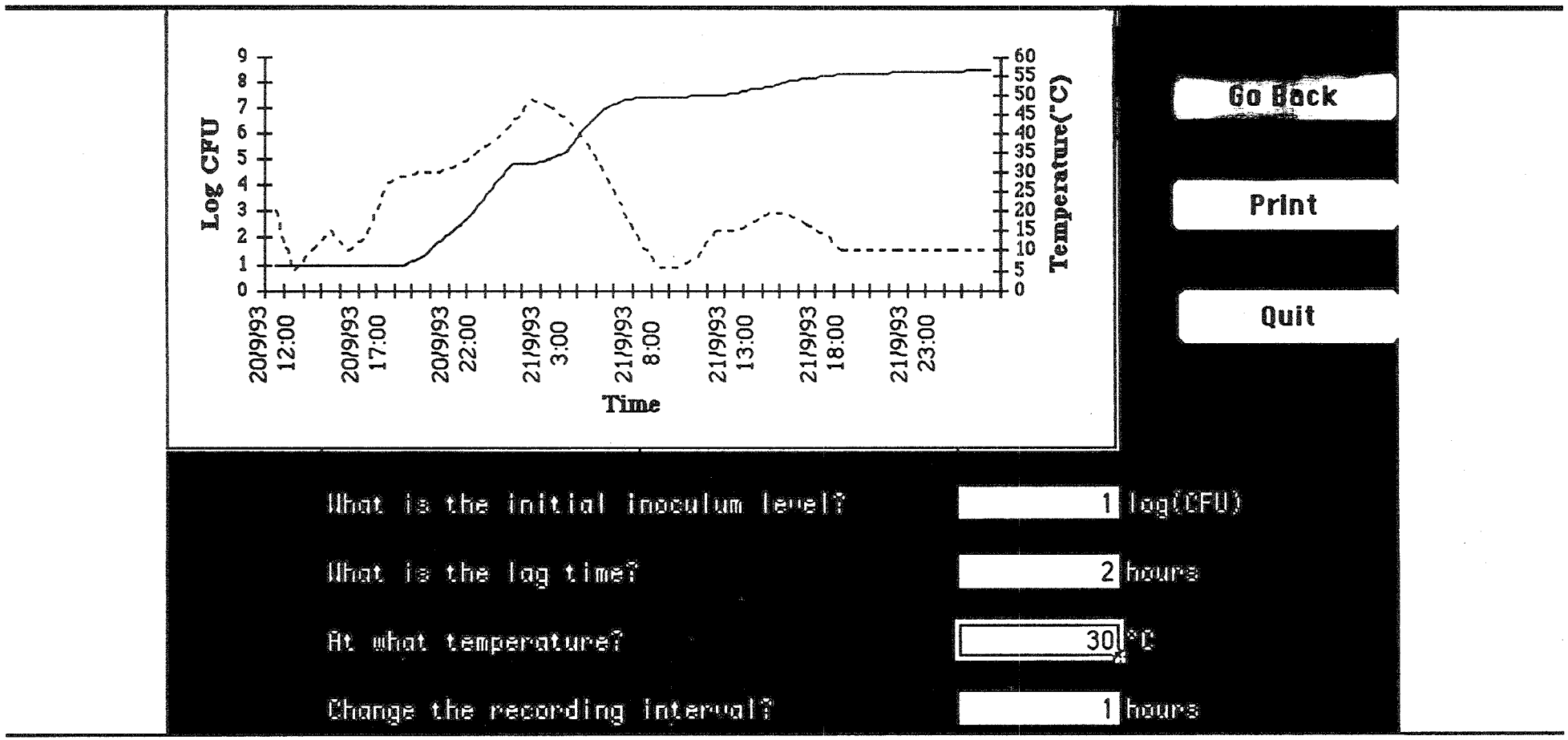


Fig. 6.4d Output from a prototype tertiary model for bacterial growth and shelf life prediction.

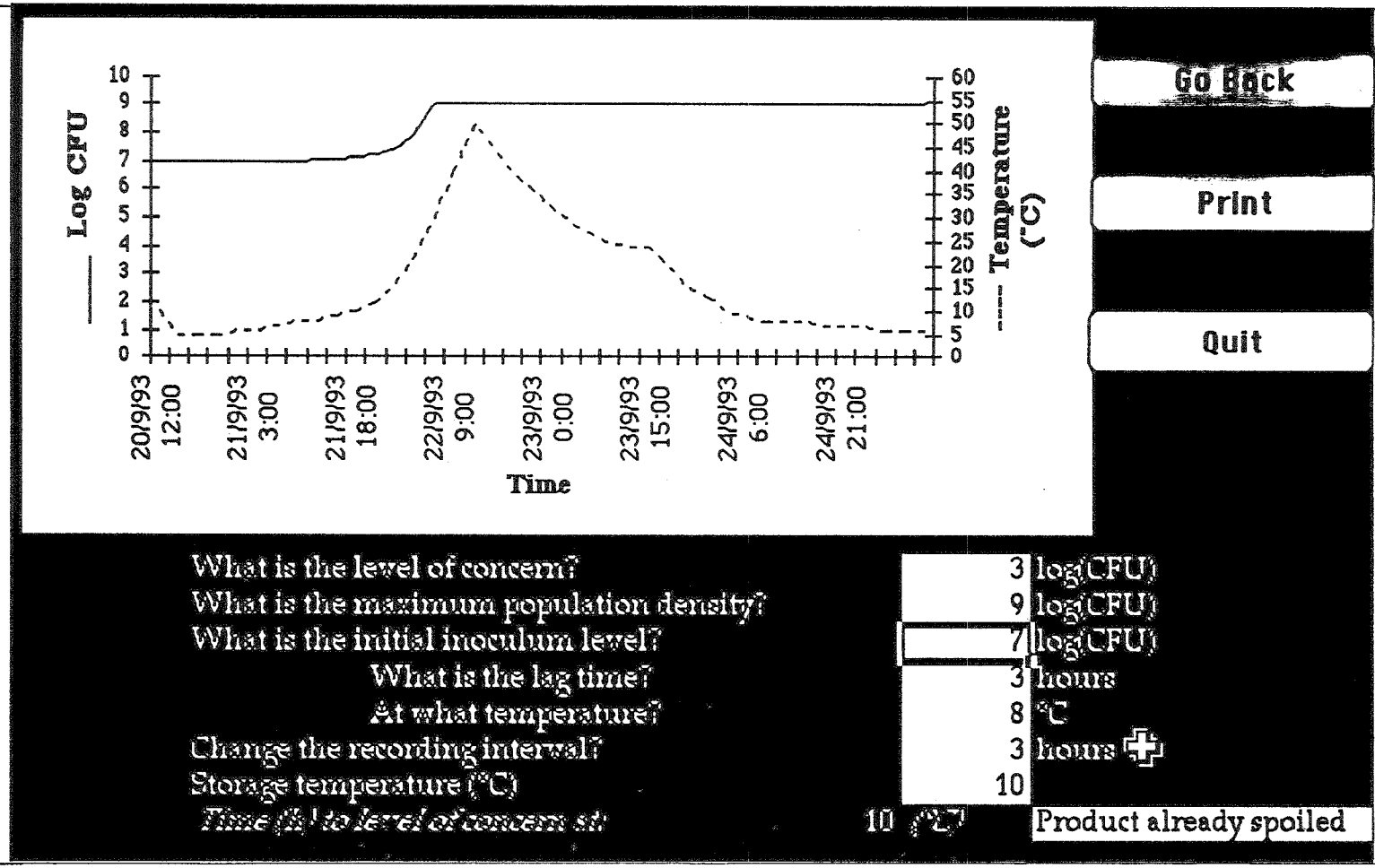


Fig. 6.4e An interactive spreadsheet for assessing the effects of various parameters on potential bacterial growth for a given temperature history.

7 MECHANISMS OF THE MICROBIAL GROWTH RESPONSE TO TEMPERATURE

> *"It is not sufficient to observe that a particular method or model appears to work; it is important to know why it works, otherwise there is no means of judging when and how it will not."* (Bratchell *et al.*, 1989).

7.1 INTRODUCTION

Square root-type models have been criticised on the basis of a reported systematic underprediction of the true growth rate at low temperatures (M. Cole, pers. comm.) and implicitly viewed as inferior because they have no demonstrated theoretical basis (Heitzer *et al.*, 1991). Draper (1981) considers mechanistic models preferable because they usually contain fewer parameters, usually fit the data better and usually extrapolate more sensibly. Ross (1993) and McMeekin *et al.* (1993) reported the development of a mechanistic model for the temperature dependence of growth rate responses of bacteria and demonstrated a possible convergence between the master reaction-type models and the square root-type models. In this chapter the mechanistic model presented in McMeekin *et al.* (1993), Chapter 10, is revised by reference to more recent literature and its utility evaluated by application to experimental data.

7.2 MODEL DEVELOPMENT

7.2.1 Starting Assumptions

During exponential growth the production of all cell components is said to be 'balanced', i.e. there is no surplus nor limiting component, and the overall behaviour of an exponentially growing population has the attributes of a first order reaction. Van Damm *et al.* (1988) concluded that emerging thermodynamic approaches to modelling of microbial growth "allow us to understand growth kinetics in terms of microbial free energy metabolism", and that the bacterial cell may be considered as a catalyst which converts substrates (nutrients) into products (more cells), and which can be characterised by the change in free energy of the substrates and products.

Cellular metabolism is highly integrated and, for bacteria during balanced growth, the production of any metabolite and the rate of all cellular reactions behave as first order reactions. Although a common starting point in the development of mechanistic models for bacterial growth rate is the assumption of the existence of an enzyme catalysed reaction which is rate limiting under all growth conditions, a more

contemporary interpretation may be that a single enzyme sets the upper and lower temperature limits for growth, and that the activity of that enzyme mimics the growth rate of the whole cell. The enzyme is presumed to exist in one of two states; the catalytically active *native*, or N-state, and the inactive *denatured*, or D-state. Provided that the enzyme is in the native state, the reaction which the enzyme catalyses is well described by the classical Van't Hoff - Arrhenius equation or its more recent formulation (Eyring, 1935). The proportion of the total enzyme present in the native state is, however, a function of temperature. Thus, the observed rate of reaction results from temperature effects on both the reaction mechanism and the amount of active enzyme. Consequently, the reversible transition of the enzyme between the native and denatured states as a function of temperature, as well as the temperature dependence of the reaction itself, must be modelled.

The transition between active and denatured states may be described as a temperature dependent reaction obeying Arrhenius kinetics. This approach is not novel, having been adopted by Johnson and Lewin (1946) to describe the high temperature growth of bacteria, and by Hultin (1955) to describe rates of enzymatic catalysis in the low temperature region. Sharpe and DeMichele (1977) synthesised these two equations to produce a model for the temperature dependence of bacterial growth rate in the entire biokinetic region. The latter model was subsequently reparameterised by Schoolfield *et al.* (1981) to overcome difficulties in fitting by nonlinear regression. One of the assumptions made by those authors, however, was that the thermodynamic functions enthalpy change (ΔH) and entropy change (ΔS) are independent of temperature for the protein folding-unfolding reaction. For simple inorganic reactants this assumption is valid. It is now known, however, that there are temperature-dependent changes in ΔH and ΔS as the largely hydrophobic interior of the protein is exposed to water in the suspending medium, and that these lead to large changes in the heat capacity (ΔC_p) of the system upon reversible denaturation of the protein. These changes are largely caused by the restructuring of water in the region of exposed non-polar amino acid sidechains. The above phenomena are well described in the many recent reviews of the thermodynamics of protein conformational structure (e.g. Creighton, 1990; Dill, 1990; Castronuova, 1991; Lee, 1991; Jaenicke, 1991).

The development of the model from these starting assumptions is analogous to that presented by the candidate in Chapter 10 of McMeekin *et al.* (1993). The following mathematical development summarises that work. It should also be borne in mind that in this treatment temperature is the only limiting factor considered.

7.2.2 Model Derivation

From the foregoing discussion, it is assumed that the rate of growth in the absence of macromolecular denaturation may be described by the empirical Arrhenius-Van't Hoff relationship:

$$rate = A\exp(\Delta E_a / RT) \tag{7.1a}$$

or its mechanistic interpretation and modification due to Eyring (1935) based in absolute reaction-rate theory:

$$rate = KT\exp(\Delta H^{\ddagger} / RT) \tag{7.1b}$$

where the parameters may be interpreted as follows:

A is a constant related to the number of collisions between reactants per unit time

E_a is the 'activation energy'[1]

R is the gas constant (8.314 J K^{-1} mol^{-1})

T is the temperature in Kelvin

K is similar to A but includes steric and entropic effects

$\Delta H^{\ddagger}$ the enthalpy difference between the transition state complex and the reactants

Equation 7.1b may be interpreted as the product of the number of collisions which could lead to reaction (KT) and the probability ($\exp(\Delta H^{\ddagger} / RT)$) of the reactants having sufficient energy to overcome the enthalpic barrier to reaction. That probability increases as a function of temperature and decreases as a function of increasing activation energy.

Gibbs free energy (G) is a concept that has been used as a measure of a system to perform useful work. If a system may exist in several states, the state with the lowest Gibbs free energy is energetically favoured, and the system will tend to move towards that state spontaneously. To move from a lower to a higher free energy state requires the input of energy. The change in Gibbs free energy (ΔG) is related to ΔH and ΔS thus:

$$\Delta G = \Delta H - T\Delta S \tag{7.1c}$$

Consider the reversible denaturation of proteins:

---

[1] The experimentally determined value of E_a in Eqn. 7.1a is related to the *enthalpy* of activation. Acccordingly, the entropic component of the free energy of activation (free energy difference between the reactants and the activated complex) is mathematically included in K in Eqn. 7.1b from the relationship Gibbs free energy = $\Delta H^{\ddagger} - T\Delta S$

Native state $\rightleftharpoons$ Denatured state

For spontaneous reaction to occur, $\Delta G < 0$. Thus, the difference in Gibbs free energy of the N-state and D-state is now defined:

$$\Delta G_{den} = G_{denatured} - G_{native}$$

Enzymes will adopt the D-state if it is energetically favourable to do so, i.e. if $\Delta G_{den} < 0$. Surprisingly, for protein molecules ΔG_{den} is a parabolic function of temperature (*see* Appendix 4), a reflection of the delicate balance of relatively large forces which determine the stability of the native conformation. Murphy *et al.* (1990) published equations which permit the description of the difference in Gibbs free energy of the native and denatured states as a function of temperature. They considered that hydrophobic interactions played the major rôle in determining the stability of the N-state. Murphy and Gill (1991) subsequently gave a slightly different interpretation of those results. Based on a 'group additivity' approach which addressed the contributions of apolar groups, hydrogen bonding and configurational entropy, they concluded that hydrogen bonding was the main stabilising force. They reiterated, however, that the temperature dependence of the changes in enthalpy and entropy between the two states of the protein molecule could be summarised in terms of the change in heat capacity, ΔC_p, of the protein upon unfolding. This was achieved by reference to ΔC_p and experimentally derived temperatures at which all globular proteins studied exhibit the same change in enthalpy and entropy upon denaturation. From the latter paper the following equations may be derived readily:

$$\Delta H = \Delta H^* + \Delta C_p (T - T^*_H) \tag{7.2}$$

$$T\Delta S = T\Delta S^* + \Delta C_p \, [T \ln(T / T^*_S)] \tag{7.3}$$

where:

ΔC_p = difference in heat capacity of the protein in the N- and D-states

T^*_H = temperature (K) at which the ΔC_p contribution to enthalpy is 0.

T^*_S = temperature (K) at which the ΔC_p contribution to entropy is 0.

ΔH^* = value of enthalpy at T^*_H

ΔS^* = value of entropy at T^*_S

n.b. To further generalise the relationships, all thermodynamic quantities are expressed per mol-amino acid-residue in the protein.

Synthesis of Eqns. 7.2, 7.3 and 7.1c generates the following expression for the temperature dependence of the change in Gibbs free energy associated with protein unfolding.

$$\Delta G_{den} = \Delta H^* - T\Delta S^* + \Delta C_p[(T - T^*_H) - T\ln(T / T^*_S)] \tag{7.4}$$

Let $[N]$ be the concentration of catalytically active enzyme, and $[D]$ be the concentration of inactive enyzme. We wish to determine the proportion of enzyme which is active at any given temperature.

That proportion is given by:

$$\frac{1}{\frac{[D]}{[N]}+1}$$
$$= \frac{1}{K_{eq}+1}$$

and as:

$$K_{eq} = \frac{[\text{products}]}{[\text{reactants}]}$$
$$= \frac{[D]}{[N]}$$

The proportion of enzyme molecules which are catalytically active is given by:

$$\frac{1}{\frac{[D]}{[N]}+1}$$
$$= \frac{1}{K_{eq}+1}$$

The relationship between the change in Gibbs free energy and the equilibrium, K_{eq}, constant is given by:

$$\Delta G = -RT\ln K_{eq}$$
$$\therefore K_{eq} = e^{-\Delta G/RT}$$

Thus:

$$proportion\ of\ active\ enzyme = \frac{1}{e^{-\Delta G_{den}/RT} + 1} \tag{7.5}$$

Substituting Eqn 7.4 into 7.5 and multiplying the resultant expression by Eqn. 7.1b generates the following equation to describe the effect of temperature on the rate of enzyme catalysed reactions:

$$rate = \frac{CT\exp(\Delta H^{\ddagger}/RT)}{1+\exp(-n(\Delta H^{*}-T\Delta S^{*}+\Delta C_p[(T-T^{*}_{H})-T\ln(T/T^{*}_{S})])/RT)} \tag{7.6}$$

where:

C = a parameter whose value must be estimated
$\Delta H^{\ddagger}$ = activation enthalpy of the reaction catalysed by the enzyme
ΔC_p = difference in heat capacity (per mol. amino acid residue) between the N- and D-state of the enzyme
T^{*}_{H} = temperature (K) at which the ΔC_p contribution to enthalpy is 0.
T^{*}_{S} = temperature (K) at which the ΔC_p contribution to entropy is 0.
ΔH^{*} = value of enthalpy at T^{*}_{H} per mol-amino acid residue
ΔS^{*} = value of entropy at T^{*}_{S} per mol-amino acid residue
T = temperature (K)
R = gas constant (8.314 J K^{-1} mol^{-1})
n = number of amino acid residues in the protein.

This equation may also be applied to model the temperature dependence of the rate of bacterial growth if the assumptions in 7.2.1 are accepted.

7.3 METHODS

To gain insight into the behaviour of the model, Eqn. 7.6 was used empirically to simulate the temperature-√growth rate curves of a range of bacteria, and also fitted by non-linear regression (Ultrafit, Biosoft®, Missouri, USA) to experimental data for the temperature dependence of:

i) rate of dephosphorylation of nitrophenol phosphate by *E. coli* alkaline phosphatase (EC 3.1.3.1, Sigma), data of Salter (1993);
ii) growth rate of *Pseudomonas putida* 1442 in Nutrient Broth (NB), data of Neumeyer (Unpublished);
iii) growth rate of *Spirillum* L9, data of Harder and Veldkamp (1971); and
iv) growth rate of *Aeromonas hydrophila* 3459 and *Escherichia coli* M23 in NB, and of *Listeria monocytogenes* Scott A in TSB (original data).

Initially, values of the constants T^*_H, T^*_S, ΔH^*, and ΔS^* used were those determined by Murphy *et al.* (1991):

T^*_H = 373.6 K

T^*_S = 385.2 K

ΔH^* = 5640 J (mol amino acid residue)$^{-1}$

ΔS^* = 18.1 J K^{-1} (mol amino acid residue)$^{-1}$

The value of ΔH^* derived by Murphy *et al.* (1991) was based on data of Privalov and Gill (1988) for 11 proteins, most from mammalian sources. To accommodate the possibility that ΔH^* is not constant for all proteins, particularly in relation to the thermal adaptation of the organism from which the protein originates, a form of Eqn. 7.6 in which ΔH^* is a parameter to be estimated was also investigated.

Square root of rate data was fitted to the square root of Eqn. 7.6 by nonlinear regression. The square root of rate was chosen to homogenise the error for those data sets for which replicated data was available, and for consistency for those data sets where it was not. The estimation behaviour of the parameters in Eqn. 7.6 was not known and the model fitted as the square root of the parameterisation shown above. Initial parameter estimates were determined empirically using a spreadsheet (Excel 4.0, Microsoft® Corporation) to calculate and graph values of the response for given parameter values: parameter values were manipulated until the graph approximated the experimental data. The fitted curves were presented as square root plots to facilitate comparison with the square root model.

7.4 RESULTS

7.4.1 Analysis of the Model

The main features of the Eqn. 7.6 are shown in Fig. 7.1 from which the modifying effect of the denominator (proportion of active enzyme) on the rate predicted by the numerator (rate in the absence of denaturation) can be more readily appreciated. Increasing values of ΔC_p tend to move the curve further to the right on the temperature axis. The 'number of amino acids' value for the growth-range-limiting enzyme controls the magnitude of the exponential term in the denominator, and in consequence has the effect of controlling the rate of denaturation as a function of temperature, i.e. the steepness of the low and high temperature 'cutoffs'. There is a strong correlation between the rate coefficient 'C' and the $\Delta H^\ddagger$ terms which interact to model the magnitude of the observed rate over most of the range. $\Delta H^\ddagger$ appears in an exponential term, thus small changes in the fitted value of $\Delta H^\ddagger$ must be compensated for by large changes in the fitted value of C.

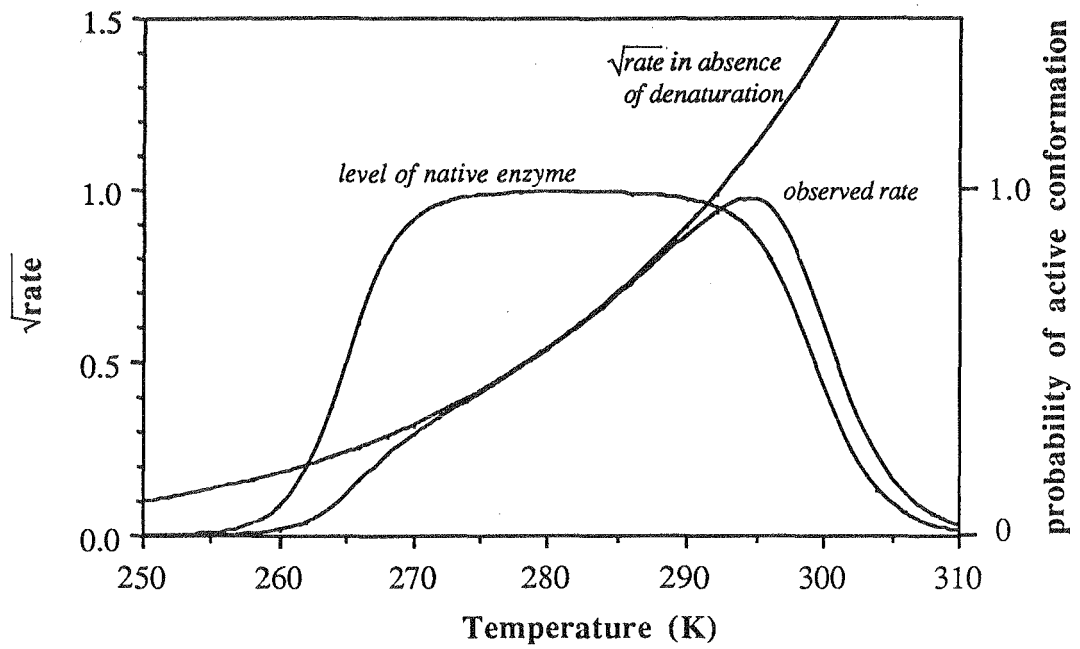


Fig. 7.1. Graphical analysis of Eqn. 7.6.

7.4.2 Enzyme Catalysis Data

The activity of alkaline phosphatase (AP) as a function of temperature is shown in Fig. 7.2. Allowing five parameters to be estimated from the data yielded the following fitted values: C = 7615 ± 18593, $\Delta H^{\ddagger}$ = 40371 ± 6115 J mol^{-1}, ΔC_p = 56.58 ± 3412 J K^{-1} (mol amino-acid res.)$^{-1}$, n = 110 ± 4109 and ΔH^* = 5636 ± 29460 J mol^{-1}. The Residual Mean Squares (RMS) value was 0.0011.

Setting ΔH^* equal to 5640 J K^{-1} (mol amino-acid res.)$^{-1}$, and allowing four parameters to be estimated from the data yielded similar values, but with much narrower confidence intervals: C = 7613 ± 12965, $\Delta H^{\ddagger}$ = 40371 ± 4357 J mol^{-1}, ΔC_p = 56.16 ± 1.40 J K^{-1} (mol amino-acid res.)$^{-1}$, and n = 109 ± 26. The RMS value was 0.0010.

E. coli alkaline phosphatase is composed of 600 - 800 amino acids (Reid and Wilson, 1971). The value of n was fixed at 590, calculated from the molecular weight of AP (Garen and Levinthal, 1960) divided by an average molecular weight of amino acids residues (135g mol^{-1}). Allowing the remaining three parameters to be estimated from the data yielded the following values: C = 13736 ± 28943, $\Delta H^{\ddagger}$ = 41818 ± 5399 J mol^{-1}, ΔC_p = 55.69 ± 0.32 J K^{-1} (mol amino-acid res.)$^{-1}$. The RMS value was 0.0017. 'Robust weighting' was used in all cases. The activation enthalpy of the same data estimated from an Arrhenius plot was 46200 J mol^{-1}.

7.4.3 Growth Curves: Simulated Data

Using thermodynamic parameter values determined by Murphy and Gill (1991) it was not possible to generate realistic bacterial growth curves. The range of thermal stability of the system predicted using Eqn. 7.6 was far wider than is observed for bacteria which are typically restricted to a biokinetic range of ~40 K (Neidhardt *et al.*, 1990). When ΔH^* also was estimated from the data, however, it was possible to produce realistic growth curves. For the simulations the number of amino acids in the enzyme growth-range-limiting enzyme was arbitrarily set at 250, and the value of C manipulated to give optimal rate values of the same magnitude to simplify visual comparison. The simulated curves are depicted in Fig. 7.3. The parameter values used to generate the curves are summarised in Table 7.1

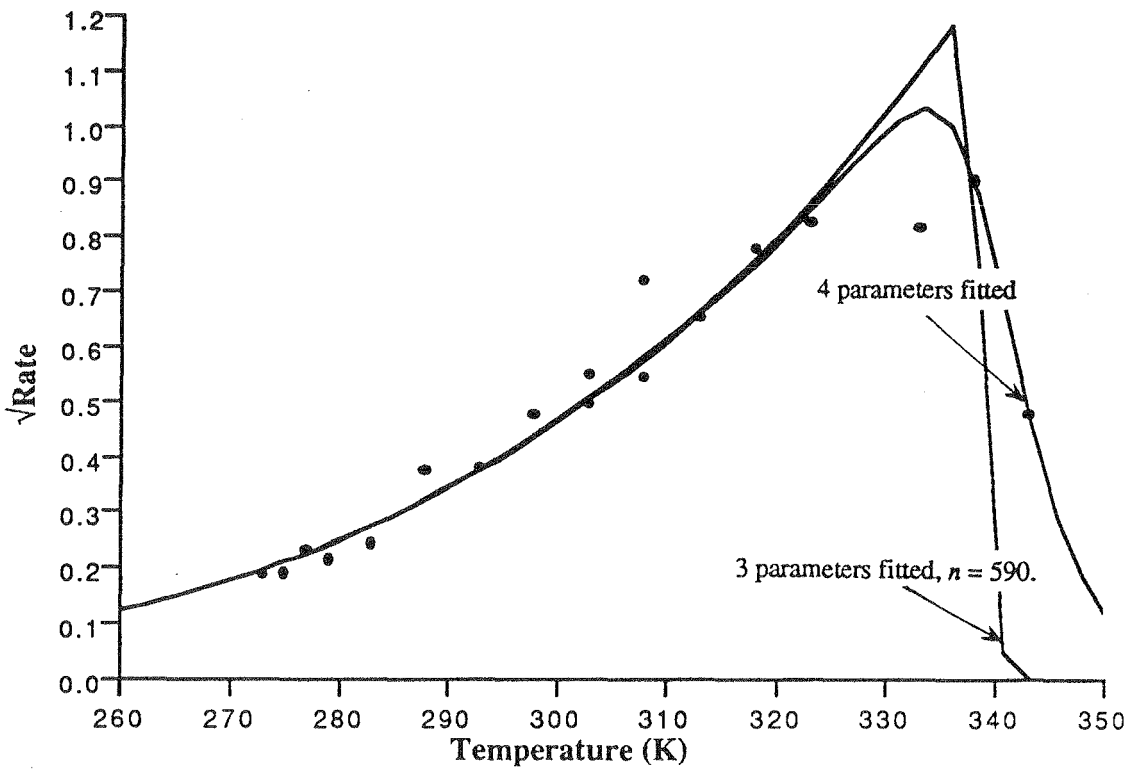


Fig. 7.2 Experimental values and fitted curves of Eqn. 7.6 for the cleavage of phosphate from nitrophenol phosphate by alkaline phosphatase. The curves shown are for the fitted parameter values given in 7.4.2, when four and three parameters of Eqn. 7.6 were allowed to be fitted to the data.

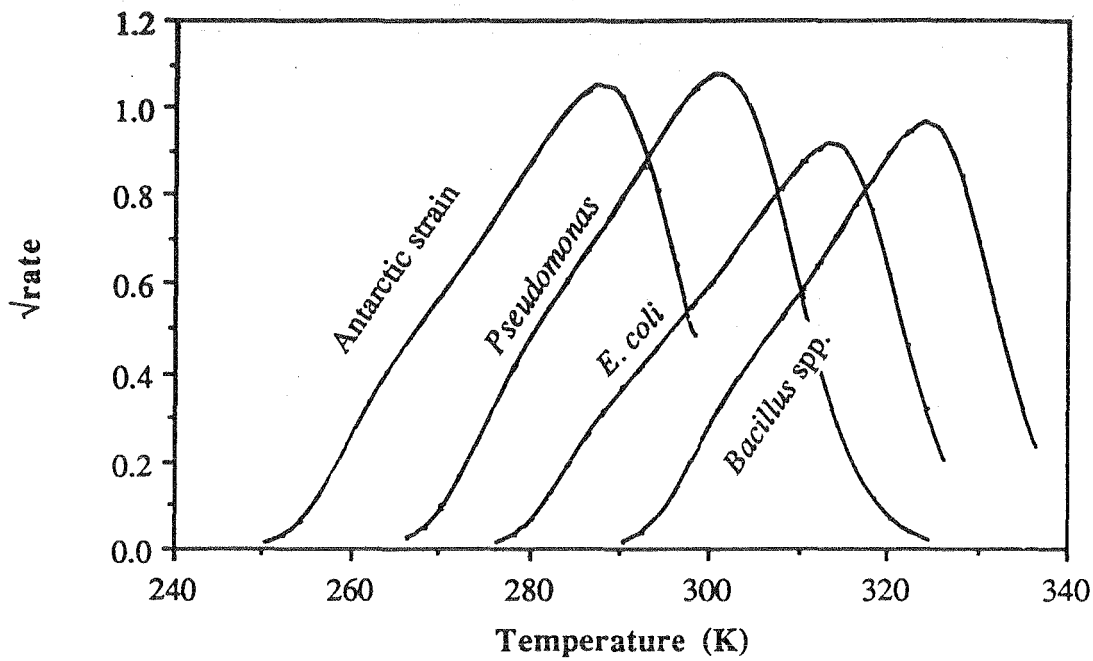


Fig. 7.3 Growth curves for a range of thermal classes of bacteria generated using Eqn. 7.6.

Table 7.1 Parameter Values for Eqn. 7.6 Used to Simulate Bacterial Growth Curves in Fig. 7.3.

Organism	Parameter Values			
	C	$\Delta H^{\ddagger}$ (J mol^{-1})	ΔC_p (J K^{-1} mol-res.$^{-1}$)	ΔH^* (J mol-res.$^{-1}$)
Antarctic strain (psychrophile)	8,000,000	51,000	54	5325
Pseudomonas Gp1 16L16 (psychrotroph)	20,000,000	55,000	63.5	5257
E. coli (mesophile)	80,000,000	62,000	73	5365
Bacillus spp. NCIB 12522 (thermophile)	2,000,000,000	75,000	86	5320

To assess the divergence between predictions of square root-type and master reaction-type models, both simple square root models (Eqn. 1.1), and 4-parameter square root models (Eqn. 1.2) were fitted to 'square root of rate' data generated at 2K intervals by each of the fitted Eqns. 7.6. Square root of rate values less than 0.1 in the suboptimal range, and less than 0.5 in the superoptimal range, i.e. those which represent very slow growth rates, were deleted from the data sets before fitting square root type models to reflect the range of data collected experimentally. Cardinal temperatures estimated from the data set simulated using Eqn. 7.6 are compared to those of the original experimental data set in Table 7.2.

Table 7.2 Fitted Square Root Model Parameters Based on Bacterial Growth Curves Simulated Using Eqn. 7.6, and Shown in Fig. 7.3.

Organism	Square root model values			
	T_{min}	T_{opt}	T_{max}	r^2
Antarctic strain	251	288		0.999
(psychrophile)	252	288	299	
	(251)	(288)	(293)	
Pseudomonas Gp1 16L16	266	288		0.997
(psychrotroph)	267	302	313	
	(266)	(302)	(310)	
E. coli	276	314		0.999
(mesophile)	277	314	323	
	(276)	(314)	(322)	
Bacillus spp. NCIB 12522	291	324		0.999
(thermophile)	291	324	333	
	(291)	(324)	(335)	

Upper row: values of parameters of simple square root models (Eqn. 1.1). Middle row: fitted 4 parameter square root model (Eqn. 1.2) values. Values in brackets are for 4 parameter square root models fitted to the original experimental data sets which were simulated. r^2 values shown are for Eqns. 1.1.

7.4.4 Growth Curves: Experimental Data

Experimental data and fitted Eqns. 7.6 for the growth of five organisms are shown in Figures 7.4a-e. Table 7.3 lists the fitted parameter values, their 95% confidence intervals, and RMS values for both Eqn. 7.6 and 4-parameter square root models (Eqn. 1.2) fitted to the data of figures 7.4. In general, fitted values of Eqn. 7.6 were very sensitive to initial parameter estimates. Convergence could be achieved from several sets of initial parameters, but did not always generate the same final values. Reduction in the number of parameters to be estimated improved both the ability to converge and confidence intervals of estimates.

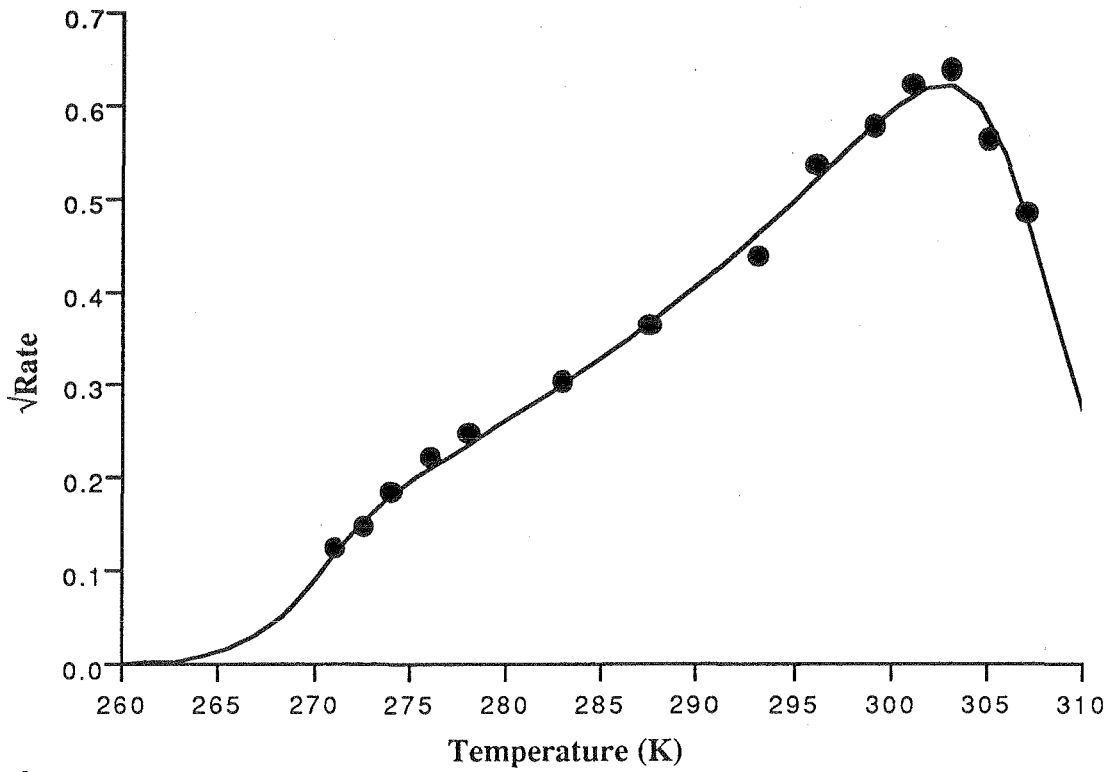
7.5 DISCUSSION

McMeekin *et al.* (1993) concluded that, if one considered the limitations of experimental data, data generated by an earlier form of Eqn. 7.6 could be described equally well by the square root model and from their analysis further concluded that there was no inherent contradiction between the two model types. That analysis was undertaken using simulated data sets. Given good experimental data sets, the ability of both the earlier master reaction-type models and the square root model to describe experimental data is firmly established in the literature. The results presented in Figs. 7.4a-e and summarised in Table. 7.3 reinforce those observations and conclusions. The experimental data presented in this chapter, and the literature concerning bacterial physiology and protein thermodynamics, enable a more objective assessment of the mechanistic bases of Eqn. 7.6 and master-reaction-type models in general.

7.5.1 AP Data

There have been several detailed studies of *E. coli* AP. Garen and Levinthal (1960) report that the activation energy for dephosphorylation of nitrophenol phosphate by *E. coli* alkaline phosphatase is 28.8 kJ mol^{-1}, and that AP is 'a reasonably stable enzyme'. Garen and Levinthal (1960) and Heppel *et al.* (1962) found that denaturation at temperatures up to 92.5 °C was reversible, albeit slowly. Garen and Levinthal (1960) also found that purified enzyme could not be stored at 85°C without loss of activity. At 60°C AP is stable (Garen and Levinthal, 1960). Heppel *et al.* (1962) noted, however, that there was very little recovery of denatured protein at 60°C despite that renaturation, which increases in rate with increasing temperature, does occur at lower temperatures. These observations suggest that at temperatures >60°C (333K) renaturation is not energetically favourable and, by inference, that temperatures > 60°C promote denaturation. Heppel *et al.* (1962) found that heating AP at 90°C for longer intervals leads to irreversible denaturation. After 60 minutes at 90°C, a maximum of ~30% of the activity could be recovered, whereas for shorter incubations more activity was subsequently recoverable.

a.



b.

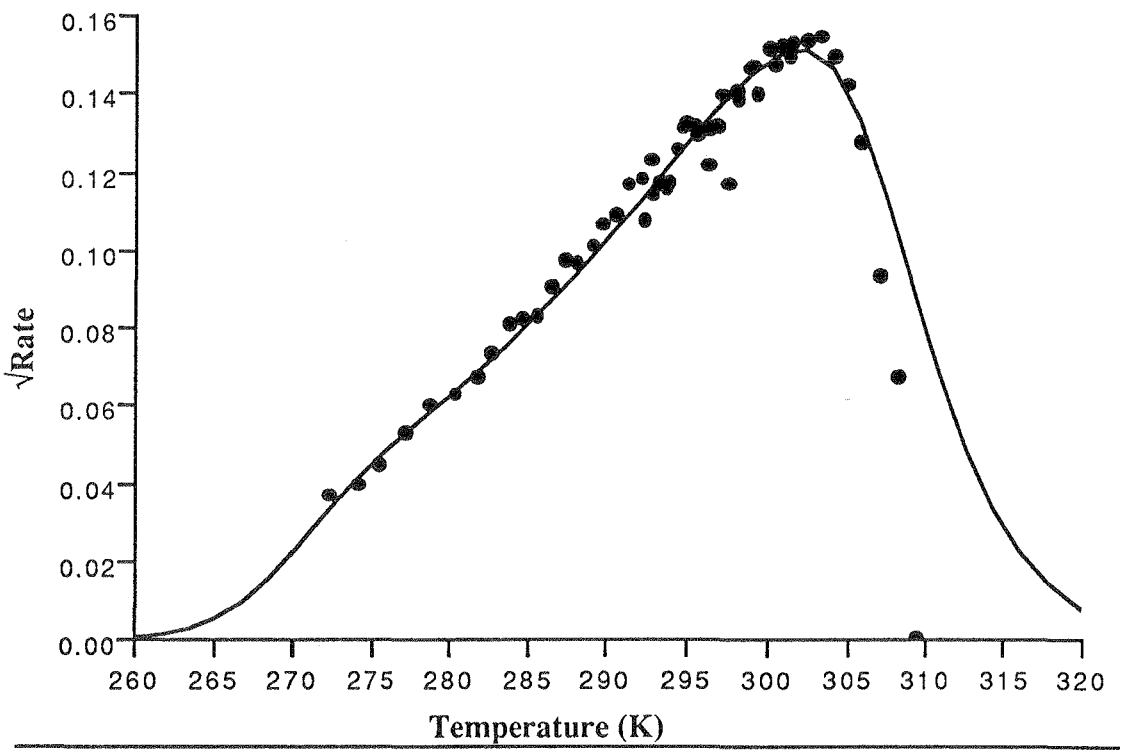
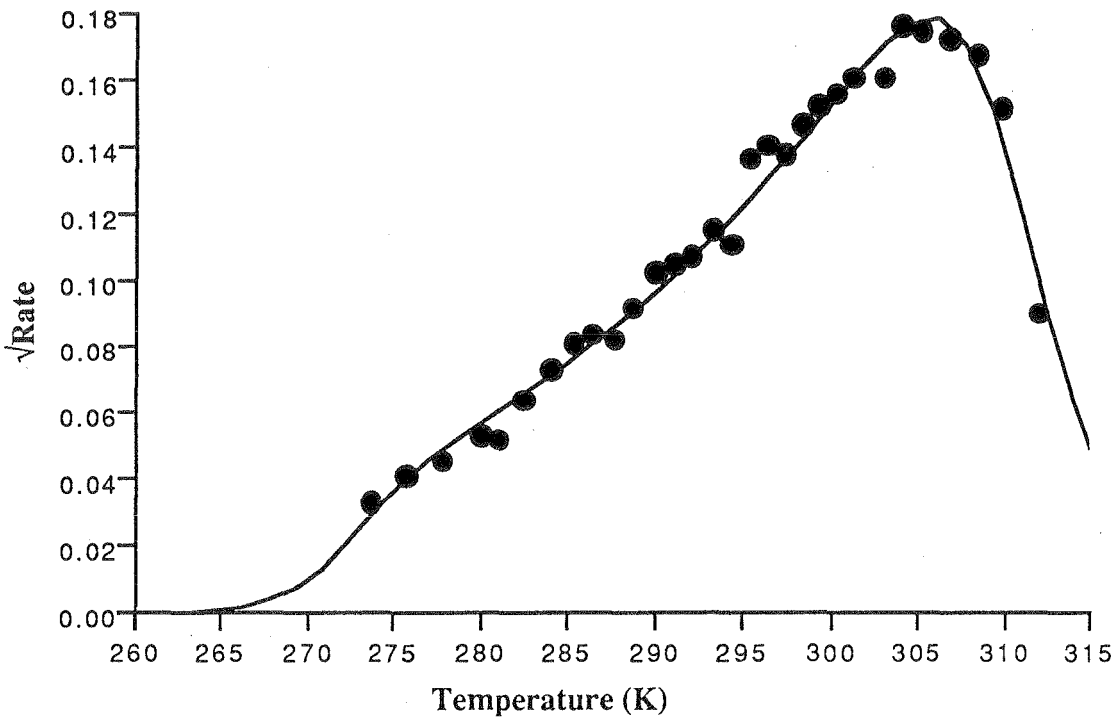


Fig. 7.4 Eqn. 7.6 fitted to the data of: a) Harder and Veldkamp (1971) for the growth of *Spirillum* L9, and b) Neumeyer (unpublished) for the growth of *Pseudomonas fluorescens* 1412.

c.



d.

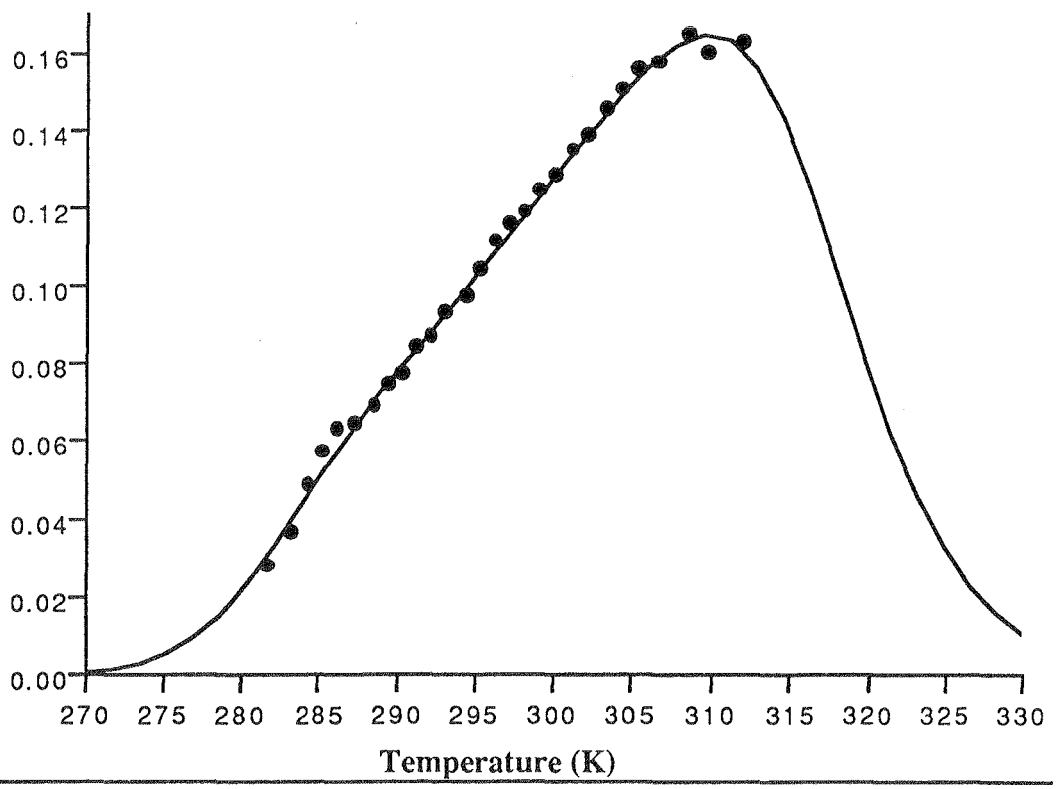


Fig. 7.4 Eqn. 7.6 fitted to growth data for: c) *Aeromonas hydrophila* 3459 and d) *Listeria monocytogenes* Scott A.

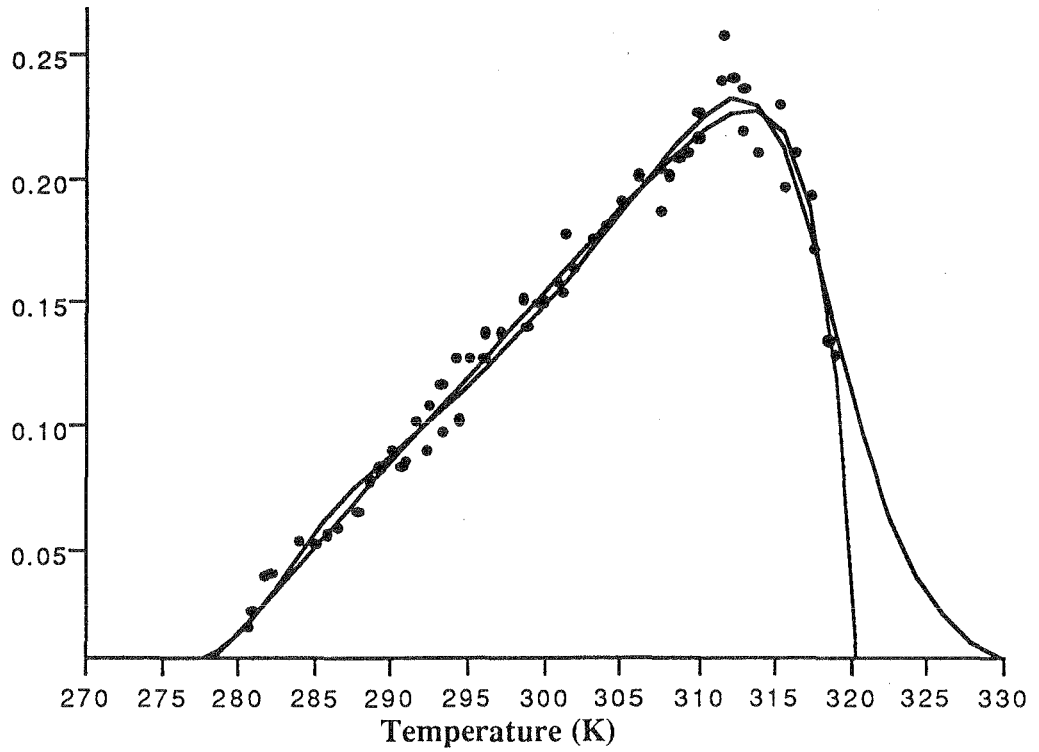


Fig. 7.4e Eqn. 7.6 fitted to data for the growth of *E. coli* M23. A 4-parameter square root model fitted to the same data is also shown for comparison.

The fitted values of Eqn. 7.6 to AP data suggest that AP is stable over a 130 K range (225 to 335 K), and that AP has an activation enthalpy of ~42 kJ mol^{-1}. With the exception of the fitted values for $\Delta H^{\ddagger}$, and n, estimates derived from Eqn. 7.6 are consistent with the above reports, lending credibility to the mechanistic bases of the model. The activation energy, analogous to $\Delta H^{\ddagger}$, estimated from the same data by Arrhenius plot was within the confidence limits of that estimated from Eqn. 7.6. When the parameter n was estimated from the data the result did not agree with literature values. The reason for the poor estimation of n may be that it is estimated from the steepness of the slope at denaturation, and in the AP data set used there are only two or three points in that range. When a literature based value for n was used, Eqn. 7.6 continued to fit the AP data well. Thus, the model theory is consistent with the data, but until it can be shown that the remaining parameter value which can be independently determined (i.e. ΔCp) is consistent with the fitted values, the evidence to support the model remains circumstantial. Furthermore, the current assessment is based on one data set only.

Table 7.3 Fitted Parameter and RMS Values for Eqns. 7.6 and 1.2 Fitted to Data Presented in Figs. 7.4a-e.

Organism (data source)	Fitted parameters and 95% confidence intervals (*italics*)										
	Equation 7.6						Equation 1.2				
	C (min^{-1})	$-\Delta H^{\ddagger}$ ($Jmol.^{-1}$)	ΔCp ($JK^{-1}mol.^{-1}$ $res.^{-1}$)	ΔH^{*} ($Jmol.^{-1}$ $res.^{-1}$)	n (res.)	RMS	RMS	b	c	T_{min}	T_{max}
Spirillum L9	1.21e+07	57320	62.56	5361	358	0.0019	0.0052	0.0160	0.5000	263.45	309.44
(Harder and Veldkamp, 1971)	*3.17e+07*	*6330*	*0.82*	*2*	*117*			*0.0017*	*0.3710*	*2.26*	*1.72*
P. putida 1412	5.66e+06	62047	62.24	5358	266	0.0028	0.0014	0.0040	0.3940	265.27	309.44
(Neumeyer, unpub.)	*1.37e+07*	*5788*	*1.32*	*3*	*85*			*0.0002*	*0.0470*	*1.40*	*0.13*
A. hydrophila 3459	2.56e+07	66054	64.53	5365	330	0.0009	0.0008	0.0049	0.4160	269.18	313.42
(Ross, unpub.)	*6.39e+07*	*6094*	*1.2*	*4*	*72*			*0.0003*	*0.103*	*1.31*	*0.43*
L. monocytogenes Scott A	2.68e+06	61097	70.78	5354	221	0.0001	0.0002	0.0053	0.2340	275.47	319.67
	5.03e+06	*4654*	*0.77*	*3*	*70*			*0.0003*	*0.1270*	*0.80*	*3.95*
E.coli M23	5.91e+07	68305	71.87	5360	308	0.0054	0.0048	0.0068	0.3630	277.41	320.47
(Ross, unpub.)	*1.48e+08*	-	*0.87*	*4*	*62*			*0.0004*	*0.0820*	*1.04*	*0.49*

7.5.2 Microbial Growth Rate Data

The apparent theoretical validity of Eqn. 7.6 for enzymatic catalysis does not appear to extend to the growth of whole cells. The results demonstrate that Eqn. 7.6 describes experimental temperature *vs.* growth rate data well with the exception of rate at superoptimal temperatures. Lowry and Ratkowsky (1983) noted that other master reaction-type models displayed the same behaviour and concluded that such models were deficient because they failed to consider irreversible protein denaturation. Furthermore, Eqn. 7.6 model cannot describe the temperature-growth rate relationship without violating one of its theoretical bases, i.e. the constancy of ΔH^* for globular proteins. It is possible that the putative rate-controlling enzyme has a unique value of ΔH^*. The constancy of the *fitted* values of ΔH^* for a range of species and thermal classes may lend some support to this view, and there is indirect evidence in the literature (Doig and Williams, 1991; Fu and Friere, 1992) that ΔH^* for globular proteins may not have a universal value of 5640 J (mol amino-acid-residue)$^{-1}$. The fitted values of the parameter $\Delta H^{\ddagger}$ are consistent with those obtained for the 'activation energy' of bacterial growth from Arrhenius plots, and within the range reported in the literature (60 - 70 kJ mol^{-1}).

7.5.3 Critique of Model Bases

A simpler interpretation is that the constancy of the fitted value reflects the similarity of the biokinetic temperature range of bacteria, and that the reason for the inability to fit the data using the theoretical ΔH^* value is that one of the starting assumptions of the master reaction type models is invalid. That Eqn. 7.6 describes the data well, however, suggests that whilst the form of the model is correct, the interpretations are not.

Examination of square root plots of experimental data often reveals evidence of upwards concavity in the suboptimal region. This is more pronounced in some data sets, e.g. Figs, 7.4 a-c. Though not apparent in the *E. coli* data presented in Fig. 7.4e (possibly due to the use of four separate data sets), the data of Gill and Harrison (1985) and Smith (1985) for *E. coli* exhibit similar curvature when presented as square root plots. These results are consistent with the original assumption that the temperature dependence of the rate of balanced cell growth is in accord with absolute reaction rate theory.

The assumption that a single enzyme limits the growth of the cell at both high and low temperatures is not supported in the literature. Neidhardt *et al.* (1990) considered the physiological factors that limit the upper temperature limit for growth, and concluded that it is the heat stability of one or more proteins which is the limiting factor. Gould (1989) considered the question in more detail and concluded that DNA damage is often the key lethal event, but that injury to other cell

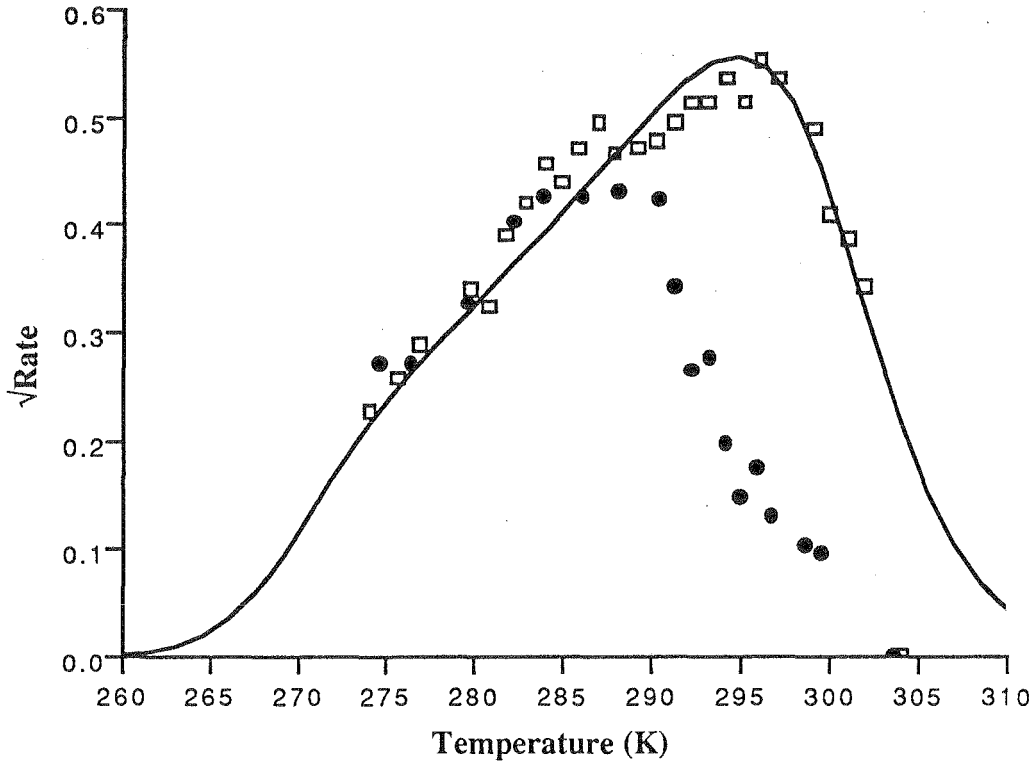


Fig. 7.5 Square root plot of an Antarctic *Carnobacterium* spp. for aerobic (●) and anaerobic (□) growth. (Unpublished data of P. Franzmann). The solid line is for Eqn. 7.6 fitted to the anaerobic data.

components may *accumulate* and eventually inhibit the initiation of growth in a manner that is very dependent upon 'environmental stress' factors. The lower temperature limit for growth is also considered by Neidhardt *et al.* (1990) who state that there is not a single cause determining the minimum temperature for growth. Nor is there evidence that it is the denaturation of a protein which sets the lower limit for growth. Studies with *E. coli* cold sensitive mutants have shown that they are unable to synthesise functional ribosomes, resulting from a mutation in the genes encoding for ribosomal proteins. Similarly, the lower limit for growth of wild-type *E. coli* was found to correspond to the temperature (7.8°C) at which ribosomes fail to assemble correctly. (Friedman *et al.*, 1969). Data for the aerobic and anaerobic growth of a *Carnobacterium* species isolated from Antarctica is shown in Fig. 7.5. These data suggest that the temperature limiting system/molecule need not necessarily be the same under all conditions or at either temperature limit. In that example, although the rate of growth in either environment is similar, the

temperature range for growth is greatly reduced in aerobic environments. It may be inferred that the thermal sensitivity of different enzymes are responsible for setting the limits of growth in either environment. In this example, the thermal sensitivity of a protein only required in aerobic environments (e.g. a peroxidase) is a possible explanation.

7.5.4 Conclusions

A fundamental purpose of a model is to assist in interpretation of observations, and from that paradigm to predict the consequences of other conditions. If the model is unsatisfactory it should be abandoned or revised. The above discussion offers a more general interpretation of the basis of the ability of Eqn. 7.6 to describe the temperature dependence of growth rate.

Although based in protein thermodynamics, the denominator of Eqn. 7.6 is a mathematical function which models an 'on/off' state as a function of temperature. Thus it describes inactivation of the cell's ability to reproduce at an upper and lower temperature, irrespective of the interpretation applied. Despite the inadequacies of Eqn. 7.6 and other master-reaction-type models, from their ability to describe the bacterial growth rate response to temperature it may be inferred that the response is a synthesis of a response which obeys Arrhenius kinetics, and some other rate modifying effect(s) which set the upper and lower temperature limits for growth. This less specific paradigm permits further interpretation of the basis of the square root models.

The consequence of fitting a straight line by least squares to square-root-of-rate data consistent with Eqn. 7.1b would explain the observation of Cole, described in 7.1, i.e. that the square root model often under predicts rate at the lowest temperatures. If the primary rate-controlling response to temperature accords with absolute reaction rate theory, then Cole's observation is expected. Another consequence of the suboptimal response obeying Arrhenius kinetics would be a systematic deviation of the estimate of T_{min} if data were removed (or not collected) at the low or high temperature 'ends' of the sub-optimal range. This was illustrated in Chapter 4.

Eqn. 7.6 also predicts that growth rate inhibiting constraints which do not alter the activation enthalpy, or the stability of the temperature-limiting systems of the cell, will generate the same T_{min} value (subject to the above considerations re: range of data). Thus, the status of T_{min} as a characteristic temperature, but with no metabolic significance, is supported. From the mechanistic viewpoint developed in this chapter T_{min} is shown to be simply the intercept of the extrapolation of the suboptimal region of a square root plot and the temperature axis.

The consequences of temperature limits for growth, however, and the manner in which their physiological basis interacts with the absolute reaction rate

predictions must also be considered. It may be appreciated by reference to Fig. 7.1, and other figures in this chapter, that there is possible, and observed, a range of responses. The interaction of the denominator and numerator of Eqn. 7.6 may lead to a square-root-of-rate response in the suboptimal region which is very well described by a straight line. Similarly, if the denominator models 'stability' over a very small range, upwards curvature of the response in the suboptimal region would be predicted.

On a purely mathematical level, Eqn. 7.6 can be used as a model of three (if n and ΔH^* are held constant) or four parameters which describes the entire biokinetic range. McMeekin *et al.* (1993) suggested that the more contemporary theory upon which Eqn. 7.6 is based may provide an explanation, for example, for previous observations (Lowry and Ratkowsky, 1983; Ratkowsky, 1990) that the six parameter Schoolfield *et al.* (1981) model is over-parameterised. That model uses four estimated parameters to describe high and low temperature inactivation. The paradigm proposed here, by treating the denominator as a temperature dependent 'on/off' function, supports this conclusion because that behaviour can be described by a function which requires the estimation of only one or two parameters (i.e. n, ΔH^*). In Eqn. 7.6, however, the rate of inactivation at both temperature extremes is strongly affected by the same parameter (n), thus Eqn. 7.6 may require further parameters to accurately model both high and low temperature 'inactivation' independently.

McMeekin *et al.* (1993) questioned the need for another 'mechanistic' model and reiterated the criticism of Heitzer *et al.* (1991) that the master reaction type models cannot be considered to be truly mechanistic because their parameters can not be experimentally determined. These criticisms apply to Eqn. 7.6 which, both philosophically (i.e. on the criteria of mechanistic description) and pragmatically (i.e. on the basis of goodness-of-fit and ease of use) can not currently be shown to be superior to the square root models. Nonetheless, Eqn. 7.6 has utility in that it has led to an interpretation of the effect of temperature on the rate of bacterial growth from which the success of the square root model may be understood, and which is complementary to the observations of McMeekin *et al.* (1993). More importantly, it has focussed attention on significant features of the response which the square root-type models may not well describe, and which require further study. The development and analysis of Eqn. 7.6 have also led to a number of experimentally testable predictions concerning the estimation of T_{min}.

Equation 7.6 appears to describe mechanistically the activity of enzymes, however, and may have utility in food processing in which control of the inactivation of enzymes is required (Adams, 1991). The parameterisation of the model is unsatisfactory, however, and must be investigated and improved before the model could be used routinely.

8 SUMMARY AND CONCLUSIONS

A number of limitations in the development of predictive models have been addressed in this thesis, and potential solutions demonstrated. The use of indirect enumeration methods offers a labour-efficient methodology for the generation of sufficient kinetic data to generate reliable models for the growth of microorganisms in foods. Growth rate estimates based on turbidimetric data have also been shown to be as reproducible as those derived from viable count methods, but to differ by a constant ratio. Thus, calibration factors to relate the rate of microbial growth determined by turbidimetric methods to those determined by viable count methods have been established. The turbidimetric method developed, based on the time for a doubling of optical density, is also general for a range of species and conditions and does not require calibration to numbers of microorganisms. Potential limitations relate to the range of sensitivity of optical density measuring devices.

Through the development of indices of bias and precision, the robustness of the method has been demonstrated by the development and evaluation of predictive models for the growth of *Staphylococcus aureus* and *Listeria monocytogenes*. Models, based on turbidimetric data from laboratory broth cultures and calibrated to viable count methods, are as accurate and unbiased as any predictive model for the growth rate of microorganisms published to date. The results of this work have provided a set of criteria by which such predictive models can be evaluated, and validated.

Primary models to describe the bacterial growth curve were also investigated. It was concluded that the modified-Gompertz function, though not an ideal description of the curve, could yield realistic kinetic parameters, through the application of a calibration factor.

The hypothesis of Chandler and McMeekin (1987b), concerning the development of kinetic models by stepwise determination of the parameters of square root type models, was also strongly supported for temperature and water activity effects on growth rate. Due to difficulties in modelling the pH response the extension of that hypothesis by McMeekin *et al.* (1992) could not be convincingly demonstrated. The independence of the effects of temperature, pH and water activity on the growth rate of microorganisms was confirmed in this study, and suggests that the hypothesis of McMeekin *et al.* (1992) will be demonstrated.

The experimental approach taken in this study has demonstrated the need for replication of data. Although the minimalistic response surface design proposed by McMeekin *et al.* (1992) was demonstrated to produce reliable models, it is vulnerable to aberrant results.

This study has also shown that the variability in microbial growth rates determined in laboratory media may be narrower than that which can be expected in foods. This issue must be addressed before realistic risk assessment (Buchanan and

Deroever, 1993), based on the accumulated knowledge summarised in predictive models can be undertaken. Integration of the probability and kinetic modelling approaches, and models, should be pursued in this regard.

Chandler (1988), in reviewing a similar research program, pointed out that the square root model remained empirical, and that detailed study in the areas of microbial physiology and genetics would be required to understand the basis of the 'square root' response. In the current study a partial explanation of that response, based on the thermodynamics of growth, and the existence of definite temperature limits for growth, has been presented. That paradigm has led to testable predictions, and provided an understanding of the square root response, so that it can be used with greater confidence.

Prototype applications software for transferring predictive microbiology technology to the food industry were described. The methodology now "validated" offers a less expensive means of developing and applying predictive models for the effects of temperature, pH and water activity, and without recourse to nonlinear regression expertise. Predictive microbiology is not a panacea to the problems of microbial food spoilage and 'food poisoning' but does offer a powerful and rational new weapon with which to understand and tackle those problems.

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APPENDIX 1 DATA SETS USED IN 3.1 AND SUMMARY

Appendices 1.1 - 1.20 (overleaf). Data used in the Comparison of Equations 3.1 and 3.5 (*see* 3.1).

Appendices 1.1 - 1.20. DATA SETS USED IN 3.1

	time	log(N)		time	log(N)		time	log(N)
1	0	1	**2**	0	1	**3**	0	1
	1	1		3	1		10	1
	3.6	2.6		7.6	2.6		21.6	2.6
	5.2	4.2		9.2	4.2		23.2	4.2
	6.8	5.8		10.8	5.8		24.8	5.8
	8.4	7.4		12.4	7.4		26.4	7.4
	11	9		17	9		38	9
	12	9		20	9		48	9
4	0	1	**5**	0	1	**6**	0	1
	0.3	1		1	1		1	1
	2.2	2.6		2.02	1.02		3.4	2.4
	3.8	4.2		2.04	1.04		4.8	3.8
	5.4	5.8		2.06	1.06		6.2	5.2
	7	7.4		2.08	1.08		7.6	6.6
	8.9	9		3.1	1.1		10	8
	9.2	9		4.1	1.1		11	8
7	0	1	**8**	0	1	**9**	0	1
	1	1		1	1		1	1
	3.2	2.2		3	2		2.8	0.8
	4.4	3.4		4	3		3.6	2.6
	5.6	4.6		5	4		4.4	3.4
	6.8	5.8		6	5		5.2	4.2
	9	7		8	6		7	5
	10	7		9	6		8	5
10	0	1	**11**	0	1	**12**	0	1
	1	1		1	1		1	1
	2.6	1.6		2.4	1.4		2.2	1.2
	3.2	2.2		2.8	1.8		2.4	1.4
	3.8	2.8		3.2	2.2		2.6	1.6
	4.4	3.4		3.6	2.6		2.8	1.8
	6	4		5	3		4	2
	7	4		6	3		5	2
13	0	8	**14**	0	1	**15**	0	1
	1	8		10	1		0.1	1
	2.2	8.2		22	3		0.22	1.02
	2.4	8.4		24	5		0.24	1.04
	2.6	8.6		26	7		0.26	1.06
	2.8	8.8		28	9		0.28	1.08
	4	9		40	11		0.4	1.1
	5	9		50	11		0.5	1.1
16	0	1	**17**	0	1	**18**	0	1
	100	1		0.625	1		0.375	1
	201.6	2.6		2.25	2		1.35	2.6
	203.2	4.2		3.25	3		1.95	2.2
	204.8	5.8		4.25	4		2.55	2.8
	206.4	7.4		5.25	5		3.15	3.4
	308	9		6.875	6		4.125	4
	408	9		7.5	6		4.5	4
19	0	1	**20**	0	1			
	0.125	1		0.0125	1			
	0.45	1.2		0.045	1.02			
	0.65	1.4		0.065	1.06			
	0.85	1.6		0.085	1.08			
	1.05	1.8		0.105	1.08			
	1.375	2		0.1375	1.1			
	1.5	2		0.15	1.1			

Appendix 1.21. Paramter Estimates and RSS Values for Data Sets 1 - 20 Fitted to the Modified-Gompertz function (Eqn. 3.1) and the Modified-Logisitc Function (Eqn. 3.5)

	Modified Logistic Values					Modified Gompertz Values					
Data Set	A	GT	D	M	RMS ($x10^3$)	A	B	D	M	t_G	RMS ($x10^3$)
1	0.978	0.299	8.044		1.394	0.923	0.328	9.207	5.298	0.335	
2	1.000	0.298	8.002	13.980	0.021	1.014	0.377	8.260	9.030	0.345	95.5
3	1.000	0.298	8.000	27.983	0.021	1.014	0.390	8.021	22.939	0.347	79.5
4	0.832	0.298	8.336	8.727	10.300	0.034	0.230	12.440	4.210	0.318	39.4
5	1.000	0.015	0.100	2.053	0.002	1.000	31.279	0.100	2.037	0.347	0.012
6	0.979	0.298	7.042	8.991	1.300	0.957	0.384	7.902	4.851	0.337	73.1
7	0.978	0.296	6.324	7.975	1.300	0.980	0.459	6.640	4.410	0.338	55.6
8	0.982	0.293	5.036	6.949	1.200	0.995	0.565	5.421	3.977	0.339	39.8
9	0.984	0.285	4.032	5.907	1.100	1.003	0.726	4.246	3.554	0.342	25.7
10	0.989	0.267	3.022	4.842	0.900	1.005	0.996	3.118	3.144	0.344	13.9
11	0.995	0.227	2.010	3.758	0.600	1.004	1.529	2.039	2.748	0.346	5.4
12	0.999	0.140	1.002	2.733	0.200	1.002	3.113	1.004	2.368	0.346	1.2
13	8.000	0.140	1.001	2.733	0.200	8.002	3.113	1.004	2.368	0.319	1.2
14	1.000	0.301	10.000	299.935	0.004	1.017	0.311	10.044	23.681	0.347	123.6
15	1.000	0.015	0.100	0.253	0.003	1.000	31.128	0.100	0.237	0.346	0.012
16	1.000	0.300	8.000	207.986	0.021	1.015	0.391	8.010	202.935	0.347	80.2
17	0.951	0.292	5.098	6.222	4.400	0.952	0.525	5.754	3.311	0.335	35.9
18	0.924	0.268	3.152	3.655	5.100	0.971	0.875	3.453	1.987	0.335	12.9
19	0.939	0.154	1.122	1.037	1.200	0.990	2.625	1.151	0.662	0.335	1.4
20	0.993	0.018	0.114	0.078	0.013	0.999	26.247	0.115	0.066	0.335	0.014

APPENDIX 2 COMMON METHODS AND MATERIALS

A2.1 MATERIALS

A2.1.1 Reagents

NaCl

Lactic Acid (Min. 88% w/w)

Univar, AR. Ajax Chemicals, Auburn, NSW, Australia.

Sucrose

Analar AR. BDH Chemicals Australia. Kilsyth, Vic., Australia.

Water

All water used in the preparation of reagents and media was prepared by glass distillation of deionised water.

A2.1.2 Organisms

Origin

The origin of Staphylococcal strains is shown in Table A2.1. Coagulase positive staphylococci were characterised by gram stain, colony morphology on BPA, and coagulase activity determined by the tube and slide method.

Listeria monocytogenes strains Scott A, and Murray B, were obtained from Dr. F. Grau, CSIRO Division of Food Processing, Brisbane, Queensland.

Table A2.1 Sources of Coagulse Positive *Staphylococcus aureus* Described in this Thesis

Strain	Source	Isolated from:
3b	Dr. J. Statham, University of Tasmania	Imported prawns
1a	〃 〃	〃
1b	〃 〃	〃
3a	〃 〃	〃
BOV 2'b		Condensed Milk
BOV 4'		〃
NCTC 6571	Mr. A. Ball, Public Health Laboratory, Hobart, Australia	
ATCC 25923	〃 〃	
Cad 010654	Mr. A. Smith, Cadbury-Schweppes, Claremont, Tas, Australia.	Milk

Maintenance

Coagulase positive staphylococci were maintained aerobically on BHIA slopes at 2°C, and periodically subcultured. Purity and identity of the culture was checked at subculture by gram reaction and colony morphology on BPA. *Listeria monocytogenes* was maintained aerobically on TSA slopes at 2°C, and periodically subcultured. Purity and identity of the culture was checked at subculture by gram reaction, 'tumbling' motility and colony morphology on OLSA..

A2.1.3 Culture Media and Diluents

Brain Heart Infusion Broth (BHIB) was prepared and sterilised according to the manufacturer's instructions from commercially prepared dehydrated media (Oxoid CM 225, Difco 0037-01-6). Brain heart infusion agar (BHIA) was prepared from BHIB by the addition of 1.5% bacteriological grade agar (Oxoid, L11) prior to sterilisation, and then sterilised by autoclaving (121°C x 15 min).

Baird-Parker Agar (Oxoid, CM 275) was prepared and sterilised according to the manufacturer's instructions, including the addition of Egg Yolk Tellurite Emulsion (Oxoid, SR 54).

Oxford Listeria Selective Agar, was prepared from Listeria Selective Agar Base (Oxoid, CM 856), sterilised according to the manufacturer's instructions, and supplemented with Selective Supplement (Oxoid, SR 140E) containing cycloheximide, colistin sulphate, acriflavine, cefotetan and fosfomycin.

Tryptone Soya Broth (TSB), (Oxoid, CM 129) was prepared and sterilised according to the manufacturer's instructions. Tryptone Soya Agar (TSA) was prepared from TSB by the addition of 1.5% bacteriological grade agar (Oxoid, L11) prior to sterilisation. It was then sterilised by autoclaving (121°C x 15 min).

Standard Plate Count Agar (PCA) (Oxoid, CM 463) was prepared and sterilised according to the manufacturer's instructions.

Diluent containing 0.1% peptone (Difco, 0118-01-8) and 0.85% NaCl was used for serial dilution and for suspension of food samples for homogenisation. It was sterilised by autoclaving (121°C x 15 min).

Broth media and diluents were stored in airtight containers in a dark place at room temperature. Agar plates were stored in airtight containers at 2°C. Media were stored for up to 2 weeks, before being discarded.

A2.1.4 Equipment

Balances

Mettler PJ 3600 Delta Range®. ± 0.01 g precision. Mettler Instrumente AG, Zurich, Switzerland.

pH metering

General.

Corning pH meter 120 (Corning Medical and Scientific, Scientific Instruments, Halstead, Essex, England) with Orion 91-06 Ag/AgCl probe.

pH measurement of cultures.

Orion Model 250A (portable) with calomel sealed flat tip probe (AEP433). Orion Research Inc., Boston, Mass., USA.

Pipettors

A range of fixed and variable volume pipettors were used throughout this study.

'Fixopet':100µl (fixed); 1 ml (fixed); 'Pluripet':200 - 1000 µl. Kartell Spa Via, Delle Industrie, 1 20082 Noviglio, Milan, Italy.

'Pipetman': 200 - -1000 µl. Gilson Medical Electronics (France) S.A., B.P. 45 - 95400 Villiers-le-Bel, France.

'Oxford Macro-set': 5- 10 ml. 'Oxford Adjustable': 40 - 200 µl. Oxford Laboratories, Inc., Calif., USA.

Dispensed volume of fixed volume pipettors was checked periodically by weighing of water at room temperature, and were typically found to be within ±1% of nominal volume. Variable volume pipettors were calibrated, by weighing of water, before use.

Spectrophotometry

Spectronic 20 (analogue display) or 20D (digital display) spectrophotometers (Milton Roy Co., USA) were used exclusively.

Temperature Gradient Incubator

Model TN 3. Advantec, Toyo Roshi International, California, USA.

Thermometry

A Fluke® 51K/J (John Fluke Manufacturing Co., Illinois, USA) electronic thermometer with Iron-Constantan thermocouple bead probe was used routinely to determine temperatures, and to calibrate other temperature metering and control

devices as necessary. Quoted accuracy: ± 0.5°C. The calibration of the instrument was periodically checked at 0°C and 100°C.

Timers

A range of electronic laboratory timers were used for all growth rate experiments. At the commencement of inoculation, the timer was set to zero, and the real time recorded in case of timer failure.

Water Activity Measurement

Novasina, 'Humidat-IC 1'. Novasina AG, Pfäffikon, Switzerland. Quoted acuracy: ± 2 % R.H., reproducibility ±0.2% R.H. The instrument was calibrated on each occasion before use by reference to standard salt (Barium Chloride, Sodium Choride, Magnesium Nitrate) solutions.

Water baths

Static.

A range of Lauda waterbaths were used:

RC 20, RM 20, M 20, RM 6. (R denotes refrigerated, the number indicates bath capacity (litres)). Temperature control: ± 0.1°C.

Lauda DR. R. Wobser GMBH & Co. K.G.

Lauda-Könighofen, West Germany.

Shaking.

Haake SWB 20 (20l). Temperature control (measured) ± 0.5°C.

Haake, Karlsruhe, West Germany.

A2.2 Methods

Counting methods

Three dilutions of samples were routinely plated. Numbers of organisms at each sampling time were predicted on the basis of models developed in broth systems. From this prediction, the sample dilition expected to yield 30 - 300 colonies on a spread plate, and the tenfold higher and tenfold lower dilutions were plated. Duplicate spread plates of each dilution were usually prepared. Sometimes, e.g. where earlier sampling times had shown good agreement between predicted and observed numbers, ony two dilutions were plated in duplicate and the next higher and next lower dilutions were plated also, without replication.

The colonies on all plates were counted and recorded, except in the case of very high numbers, for which an estimate based on the number of colonies within a subsection of the plate was used. All plates having between 30 and 300 colonies were included in the calculation of the number of organisms present in the sample,

using the method of Farmiloe *et al.* (1954). That method gives only 1/10 as much weight to the higher tenfold dilution (which has lower precision) whilst acknowledging that the overall precision of the mean count is increased by counting all countable colonies. Since a larger number of colonies is used to derive the weighted mean, the limiting precision of the mean is better than for either the mean count of colonies on the first countable plates or the *arithmetic* mean of colonies from two countable dilutions.

APPENDIX 3 DATA SETS USED FOR MODEL GENERATION.

The data sets upon which Eqns. 4.1 to 4.3 are based are presented in the following tables. The variables space covered by the data sets is shown diagrammatically in Figs. A2 and A3.

Appendix 3.1 *S. aureus* 3b Data Set

Temperature (°C)	Water Activity	Gen. Time (min) (Eqn. 3.15)
13.6	0.997	497.59
14.6	0.997	487.74
15.7	0.997	340.86
16.7	0.997	234.31
17.5	0.997	196.98
18.6	0.997	176.92
19.6	0.997	150.28
20.6	0.997	121.28
21.9	0.997	105.10
23.1	0.997	95.20
24.0	0.997	78.66
25.0	0.997	68.77
25.9	0.997	64.06
29.7	0.997	42.26
30.6	0.997	38.84
31.6	0.997	35.00
32.6	0.997	33.75
33.8	0.997	29.04
34.8	0.997	28.47
8.9	0.986	6639.14
9.9	0.986	4694.65
10.7	0.986	3073.57
11.7	0.986	2185.68
12.7	0.986	1430.58
14.1	0.986	875.82
15.1	0.986	594.26
16.0	0.986	477.45
17.0	0.990	311.92
17.9	0.986	255.44
18.9	0.986	202.62
19.7	0.986	155.38
20.6	0.986	130.90
22.3	0.986	117.57
25.8	0.986	65.35
27.4	0.986	52.47
29.1	0.986	44.16
30.7	0.986	37.69
32.7	0.986	34.51

Temperature (°C)	Water Activity	Gen. Time (min) (Eqn. 3.15)
10.6	0.963	2700.56
11.5	0.963	1487.13
13.4	0.963	855.68
14.3	0.963	687.75
15.3	0.963	561.12
16.4	0.963	425.40
17.4	0.963	296.28
18.1	0.963	259.89
20.0	0.963	216.78
21.6	0.963	157.93
23.2	0.963	115.77
24.6	0.963	94.92
26.0	0.963	77.50
27.8	0.963	68.90
29.1	0.963	56.84
30.7	0.963	47.33
32.4	0.963	43.71
9.5	0.963	6576.02
9.6	0.949	3152.45
11.2	0.949	2082.22
12.4	0.949	1375.12
13.5	0.949	814.54
14.1	0.949	675.31
15.2	0.949	550.49
16.2	0.949	440.43
17.3	0.949	375.68
18.2	0.949	266.48
18.9	0.949	245.54
19.8	0.949	224.33
20.7	0.949	196.12
21.5	0.949	171.38
22.2	0.949	150.20
23.1	0.949	147.35
24.5	0.949	112.14
27.6	0.949	90.34
29.0	0.949	66.28
30.8	0.949	57.76
32.5	0.949	48.68
34.5	0.949	39.48

Appendix 3.1 (cont.) *S. aureus* 3b Data Set

Temperature (°C)	Water Activity	Gen. Time (min) (Eqn. 3.15)
9.8	0.974	4007.16
11.0	0.974	2917.90
12.0	0.974	1731.57
12.6	0.974	1195.45
13.6	0.974	859.66
15.2	0.974	556.45
16.2	0.974	501.43
17.1	0.974	338.30
18.1	0.974	250.57
18.8	0.974	185.91
19.8	0.974	153.03
20.6	0.974	131.54
21.5	0.974	120.41
22.3	0.974	106.51
23.8	0.974	79.59
25.5	0.974	66.53
27.2	0.974	52.17
28.9	0.974	47.08
30.6	0.974	41.99
32.5	0.974	33.32
34.8	0.974	32.70
10.1	0.969	2317.11
11.0	0.969	1348.50
12.0	0.969	893.25
13.0	0.969	663.48
14.0	0.969	535.55
15.0	0.969	416.48
16.0	0.969	360.32
19.0	0.969	193.29
17.2	0.969	288.98
18.2	0.969	245.78
19.8	0.969	164.26
20.5	0.969	123.37
22.1	0.969	149.31
23.9	0.969	105.14
25.2	0.969	87.39
25.8	0.969	70.56
27.8	0.969	49.97
29.9	0.969	44.02
32.3	0.969	35.18
34.8	0.969	31.87

Temperature (°C)	Water Activity	Gen. Time (min) (Eqn. 3.15)
11.9	0.935	1783.35
13.2	0.935	1410.52
17.1	0.935	347.86
14.2	0.935	1080.05
15.1	0.935	810.15
16.1	0.935	649.16
18.9	0.935	347.86
20.6	0.935	222.51
24.0	0.935	135.78
25.5	0.935	154.33
27.1	0.935	131.93
28.8	0.935	103.80
30.5	0.935	72.05
32.3	0.935	59.01
34.5	0.935	52.18
12.6	0.914	4956.43
13.9	0.914	3215.07
14.7	0.914	2106.15
15.6	0.914	1366.95
16.6	0.914	903.37
17.3	0.914	683.87
18.5	0.914	551.49
19.3	0.914	411.47
20.2	0.914	373.59
21.1	0.914	338.10
23.0	0.914	182.97
26.8	0.914	120.93
28.8	0.914	124.24
31.0	0.914	89.87
33.3	0.914	70.77
36.2	0.914	61.68

Appendix 3.1 (cont.) *S. aureus* 3b Data Set

Temperature (°C)	Water Activity	Gen. Time (min) (Eqn. 3.15)
14.6	0.969	392.76
15.7	0.969	331.68
16.6	0.969	273.54
18.9	0.969	186.06
20.0	0.969	124.20
20.7	0.969	143.87
21.6	0.969	120.02
22.7	0.969	97.23
23.7	0.969	90.77
24.7	0.969	79.62
25.8	0.969	69.33
27.7	0.969	55.26
28.7	0.969	51.31
29.7	0.969	47.13
30.8	0.969	44.97
31.7	0.969	37.22
32.8	0.969	38.90
33.8	0.969	33.32
35.0	0.969	34.23
34.0	0.997	32.34
34.0	0.990	34.88
34.0	0.982	37.65
34.0	0.974	40.53
34.0	0.966	47.62
34.0	0.958	50.27
34.0	0.949	59.13
34.0	0.939	71.98
34.0	0.929	80.84
34.0	0.919	98.33
34.0	0.908	115.61
34.0	0.898	147.28
34.0	0.885	182.94
34.0	0.873	272.30
34.0	0.860	256.25

Temperature (°C)	Water Activity	Gen. Time (min) (Eqn. 3.15)
30.0	0.997	52.81
30.0	0.990	58.63
30.0	0.982	63.23
30.0	0.974	72.61
30.0	0.966	82.11
30.0	0.958	84.97
30.0	0.949	109.34
30.0	0.939	134.77
30.0	0.929	141.76
30.0	0.919	177.77
30.0	0.908	226.26
30.0	0.898	304.12
30.0	0.885	394.86
30.0	0.873	612.88
26.0	0.997	70.57
26.0	0.990	73.11
26.0	0.982	78.71
26.0	0.966	79.11
26.0	0.958	89.56
26.0	0.949	98.83
26.0	0.939	117.12
26.0	0.929	128.54
26.0	0.919	147.93
26.0	0.908	186.61
26.0	0.898	235.56
26.0	0.885	305.63
26.0	0.873	477.82
26.0	0.860	870.83

Appendix 3.2 *Listeria monocytogenes* Scott A Data set

Temperature (°C)	Water Activity	pH	Gen. Time (h) (Eqn. 3.15)
6.80	0.997	7.20	19.35
8.80	0.997	7.20	11.48
10.60	0.997	7.20	6.34
12.20	0.997	7.20	4.62
13.40	0.997	7.20	3.80
14.30	0.997	7.20	3.76
15.60	0.997	7.20	3.13
16.60	0.997	7.20	2.71
17.40	0.997	7.20	2.51
18.30	0.997	7.20	2.13
19.20	0.997	7.20	2.00
20.20	0.997	7.20	1.73
21.50	0.997	7.20	1.59
22.40	0.997	7.20	1.39
23.30	0.997	7.20	1.21
24.20	0.997	7.20	1.12
25.20	0.997	7.20	1.07
26.20	0.997	7.20	0.97
27.20	0.997	7.20	0.92
28.30	0.997	7.20	0.86
29.20	0.997	7.20	0.78
30.40	0.997	7.20	0.71
31.40	0.997	7.20	0.67
32.50	0.997	7.20	0.62
33.70	0.997	7.20	0.61
35.60	0.997	7.20	0.56
36.80	0.997	7.20	0.59
38.90	0.997	7.20	0.57
19.50	0.997	7.20	1.76
19.00	0.997	7.20	1.85
19.50	0.997	7.20	2.38
19.50	0.997	7.20	2.76
19.50	0.997	7.20	1.70
19.50	0.997	7.20	1.60
19.50	0.997	7.20	1.65
19.50	0.997	7.20	2.07
19.50	0.997	7.20	2.21
19.50	0.997	7.20	1.96
19.50	0.997	7.20	2.21
19.50	0.997	7.20	1.65
19.50	0.997	7.20	1.73
19.50	0.997	7.20	1.51
19.50	0.997	7.20	1.60

Appendix 3.2 (cont.) *Listeria monocytogenes* Scott A Data set

Temperature (°C)	Water Activity	pH	Gen. Time (h) (Eqn. 3.15)
28.00	0.997	7.20	0.88
28.00	0.997	7.20	1.00
27.00	0.997	7.20	0.95
27.00	0.997	7.20	1.15
13.00	0.997	7.20	4.17
13.00	0.997	7.20	4.53
9.00	0.997	7.20	9.54
9.00	0.997	7.20	8.00
6.50	0.997	7.20	10.07
			0.00
19.50	0.990	7.20	2.82
19.50	0.984	7.20	2.86
19.50	0.979	7.20	3.32
19.50	0.973	7.20	3.97
19.50	0.967	7.20	4.25
19.50	0.960	7.20	5.38
19.50	0.954	7.20	5.90
19.50	0.947	7.20	8.49
19.50	0.940	7.20	12.05
19.50	0.933	7.20	30.36
			0.00
19.50	0.990	7.60	1.96
19.50	0.990	7.45	1.87
19.50	0.990	7.25	1.86
19.50	0.990	6.90	2.00
19.50	0.990	6.55	1.85
19.50	0.990	6.20	2.07
19.50	0.990	6.00	2.41
19.50	0.990	5.85	3.41
19.50	0.990	5.80	5.80
19.50	0.990	5.65	10.60

Appendix 3.3 *Listeria monocytogenes* Murray B Data Set

Temperature (°C)	Water Activity	pH	Gen. Time (h) (Eqn. 3.15)
6.90	0.997	7.20	17.83
8.80	0.997	7.20	10.03
10.60	0.997	7.20	5.65
12.60	0.997	7.20	4.73
13.50	0.997	7.20	4.03
14.90	0.997	7.20	3.55
15.90	0.997	7.20	3.70
17.20	0.997	7.20	2.72
18.00	0.997	7.20	2.88
18.90	0.997	7.20	2.27
19.70	0.997	7.20	1.97
20.80	0.997	7.20	1.72
21.90	0.997	7.20	1.53
22.80	0.997	7.20	1.49
23.40	0.997	7.20	1.25
24.20	0.997	7.20	1.19
25.00	0.997	7.20	1.07
26.30	0.997	7.20	1.01
27.30	0.997	7.20	0.93
28.40	0.997	7.20	0.86
29.30	0.997	7.20	0.84
30.50	0.997	7.20	0.73
31.80	0.997	7.20	0.70
32.70	0.997	7.20	0.67
33.90	0.997	7.20	0.64
35.60	0.997	7.20	0.63
37.20	0.997	7.20	0.58
38.80	0.997	7.20	0.58
19.50	0.997	7.20	1.62
19.00	0.997	7.20	1.73
19.50	0.997	7.20	1.75
19.50	0.997	7.20	2.30
19.50	0.997	7.20	1.90
19.50	0.997	7.20	1.77

Appendix 3.3 (cont.) *Listeria monocytogenes* Murray B Data Set

Temperature (°C)	Water Activity	pH	Gen. Time (h) (Eqn. 3.15)
19.50	0.990	7.20	3.77
19.50	0.984	7.20	3.50
19.50	0.979	7.20	3.97
19.50	0.973	7.20	4.48
19.50	0.967	7.20	4.73
19.50	0.960	7.20	5.78
19.50	0.954	7.20	7.23
19.50	0.947	7.20	8.77
19.50	0.940	7.20	13.20
19.50	0.933	7.20	23.83
19.50	0.990	7.60	1.88
19.50	0.990	7.50	1.87
19.50	0.990	7.30	1.83
19.50	0.990	6.90	1.90
19.50	0.990	6.55	1.97
19.50	0.990	6.10	2.13
19.50	0.990	5.90	2.27
19.50	0.990	5.80	2.98
19.50	0.990	5.75	4.35
19.50	0.990	5.70	8.23

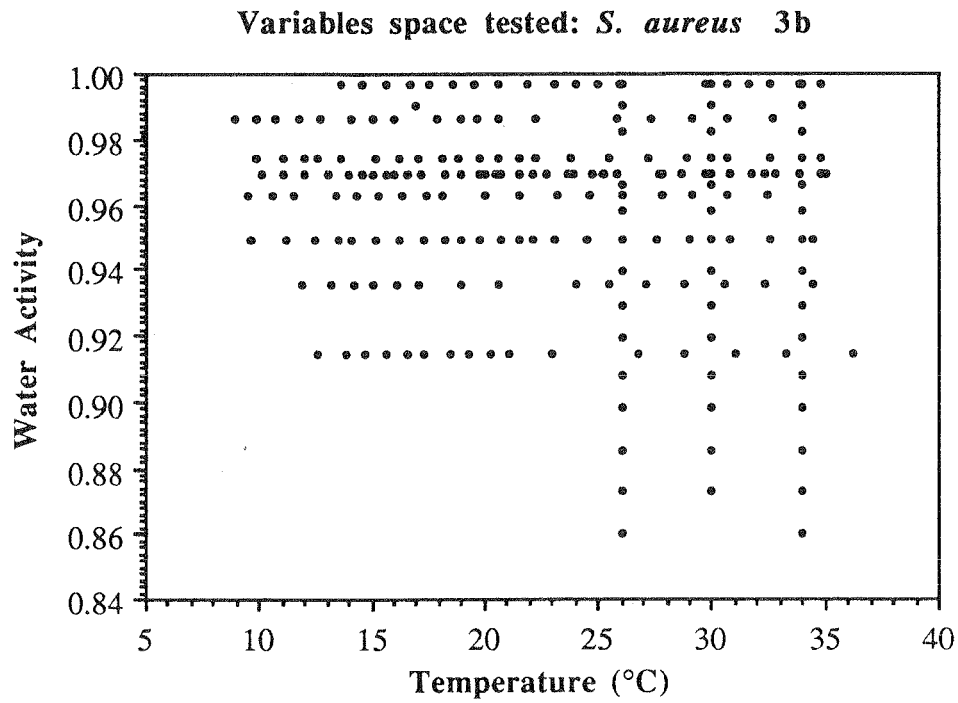

Fig. A1 Diagrammatic representation of variables combinations tested in the generation of kinetic models for *S. aureus* 3b Appendices 3.1

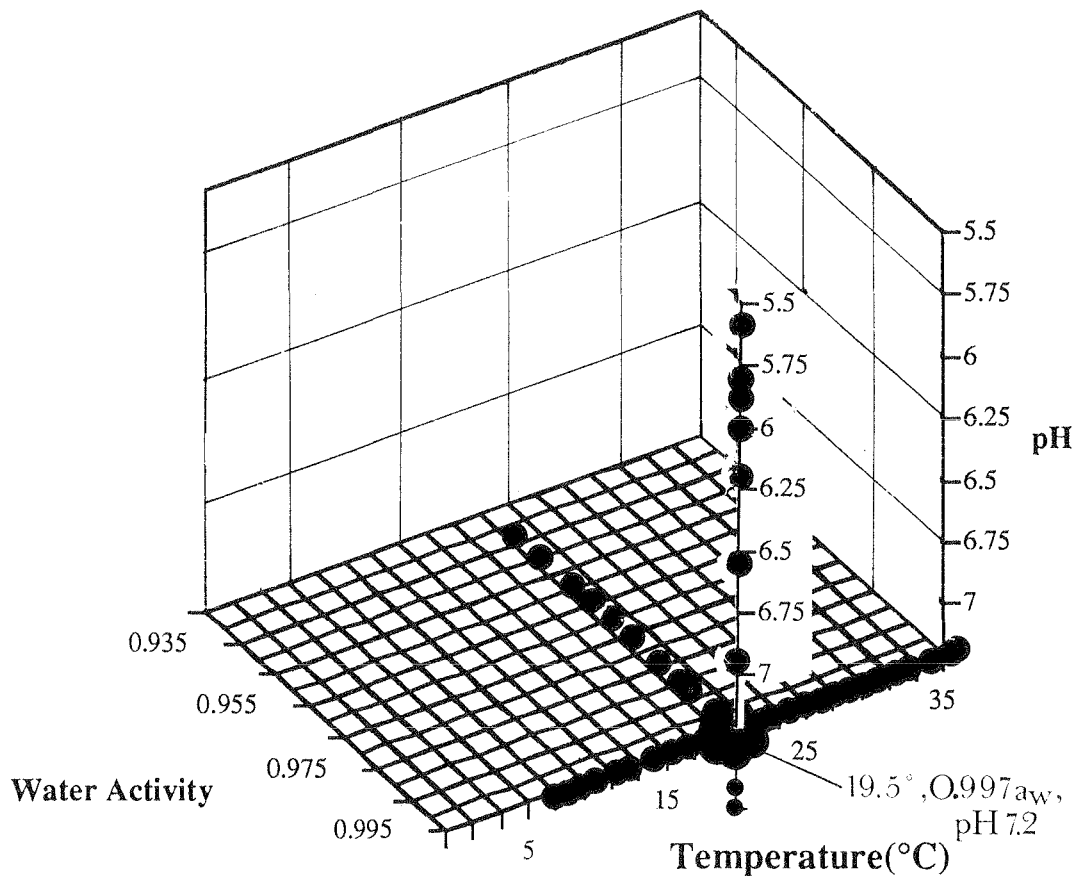

Fig. A2 Diagrammatic representation of variables combinations tested in the generation of kinetic models for *L. monocytogenes*, Appendices 3.2 - 3.3

APPENDIX 4 GIBBS FREE ENERGY OF DENATURATION OF PROTEINS AS A FUNCTION OF TEMPERATURE.

Figure A3 shows the effect of temperature on the Gibbs Free Energy of denaturation of an imaginary protein with the following characteristics:

ΔH^* = 5358 J mol-res.$^{-1}$

n = 330 amino acid res.

S^* = 18.1 JK^{-1} mol -res^{-1}

Curves for three values of the change in heat capacity of the protein upon denaturation, ΔC_p, are shown.The values are 50, 65 and 80 J K^{-1} mol-res.$^{-1}$. In the figure, positive values of ΔG_{den} represent those temperatures at which the protein is in catalytically active.

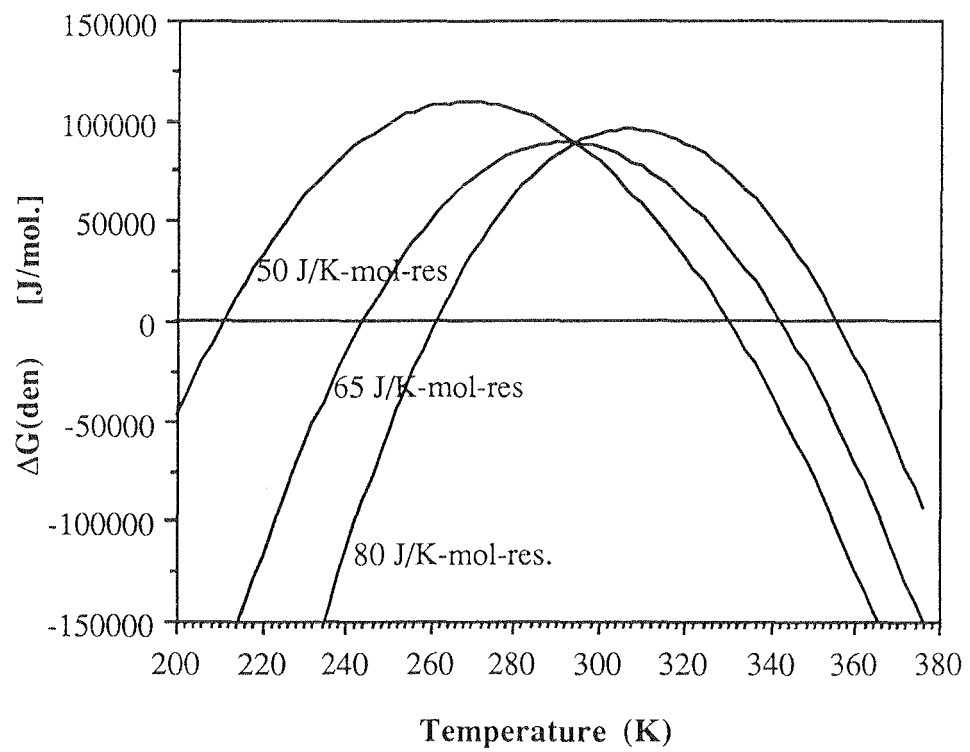

Fig. A3 Relationship between ΔG_{den} and temperature for an imaginery protein, and showing the effect of ΔC_p on the range of activity.