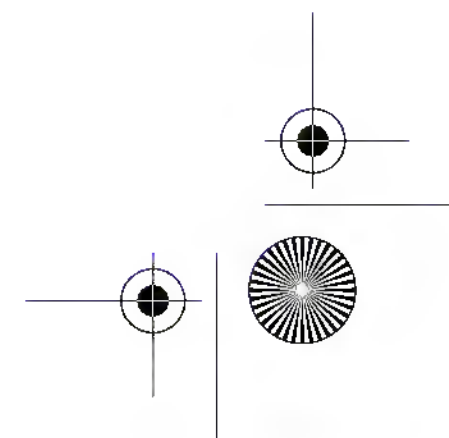
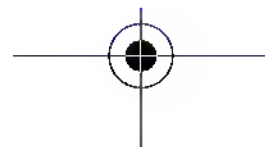
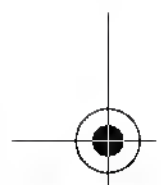
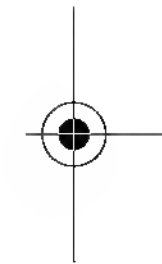
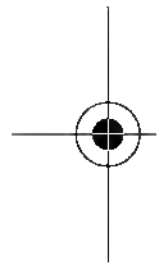


2 Primary Models

Robin C. McKellar and Xuewen Lu

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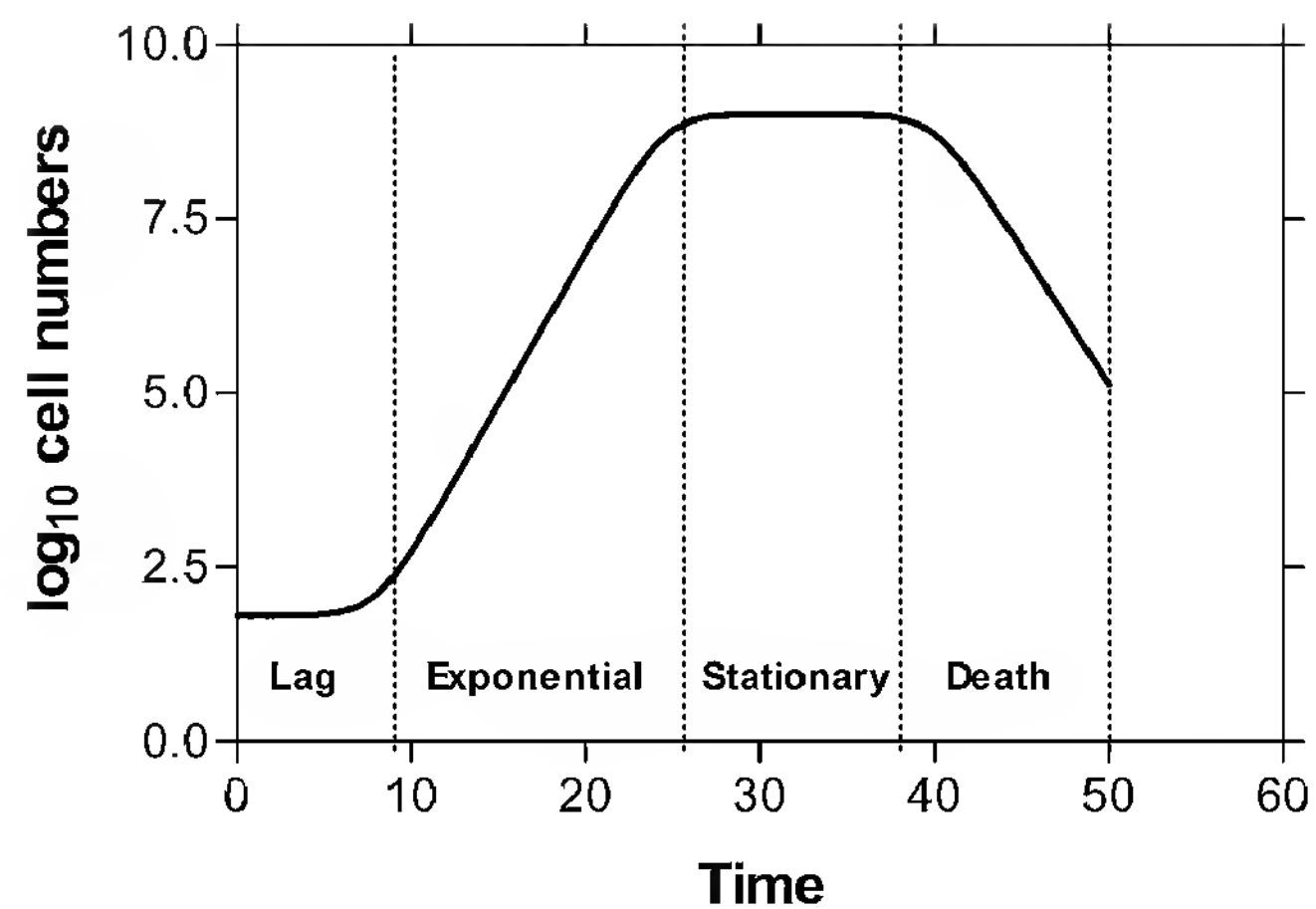
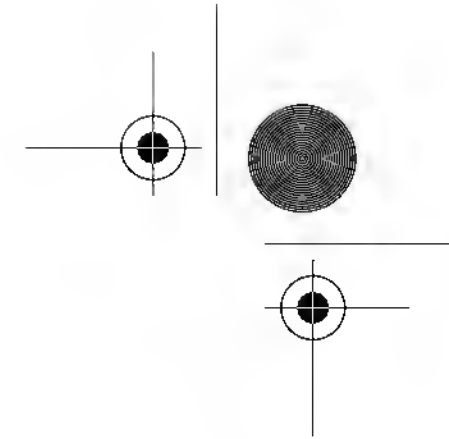
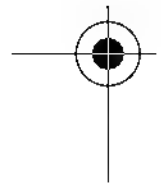


FIGURE 2.1 Stages of a bacterial growth curve.

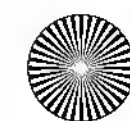
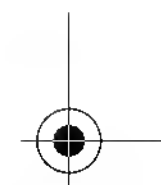
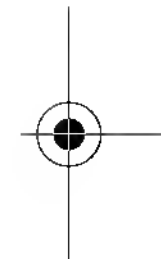
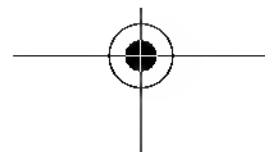
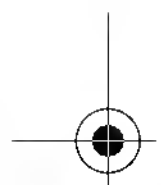
2.1 GROWTH MODELS

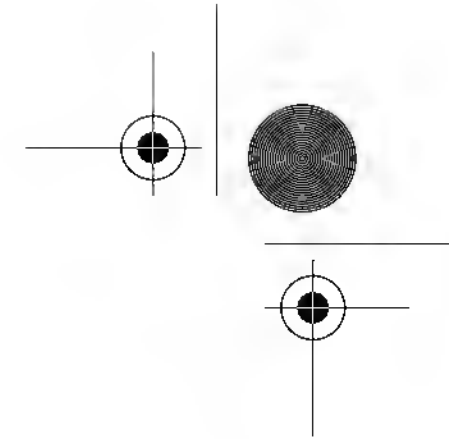
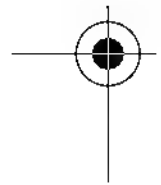
2.1.1 INTRODUCTION

The concept of the primary model is fundamental to the field of predictive microbiology (see the definition of a model in the Preface). A primary model for microbial growth aims to describe the kinetics of the process with as few parameters as possible, while still being able to accurately define the distinct stages of growth. A typical bacterial growth curve is shown in Figure 2.1. When the increase in population density (usually defined as the base 10 logarithm of cell numbers) is plotted against time, the resulting curve usually has four phases, referred to respectively as the lag, exponential, stationary, and death or decline phases.

In the only book published thus far that is devoted exclusively to the field of predictive microbiology, McMeekin et al.¹ provide an excellent review and discussion of the classical sigmoid growth functions, especially the modified logistic and Gompertz equations. As they point out, these are empirical applications of the original logistic and Gompertz functions. They lack mechanistic interpretability though the original logistic and Gompertz functions are considered mechanistic models. Over the last decade, a new generation of bacterial growth curve models have been developed that are purported to have a mechanistic basis: for example, the Baranyi model,^{2,3} the Hills model,^{4,5} the Buchanan model,⁶ and the heterogeneous population model.⁷ In addition to the book by McMeekin et al., other authors have provided reviews of microbial growth models.^{3,8-11}

In this chapter, we will review the modified logistic and Gompertz equations as well as the new models that were not covered by McMeekin et al.¹ and discuss their applications. We will compare these models based on their performance in predictive microbiology applications.





2.1.2 THE LOGISTIC AND THE GOMPERTZ FUNCTIONS

Sigmoidal functions have been the most popular ones used to fit microbial growth data since these functions consist of four phases, similar to the microbial growth curve.⁹ The most commonly used are the modified logistic (Equation 2.1) and the modified Gompertz (Equation 2.2) introduced by Gibson et al.¹²:

$$\log x(t) = A + \frac{C}{(1 + e^{(-B(t-M))})} \quad (2.1)$$

$$\log x(t) = A + C \exp\{-\exp[-B(t - M)]\} \quad (2.2)$$

where $x(t)$ is the number of cells at time t , A the asymptotic count as t decreases to zero, C the difference in value of the upper and lower asymptote, B the relative growth rate at M , and M is the time at which the absolute growth rate is maximum.^{1,9}

The above functions use $\log x(t)$ instead of $x(t)$ as the response variable. Thus, they are not simply reparameterizations of the original logistic^{13,14} and Gompertz¹⁵ functions, but are “modified” functions. The original logistic and Gompertz functions are considered to be mechanistic; however, the modified functions are empirical.

The parameters of the modified Gompertz equation can be used to characterize bacterial growth as follows¹:

$$e = 2.718 \dots$$

$$\text{lag time} = M - (1/B) + \frac{\text{Log}N(0) - A}{BC/e} \quad (2.3)$$

$$\text{exponential growth rate} = BC/e$$

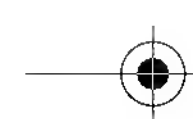
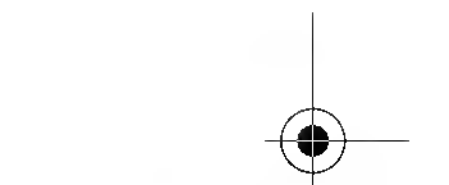
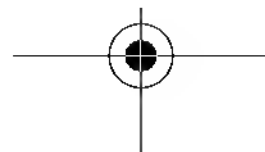
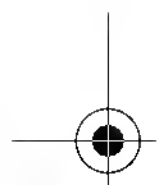
$$\text{generation time} = \log(2)e / BC = 0.8183 / BC$$

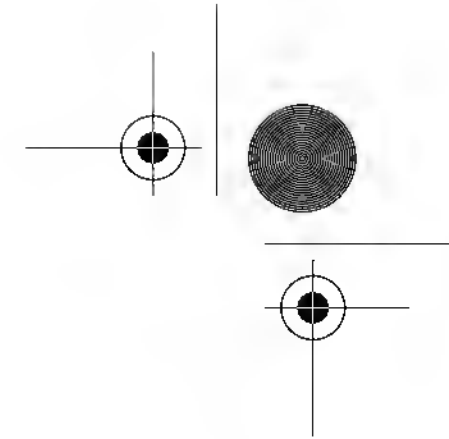
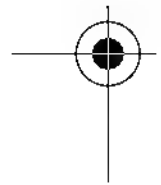
The expression in Equation 2.3 for lag time is different from the following Equation 2.4 proposed by Gibson et al.¹² and other workers^{16,17}:

$$\text{lag time} = M - \frac{1}{B} \quad (2.4)$$

As explained by McMeekin et al.,¹ Equation 2.3 is a more general and correct expression for the lag time.

In order to simplify the fitting process, reparameterized versions of the Gompertz equation have been proposed^{18,19}:





$$\log_{10} x = A + C \exp \left(- \exp \left(2.71 \left(\frac{R_g}{C} \right) (\lambda - t) + 1 \right) \right) \quad (2.5)$$

where $A = \log_{10} x_0$ (\log_{10} cfu \times ml⁻¹), x_0 is the initial cell number, C the asymptotic increase in population density (\log_{10} cfu \times ml⁻¹), R_g the growth rate (\log_{10} cfu h⁻¹), and λ is the lag-phase duration (h).

2.1.2.1 Applications of the Logistic Model

There have been limited examples of fitting of microbial growth data using the logistic function, since the Gompertz function, which is asymmetric about the point of inflection unlike the logistic function,^{9,20,21} is generally preferred. Some recent examples include modeling of fish spoilage²²⁻²⁴ and colony diameter of fungi.²⁵ A variation of the logistic model with a breakpoint at the transition between the lag phase and the exponential phase has also been used to model the lag phase of *Listeria monocytogenes*.²⁶

2.1.2.2 Applications of the Gompertz Equation

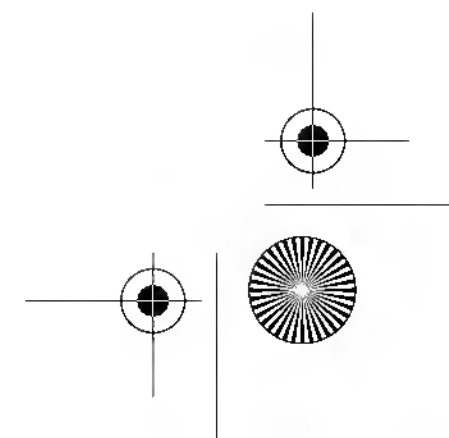
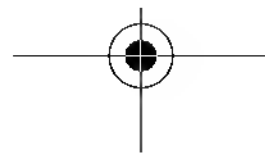
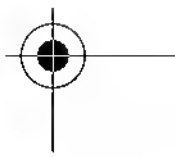
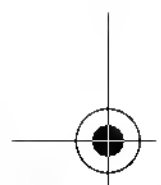
The Gompertz equation has been used extensively by researchers to fit a wide variety of growth curves from different microorganisms. Some of the recent models developed with the Gompertz function include those for *Yersinia enterocolitica*,²⁷ *Staphylococcus aureus*,^{28,29} *L. monocytogenes*,³⁰ *Vibrio parahaemolyticus*,³³ and *Bacillus cereus*.^{32,33}

The Gompertz function has also been applied to growth curves based on turbidity data³⁴; mixed cultures of *Pseudomonas* spp. and *Listeria* spp.^{35,36}; *Lactobacillus curvatus*³⁷; spoilage of vegetables,³⁸ beer,³⁹ and meat⁴⁰; and germination and growth of *Clostridium botulinum*.⁴¹

There are, however, some limitations associated with the use of the Gompertz function. The Gompertz rate (μ_{\max}) is always the maximum rate and occurs at an arbitrary point of inflection⁴²⁻⁴⁴; thus the generation time can be underestimated by as much as 13%.³¹ In addition, since the slope of the function cannot be zero, the lower asymptote must be lower than the inoculum level, giving a negative λ for some data sets.⁴³ Another limitation is that, in order to get a good fit, experimental data are required over the whole growth range.^{1,21}

2.1.3 BARANYI MODEL

In a series of papers,^{2,3,10} Baranyi and coworkers introduced a mechanistic model for bacterial growth. Briefly, the lag phase is attributed to the need to synthesize an unknown substrate q that is critical for growth. Once cells have adjusted to the new environment, they grow exponentially until limited by restrictions dictated by the growth medium; thus:



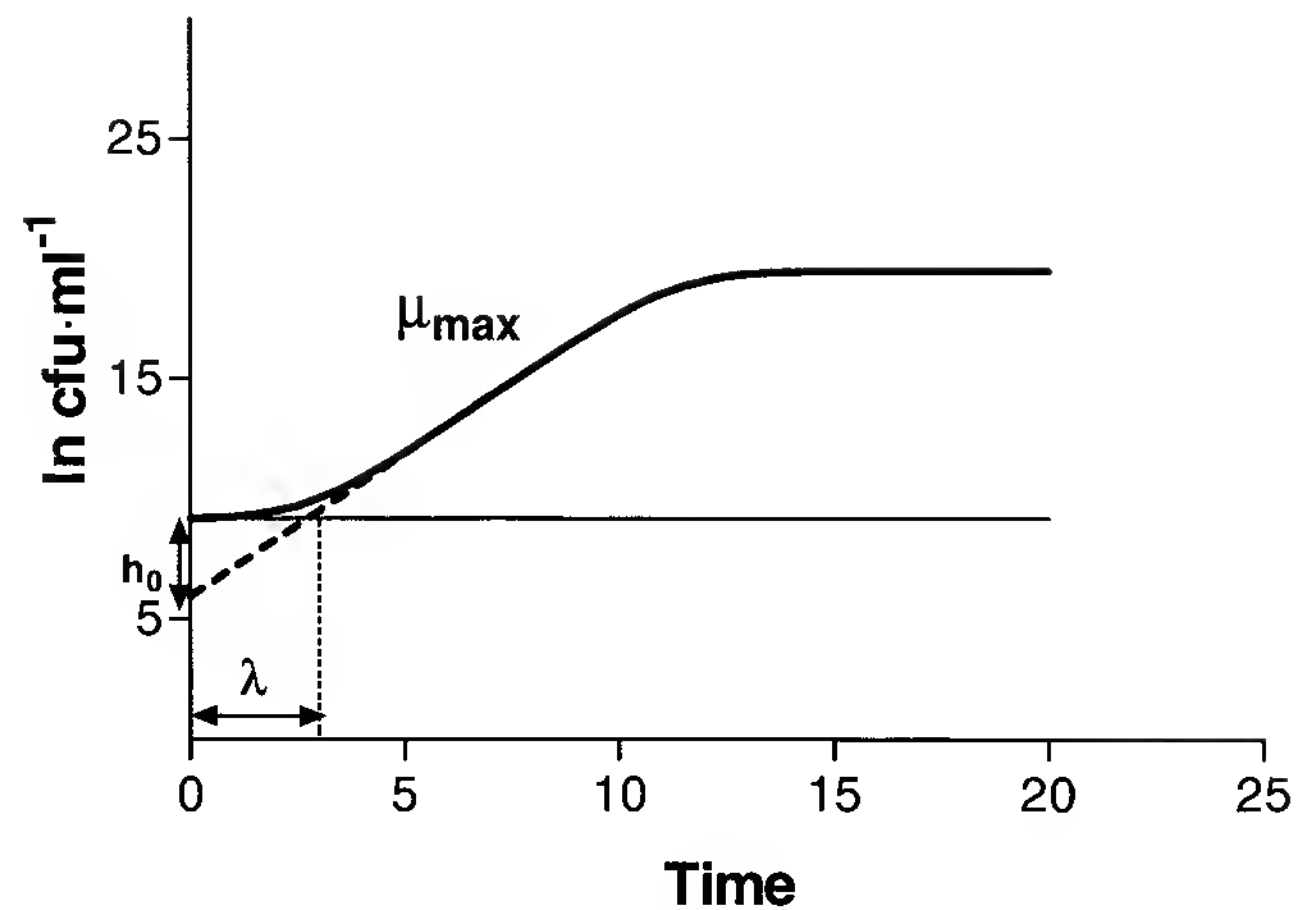
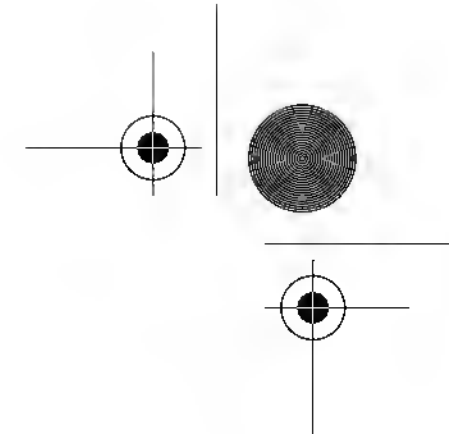
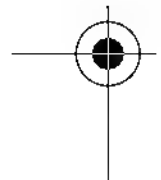


FIGURE 2.2 Example of a growth curve generated by the Baranyi and McKellar models. Parameters are defined in the text.

$$\frac{dx}{dt} = \frac{q(t)}{q(t)+1} \cdot \mu_{\max} \cdot \left(1 - \left(\frac{x(t)}{x_{\max}} \right)^m \right) x(t) \quad (2.6)$$

where x is the number of cells at time t , x_{\max} the maximum cell density, and $q(t)$ is the concentration of limiting substrate, which changes with time:

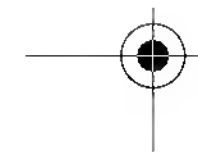
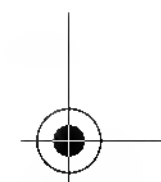
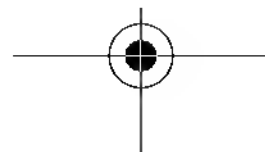
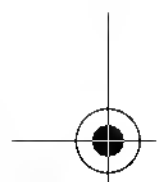
$$\frac{dq}{dt} = \mu_{\max} \cdot q(t) \quad (2.7)$$

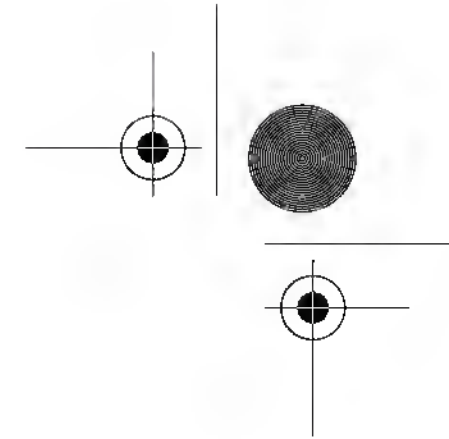
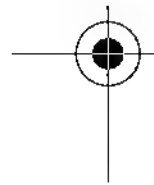
The initial value of q (q_0) is a measure of the initial physiological state of the cells. A more stable transformation of q_0 may be defined as:

$$h_0 = \ln \left(1 + \frac{1}{q_0} \right) = \mu_{\max} \lambda \quad (2.8)$$

The parameter m characterizes the curvature before the stationary phase. When $m = 1$ the function reduces to a logistic curve, a simplification of the model that is often assumed. Thus, the final model has four parameters: x_0 , the initial cell number; h_0 ; x_{\max} ; and μ_{\max} . The output of this model (and the relationship between h_0 , λ , and μ_{\max}) is shown in Figure 2.2.⁴⁵

An explicit version of the Baranyi model has also been derived:





$$y(t) = y_0 + \mu_{\max} A(t) - \frac{1}{m} \ln \left(1 + \frac{e^{m\mu_{\max} A(t)} - 1}{e^{m(y_{\max} - y_0)}} \right) \quad (2.9)$$

$$A(t) = t + \frac{1}{v} \ln \left(\frac{e^{-vt} + q_0}{1 + q_0} \right) \quad (2.10)$$

where $y(t) = \ln x(t)$, $y_0 = \ln x_0$, and v is the rate of increase of the limiting substrate, generally assumed to be equal to μ_{\max} .

2.1.3.1 Applications of the Baranyi Model

Since its inception in the early 1990s, the Baranyi model has been used extensively to model microbial growth. The popularity of this model has been facilitated by the availability of two programs: DMFit, an Excel add-in; and MicroFit, a stand-alone fitting program, distributed by the Institute of Food Research in the U.K. (<http://www.ifr.bbsrc.ac.uk/Safety/DMFit/default.html>). The model was used for growth modeling of a wide variety of microorganisms, the results of which are included in the Food MicroModel software.⁴⁶ Some recent applications were related to *Listeria monocytogenes*,^{47,48} *B. cereus*,⁴⁹ *Escherichia coli*,⁵⁰ *Y. enterocolitica*,⁵¹ increasing colony diameter of heat-resistant fungi,⁵² and spoilage in green asparagus and vegetable salad.^{53,54}

One of the advantages of the Baranyi model is that it is readily available as a series of differential equations that allow modeling in a dynamic environment, generally resulting from nonisothermal temperature profiles. This form of the model was used to describe the behavior of *E. coli* at suboptimal temperatures,⁵⁵ and to develop and validate a dynamic growth model for *L. monocytogenes* in fluid whole milk.^{56,57} It has also been used to study the influence of either slowly⁵⁸ or rapidly⁵⁹ changing temperature on the growth of *L. monocytogenes* and *Salmonella*.

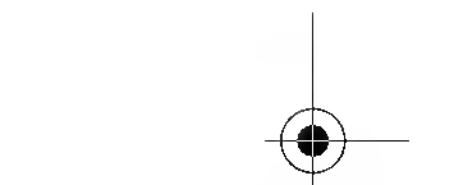
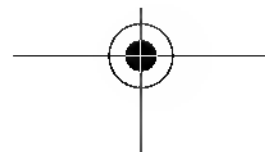
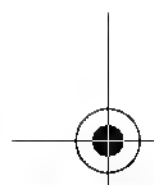
2.1.4 HILLS MODEL

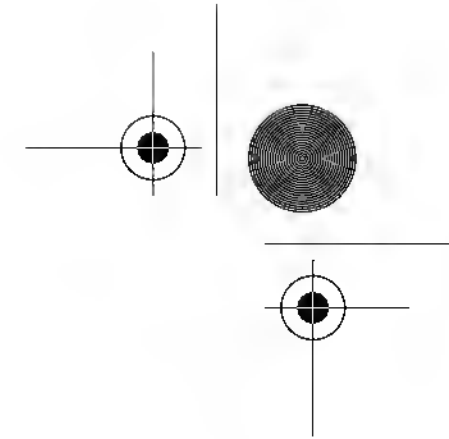
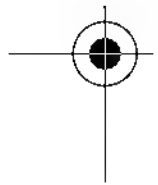
A general theory of spatially dependent bacterial growth in heterogeneous systems was developed by Hills and coworkers.^{4,5} This was achieved by combining a structured-cell kinetic model with reaction-diffusion equations describing transport of nutrients.⁴ The model was based in part on the concept of DNA synthesis and cell division being dependent on the excess cell biomass.

Assume M is the total biomass in the culture and N is the total number of cells in the culture. It can be shown that for inoculation with stationary-phase cells,

$$\begin{aligned} M(t) &= M(0) \exp(At) \\ N(t) &= N(0) [k_n \exp(At) + A \exp(-k_n t)] / (A + k_n) \end{aligned} \quad (2.11)$$

A and k_n are rate constants; in general, they depend on all the environment factors. The expression for $N(t)$ in Equation 2.11 has a much simpler form than the empirical





Gompertz function for fitting population growth, being a biexponential function where the second term, involving the rate of DNA synthesis, gives rise to the observed lag behavior. The lag time and the doubling time have the following relationships:

$$\begin{aligned}t_{LAG} &= A^{-1} \log[1 + (A / k_n)] \\t_{LAG} / t_D &= (\ln 2)^{-1} \log[1 + (A / k_n)]\end{aligned}\quad (2.12)$$

This shows that if the rate constants A and k_n have similar activation energies, the ratio of lag to doubling time should be nearly independent of temperature. This model takes no account of possible lag behavior in the total biomass (M).

The above model can also be generalized to spatially inhomogeneous systems such as food surfaces.⁴ If more detailed kinetic information on cell composition is available, more complex multicompartiment kinetic schemes can be incorporated. A two-compartment kinetic model of bacterial population dynamics has been developed that is capable of describing the phenomena of lethal and sublethal injury, resuscitation, and transient conditions. A more general three-compartment kinetic model has been developed to interpret lag behavior in total biomass. These models can be further generalized to describe growth in spatially heterogeneous systems.⁵

2.1.4.1 Applications of Hills Model

There have been few applications of the Hills model. The above two-compartment kinetic cell model was shown to fit batch-growth data for *L. monocytogenes*⁴ and for *Salmonella typhimurium*.⁵ More recently, the model was used for modeling viable counts of *S. typhimurium* in gel cassettes.⁶⁰

2.1.5 BUCHANAN THREE-PHASE LINEAR MODEL

Buchanan et al.⁶ proposed a three-phase linear model. It can be described by three phases: lag phase; exponential growth phase; and stationary phase:

Lag Phase:

$$\text{For } t \leq t_{LAG}, \quad N_t = N_0$$

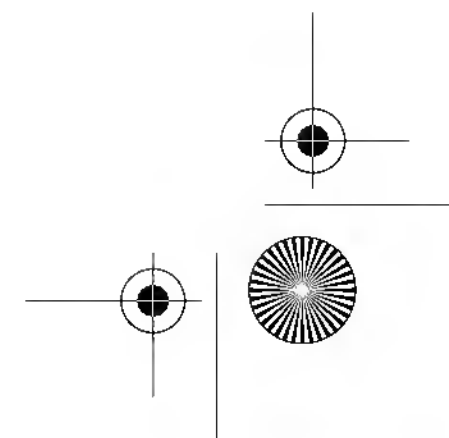
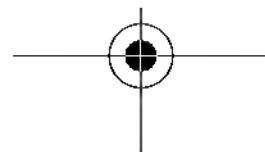
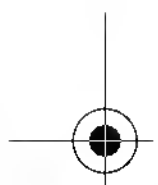
Exponential Growth Phase:

$$\text{For } t_{LAG} < t < t_{MAX}, \quad N_t = N_0 + \mu(t - t_{LAG}) \quad (2.13)$$

Stationary Phase:

$$\text{For } t \geq t_{MAX}, \quad N_t = N_{MAX}$$

where N_t is the log of the population density at time t (log cfu ml⁻¹); N_0 the log of the initial population density (log cfu ml⁻¹); N_{MAX} the log of the maximum population



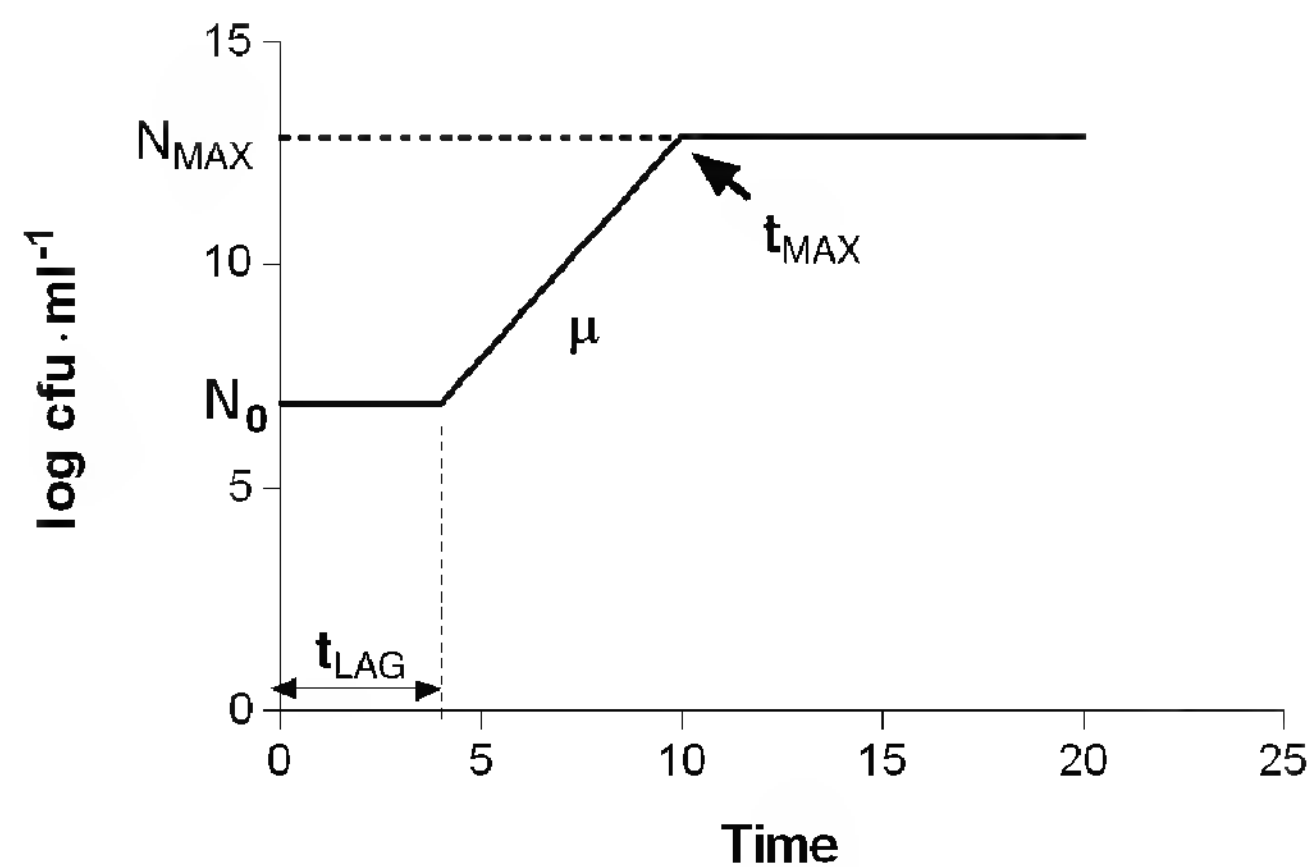
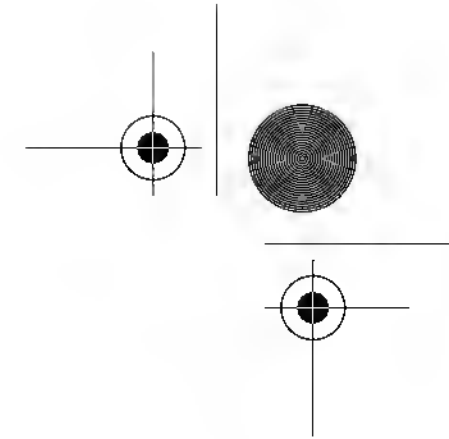
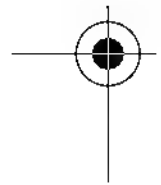


FIGURE 2.3 Example of a growth curve generated by the Buchanan model. Parameters are defined in the text.

density supported by the environment ($\log \text{cfu ml}^{-1}$); t the elapsed time; t_{LAG} the time when the lag phase ends (h); t_{MAX} the time when the maximum population density is reached (h); and μ is the specific growth rate ($\log \text{cfu ml}^{-1} \text{h}^{-1}$). The three-phase model is illustrated in Figure 2.3.

In this model, the growth rate was always at maximum between the end of the lag phase and the start of the stationary phase. The μ was set to zero during both the lag and stationary phases. The lag was divided into two periods: a period for adaptation to the new environment (t_a) and the time for generation of energy to produce biological components needed for cell replication (t_m). Thus, the lag phase is given by:

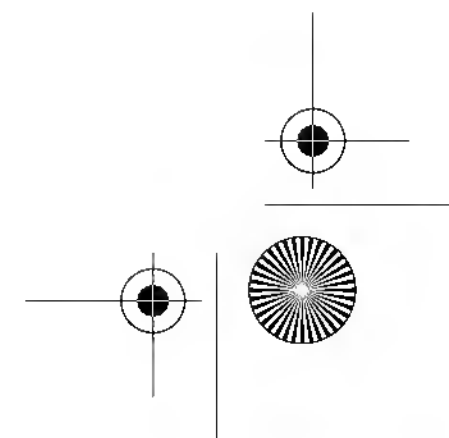
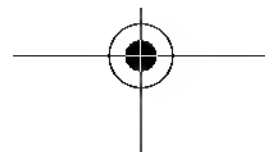
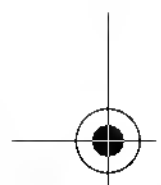
$$t_{\text{LAG}} = t_a + t_m \quad (2.14)$$

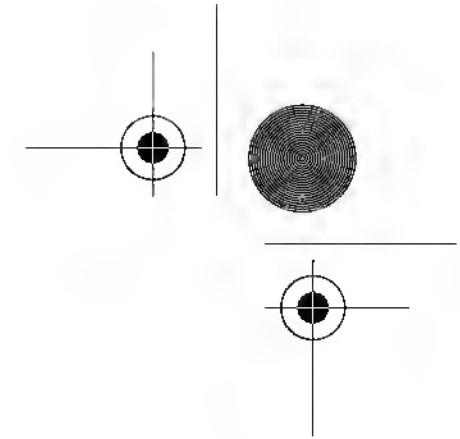
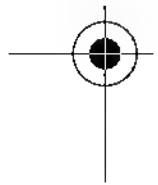
This implies that t_a and t_m can be estimated from data fitted with the linear model using the following relationships⁶:

$$\begin{aligned} t_m &= \text{generation time} \\ t_a &= t_{\text{LAG}} - \text{generation time} \end{aligned} \quad (2.15)$$

2.1.5.1 Applications of the Buchanan Model

Surprisingly, this simple model has not been used extensively for fitting growth data. The original authors used the three-phase version of the model to fit experimental data for *E. coli* O157:H7.⁶ As there is often little interest in modeling the stationary phase, a modified two-phase version has been proposed that fits only the lag and exponential phases. In a series of papers published in 1999, Oscar used the two-phase model to fit growth data for *S. typhimurium* in brain heart infusion broth,⁶¹





and on cooked chicken⁶² and ground chicken⁶³ breast meat. Fitting was accomplished using a useful nonlinear regression software package called Prism (GraphPad Software, San Diego, CA) in which an if-then statement defines the model:

$$N_t = N_0 + IF[t \leq t_{LAG}, 0, \mu \cdot (t - t_{LAG})] \quad (2.16)$$

with symbols defined in Equation 2.13. A two-phase model was also used to model growth of *E. coli* O157:H7.⁶⁴

2.1.6 McKellar Model

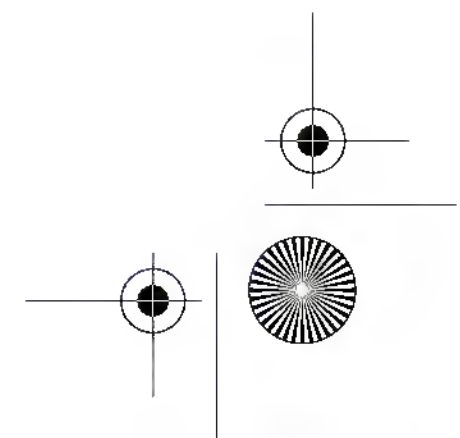
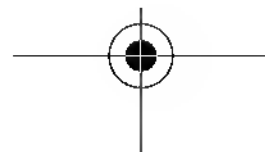
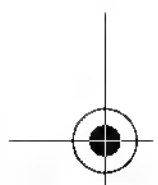
One of the limitations of existing models is that they all assume a homogeneous population of cells. A heterogeneous population model was recently proposed in which growth was expressed as a function of two distinct cell populations.⁷ Cells can exist in one of two “compartments” or states: growing or nongrowing. All growth was assumed to originate from a small fraction of the total population of cells that are present in the growing compartment at $t = 0$. Subsequent growth is based on the following logistic equation:

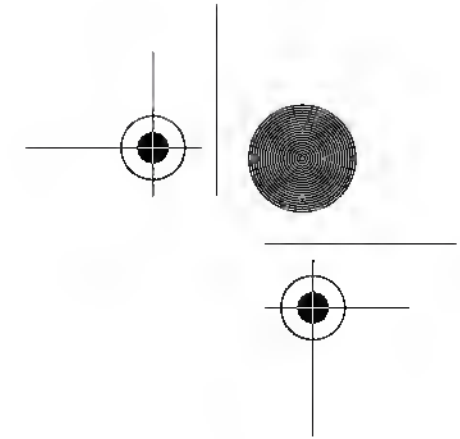
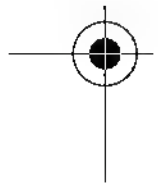
$$\frac{dG}{dt} = G \cdot \mu \cdot \left(1 - \frac{G}{N_{MAX}}\right) \quad (2.17)$$

where G is the number of growing cells in the growing compartment. The majority of cells were considered not to contribute to growth, and remained in the nongrowing compartment, but were included in the total population. While this is an empirical model, it does account for the observation that growth in liquid culture is dominated by the first cells to begin growth, and that any cells that subsequently adapt to growth are of minimal importance.⁷

This model has an interesting relationship with the Baranyi model. It is derived from a different initial premise, that microbial populations are heterogeneous rather than homogeneous. It is based on two populations of cells that behave differently, rather than a single population. The sum of the two populations effectively describes the transition from lag to exponential phase, and defines a new parameter G_0 , the initial population capable of growth. Reparameterization of the model led to the finding that a relationship existed between μ_{max} and λ , which is shown in Figure 2.2,⁷ and which had been derived by Baranyi from a more mathematical argument.³ Baranyi⁶⁵ later supported the geometric relationship in Figure 2.2, and stated that the initial physiological state of the whole population could reside in a small subpopulation. Thus, the McKellar model constitutes a simplified version of the Baranyi model, and has the same parameters.

The concept of heterogeneity in cell populations was extended further to the development of a combined discrete-continuous simulation model for microbial growth.⁶⁶ At the start of a growth simulation, all of the cells were assigned to the nongrowing compartment. A distribution of individual cell lag times was used to generate a series of discrete events in which each cell was transferred from the





nongrowing to the growing compartment at a time corresponding to the lag time for that cell. Once in the growing compartment, cells start growing immediately according to Equation 2.17. The combination of the discrete step with the continuous growth function accurately described the transition from lag to exponential phase. This model was further modified to include a continuous adaptation phase prior to the discrete event.⁶⁷ A new physiological state parameter p_0 was proposed that represents the mean of the initial individual cell physiological states. This model is dynamic in both the lag and exponential phases, and thus is useful for simulating the behavior of individual cells in a changing environment.

2.1.6.1 Applications of the McKellar Model

This model has not been used extensively for modeling microbial growth partly because of its similarity to the Baranyi model. It is also a compartmental model, and as such cannot be fitted easily using conventional nonlinear regression programs. This model was fitted to data for growth of *L. monocytogenes* at 5 to 35°C, and compared to the Gompertz model.⁷ Values for μ_{\max} were slightly higher with this model, and λ were generally shorter than found with the Gompertz model. Goodness-of-fit analysis suggested that the McKellar model generally fit the data better than the Gompertz.

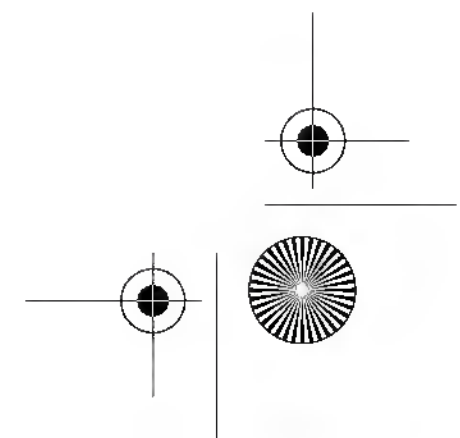
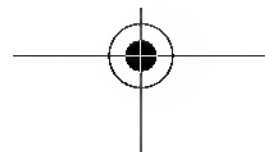
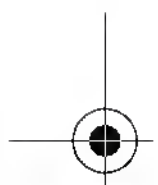
2.1.7 OTHER MODELS

There have been a large number of alternative models proposed for modeling microbial growth. Many of the earlier ones have been thoroughly discussed by McMeekin et al.,¹ and will not be discussed further here.

Whiting and Cygnarowicz-Provost⁶⁸ suggested a quantitative four-parameter model for the germination, growth, and decline of *C. botulinum*, and the growth of *L. monocytogenes*. Jones and Walker⁶⁹ developed an equation to predict growth, survival, and death of microorganisms based on data obtained using *Y. enterocolitica* in varying pH and sodium chloride concentrations at different temperatures. Van Impe et al.⁷⁰ proposed a dynamic first-order differential equation to predict both microbial growth and inactivation, with respect to both time and temperature. We are expecting more accurate and more mechanistic primary models when people gain more knowledge on the kinetics of individual cells and behavior of bacteria. Recently, a series of three models has been proposed in which μ can increase, remain constant, or decrease with time.⁷¹ The latter two models bear some resemblance to those discussed earlier; however, the concept of μ increasing with time was designed to accommodate the observation that recombinant *E. coli* initially grew rapidly in a bioreactor because of high substrate concentrations.

2.1.8 EXAMPLES OF GROWTH MODEL FITTING

It seems appropriate at this point to provide an example of how some of the more popular and useful functions may be used to fit experimental growth data. The data selected (taken from an earlier study⁷) were for the growth of *L. monocytogenes* at 5°C (Table 2.1). The models used in this comparison were Gompertz using Equation



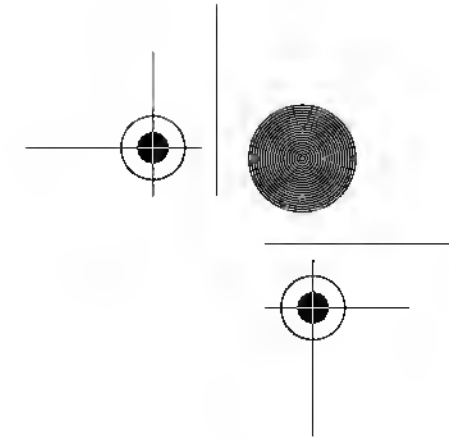
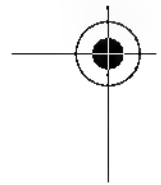
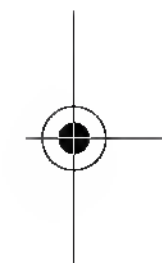
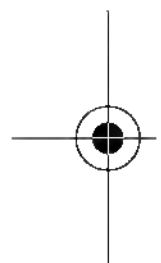
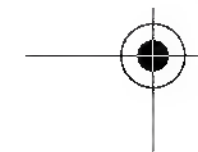
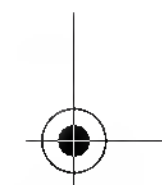
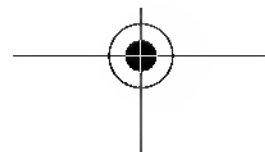
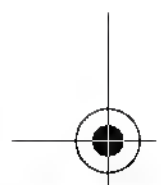


TABLE 2.1
Growth Data for *Listeria monocytogenes*
at 5°C

Time (d)	log cfu ml ⁻¹
0	4.8
6	4.7
24	4.7
30	4.7
48	4.9
54	5.1
72	5.3
78	5.4
99	5.9
126	6.3
144	6.9
150	6.9
168	7.2
174	7.3
191	7.7
198	7.8
216	8.3
239	8.8
266	9.1
291	9.2
316	9.3
336	9.7
342	9.7
360	9.7
384	9.5



2.5, Baranyi using Equation 2.6 and Equation 2.7, McKellar using Equation 2.17, and Buchanan using Equation 2.13. Nonlinear regression analysis was done using the ModelMaker[®] software (Modelkinetix, Old Beaconsfield, Bucks, U.K., www.modelkinetix.com), which uses the Runge–Kutta method for solving differential equations. Initial parameter estimates were made using the simplex method, and regression was performed using the Marquardt algorithm. The Baranyi and McKellar models gave values for μ_{\max} directly, since they were in the form of differential equations, and modeled the cell number rather than \log_{10} cfu ml⁻¹. The Gompertz and Buchanan models were applied directly to \log_{10} cfu ml⁻¹ data, and thus the rate parameter (R_g) obtained from the fitting had to be converted to μ_{\max} using the relationship $\mu_{\max} = R_g \cdot \ln 10$. The λ parameter for the Gompertz and Buchanan models was obtained directly from the fitting, while the values for the Baranyi and McKellar models were derived from the h_0 parameter values using the following relationship: $h_0 = \mu_{\max} \cdot \lambda$. The Baranyi model (Baranyi_{MF}) was also fitted using the MicroFit software, in which the model was reparameterized to fit λ directly. The



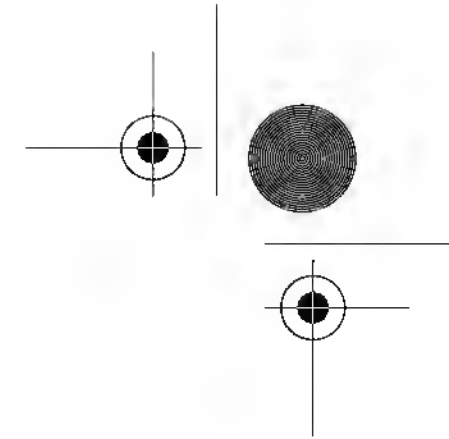
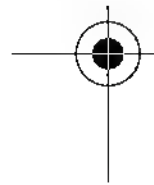


TABLE 2.2
Results of Model Fitting to Growth Data

Model ^a	μ (h ⁻¹)	λ (d)	Log x_0	Log x_{\max}	DF	RMSE
Baranyi _{MF}	0.050	46.9	4.68	9.57	21	0.100
McKellar	0.049	44.9	4.63	9.57	21	0.112
Gompertz _{PZ}	0.054	54.7	4.68	10.0	21	0.119
Gompertz	0.058	68.4	4.76	9.87	21	0.139
Buchanan	0.048	53.8	4.84	9.49	20	0.157
Baranyi	0.056	61.4	4.72	9.32	21	0.179

Note: DF = residual degrees of freedom; RMSE = root mean square error.

^a The McKellar, Gompertz, Buchanan, and Baranyi models were fit using the ModelMaker software. Baranyi_{MF} is the Baranyi model fit using the MicroFit software. Gompertz_{PZ} is the Gompertz model fit using the Prism software.

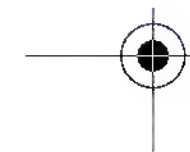
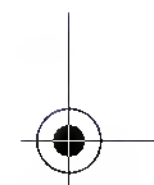
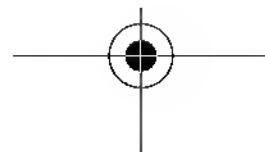
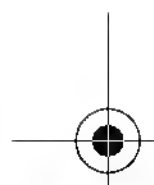
Gompertz model (Gompertz_{PZ}) was also fitted using Prism™ Version 3.03 (GraphPad Software, San Diego, CA, www.graphpad.com).

The results of the various fitting approaches are given in Table 2.2. The root mean square error (RMSE) was taken as the measure of goodness of fit, as suggested by Ratkowsky (Chapter 4). The models are placed in order of increasing RMSE.

The best model was Baranyi_{MF} with the lowest RMSE. In contrast, the poorest fit was with the Baranyi model using ModelMaker, which also gave a higher μ_{\max} and λ than did the Baranyi_{MF} model. The next best model was the McKellar, with parameter values close to those for Baranyi_{MF}. The Gompertz model fitted using either Prism or ModelMaker gave larger μ_{\max} and λ values than did the Baranyi_{MF} and McKellar models, and the highest values of log x_{\max} among all other models. The Buchanan model gave the lowest value of μ_{\max} of all the models, and a shorter λ than all except the Baranyi_{MF} and McKellar models.

The output of the four models fitted with ModelMaker is also shown in Figure 2.4. The steeper slope (μ_{\max}) of the Gompertz and Baranyi models may be observed. The greatest difference between models occurred during the late-log early-stationary phase. The Gompertz model never reaches a plateau, which reflects its higher log₁₀ x_{\max} (Table 2.2). As expected, the Buchanan model has a sharp breakpoint, while the transition to the stationary phase appears smoother with both the McKellar and Baranyi models.

The results of the nonlinear regression fitting described above emphasize an important point: there is no single solution for nonlinear regression, in contrast to linear regression. The iterative approach used in nonlinear regression is dependent on the parameter starting values, and may find local, rather than global, optimum values. In addition, different software packages use different procedures for fitting, and thus the results obtained (such as those above) should be considered comparative rather than absolute. The fitting results do show that, while there are differences between the models and the software used, the parameter differences are often slight. It is worth noting that estimates of λ range from 44.9 to 68.4 days. Further discussion



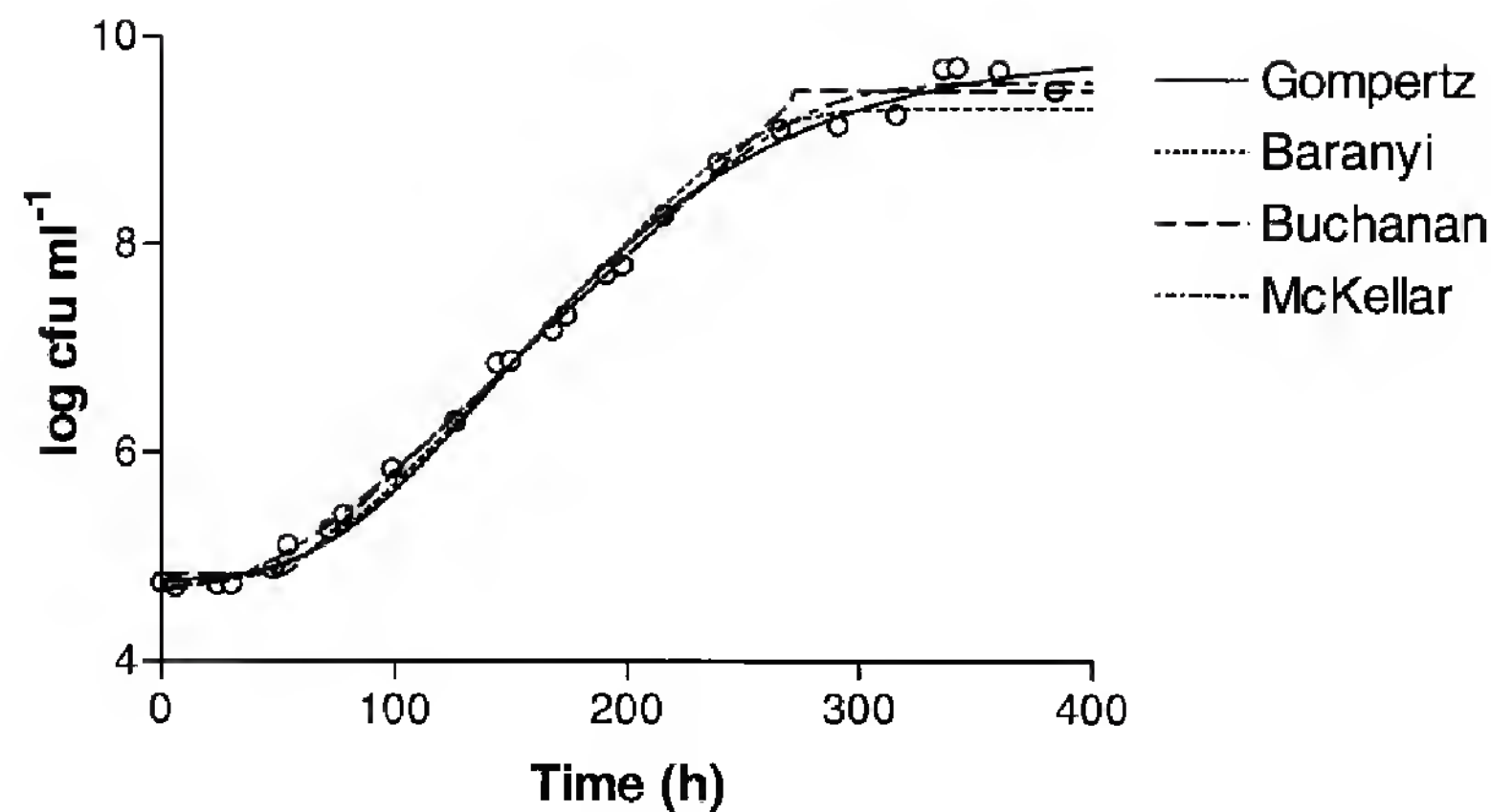
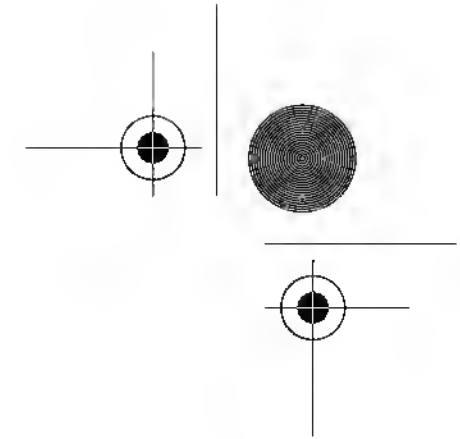
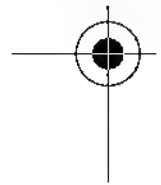


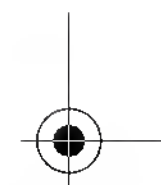
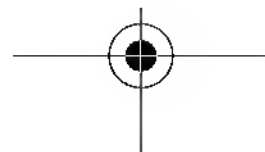
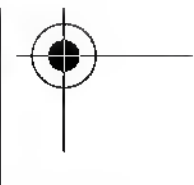
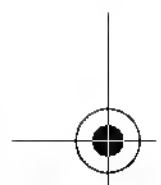
FIGURE 2.4 Comparison of growth models fitted to viable count data of *Listeria monocytogenes* grown at 5°C.

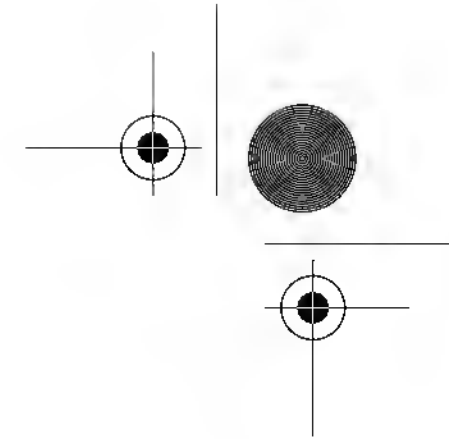
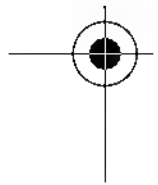
on the difficulties in modeling λ , and the role of the physiological state, can be found in [Chapter 9](#), and a more complete discussion of model fitting can be found in [Chapter 4](#).

2.1.9 COMPARISON OF EXISTING MODELS

Zwietering et al.¹⁸ statistically compared several different modified sigmoidal functions (Logistic, Gompertz, Richards, Schnute, and Stannard) using the *t*-test and the *F*-test. In most of the cases, the modified Gompertz expression was regarded as the best model to describe the growth data both in terms of statistical accuracy and ease of use when compared to other sigmoidal functions.

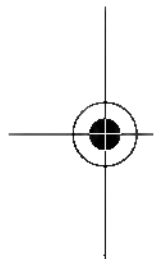
Baranyi et al.² compared the output of their model with that of the Gompertz, and concluded that the goodness of fit was generally at least as good. They also showed that their model gave estimates for lag and growth rate that were slightly lower than in the Gompertz case. Baranyi et al. also compared their model to those of Hills¹⁰ and Buchanan⁷² and stated that these models are special cases of the Baranyi model. Baranyi argues that the Buchanan model has merit in its simplicity, but that the model lacks the capability of simulating dynamic behavior.⁷² Buchanan et al.⁶ asserted that their three-phase model is comparable to, and more robust than, either the Gompertz or the Baranyi models, especially when experimental data were minimal. The three-phase linear and Baranyi models predicted similar maximum population densities. These values were typically smaller than the values provided by the Gompertz model. Garthright⁴⁴ strongly supports the three-phase model, and points out its superiority in describing the lag and exponential phases as compared to the Gompertz. He concludes that the nonlinear approach does not achieve any advantage over the three-phase linear approach for environmental applications. This model appears particularly appropriate for modeling conditions where growth is poor, and an upper asymptote cannot be accurately fixed. The Baranyi model and the McKellar model can also be used when stationary-phase data are lacking.





Other comparisons between growth models have been made. A comparison of the logistic, Gompertz, and Baranyi models for fish spoilage showed that the logistic function was similar to the Baranyi but easier to fit.⁷³ A comparison between Gompertz and Baranyi models gave better fit with the Baranyi model, and a higher growth rate with Gompertz.⁷⁴ The Gompertz function was found to be more appropriate than the Baranyi model for monitoring CO₂ evolution as an indicator of bacterial growth.⁷⁵ Other workers have compared the Baranyi and Gompertz models, and have concluded that the Baranyi function gave better parameter estimates as compared to the Gompertz.⁷⁶

At the present time it is not possible to select one growth model as the most appropriate representation of bacterial growth. If simple is better, then the three-phase model is probably sufficient to represent fundamental growth parameters accurately.^{44,77} There does appear to be general agreement in relationship to underlying principles, and emphasis should be placed on the development and use of models and parameters that can be easily understood by food microbiologists.⁷⁷ However, in spite of Garthright's assertion that straight line simplicity is sufficient to model growth,⁴⁴ the development of more complex models (and subsequently more mechanistic models) will depend on an improved understanding of cell behavior at the physiological level.



2.2 SURVIVAL MODELS

2.2.1 INTRODUCTION

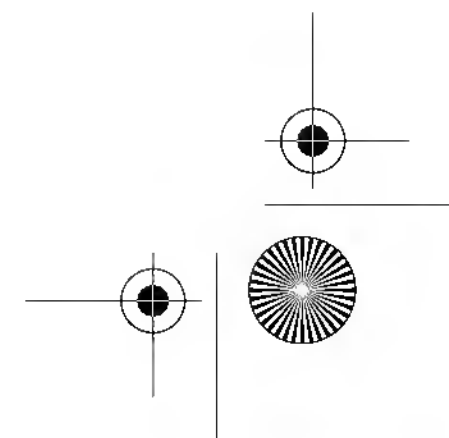
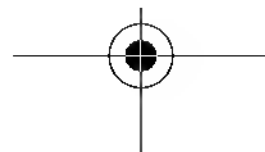
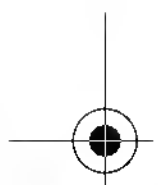
Our ability to understand and model the survival of pathogens in foods or during processing of food is critical to the safety of the food supply. Thus, models to describe microbial death due to heating have been used since the 1920s, and constitute one of the earliest forms of predictive microbiology. Much of the early work centered around the need to achieve destruction of *C. botulinum* spores in low acid canned foods,^{17,78,79} and much effort has been put towards characterizing the kinetics of spore inactivation. In this section of the chapter we will focus on the evolution of survival modeling from the classical linear approach to the more complex models required to describe inactivation curves that deviate from linearity.

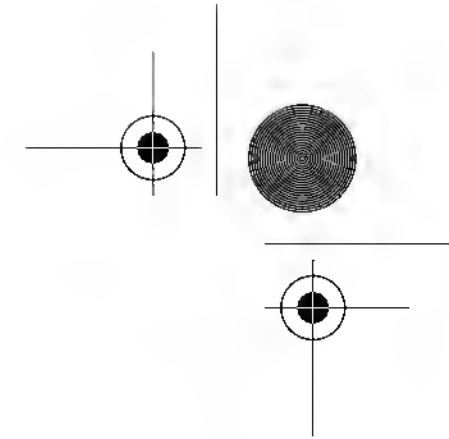
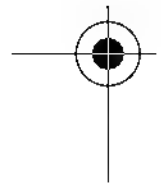
2.2.2 CLASSICAL LINEAR MODELS

It has always been assumed that spore inactivation follows simple first-order reaction kinetics under isothermal conditions:

$$\frac{dS_t}{dt} = -k'S_t \quad (2.18)$$

where S_t is the survival ratio (N_t/N_0) and k' is the rate constant. Thus the number of surviving cells decreases exponentially:





$$S_t = e^{-k't} \quad (2.19)$$

and when expressed as \log_{10} , gives:

$$\log S_t = -kt \quad (2.20)$$

where $k = k'/\ln 10$. The well-known D -value (time required for a 1-log reduction) is thus equal to $1/k$, where k is the slope (Figure 2.5). The D -values can also be expressed as:

$$D\text{-value} = \frac{t}{\log N_0 - \log N_t} \quad (2.21)$$

When $\log D$ -values are plotted against the corresponding temperatures, the reciprocal of the slope is equal to the z -value, which is the increase in temperature required for a 1-log decrease in D -value (Figure 2.5; inset). The rate constant can also be related to the temperature by the Arrhenius equation:

$$k = N_0 e^{\left(\frac{-E_a}{RT}\right)} \quad (2.22)$$

where E_a is the activation energy, R the universal gas constant, and T is the temperature in Kelvin.

From the first-order reaction it is not possible to achieve complete destruction of all *C. botulinum* spores in a given volume of product; one spore will always be

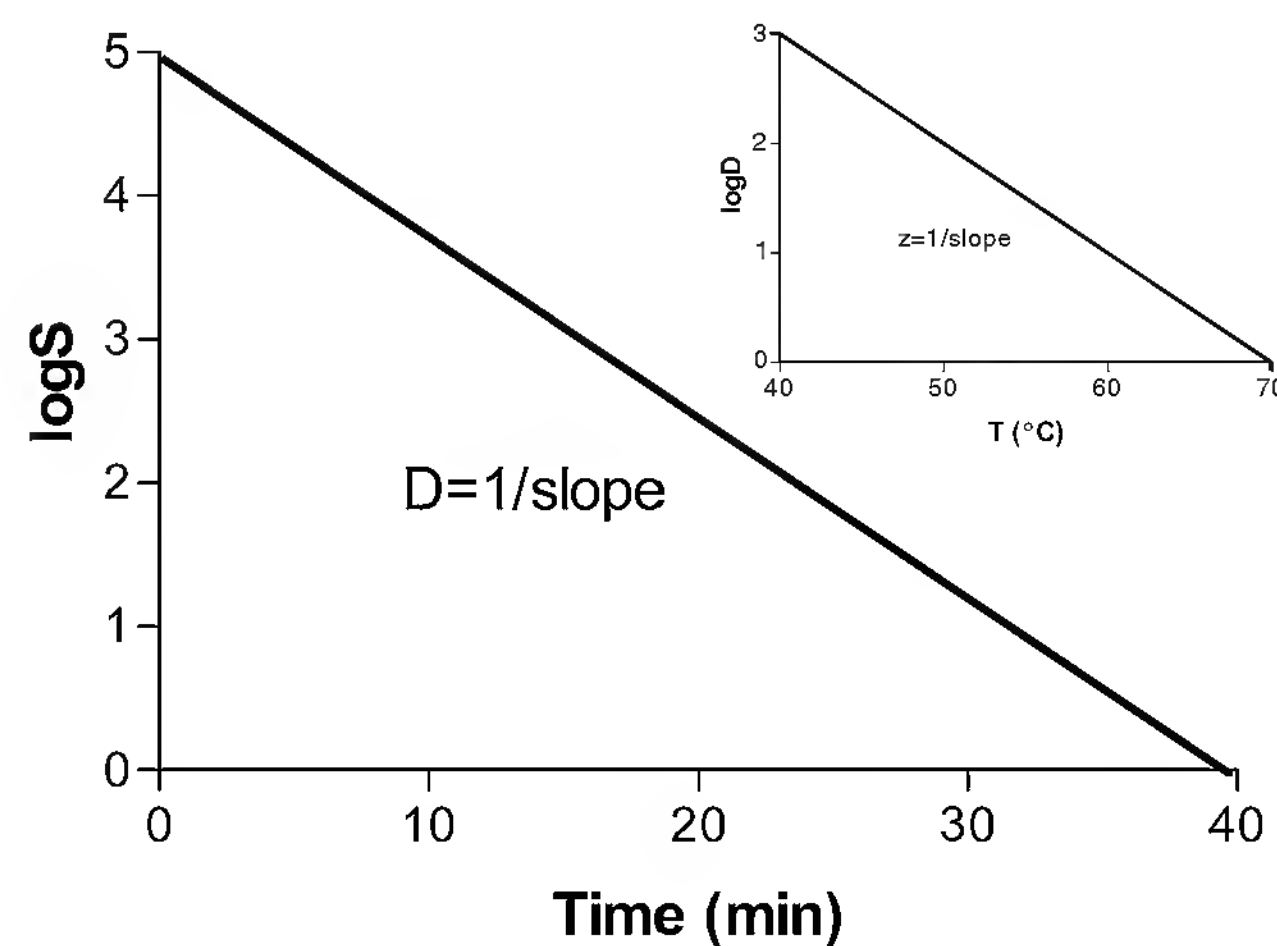
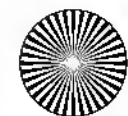
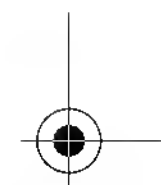
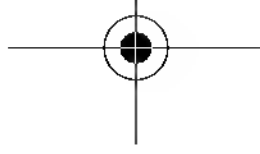
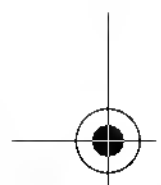
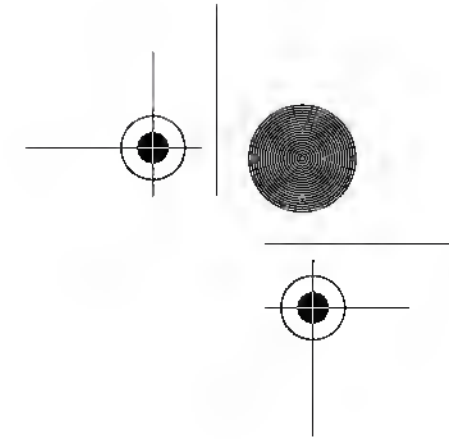
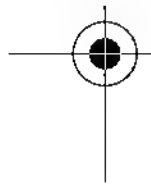


FIGURE 2.5 Geometric description of D - and z -values. (From McKellar, R.C., Modelling the effectiveness of pasteurization, in *Dairy Processing: Maximizing Quality*, Smit, G., Ed., CRC Press Inc./Woodhead Publishing, 2003 pp. 104–129. With permission.)





left in a can if a sufficient number of cans are examined. Thus it is generally assumed that a 12-log reduction (also known as 12D) is sufficient to achieve “commercial sterility,” or an acceptable level of risk of survival of *C. botulinum*. Knowledge of the *D*-values of representative strains allows the determination of the F_0 -value, which is the time required to achieve 12D, assuming a *z*-value of 10°C. At 121°C, F_0 is equal to 2.5 min for most strains of *C. botulinum*.¹⁷

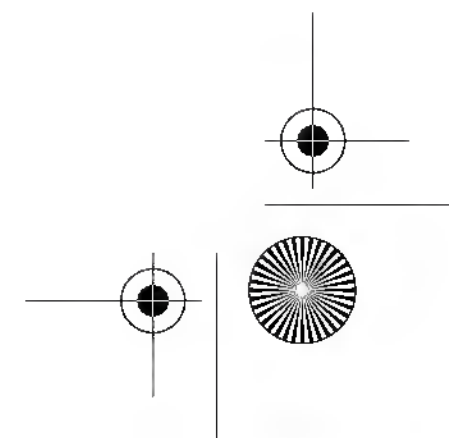
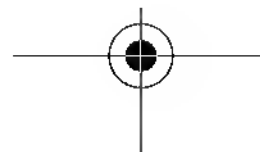
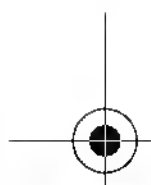
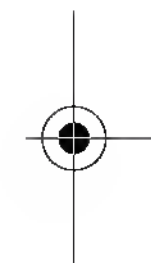
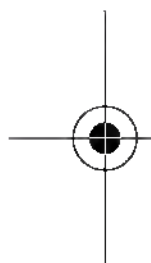
Comparable standards for other food-borne pathogens do not exist; however, it is generally accepted that a 4- or 5-log reduction is considered adequate, depending on the product. An extensive amount of work has gone into the determination of *D*- and *z*-values for various pathogens. Thermal stability of pathogens such as *L. monocytogenes*,⁸⁰ salmonellae,⁸¹ and *E. coli* O157:H7⁸² has been well documented and summarized in recent reviews.

2.2.3 NONLINEAR MODELS

2.2.3.1 Nonlinearity Issues

The canning industry has enjoyed an enviable record of safety, and thus the concept of logarithmic death of microorganisms has persisted, and is now considered accepted dogma. In spite of this, nonlinear survival curves were reported for some bacteria almost 100 years ago.⁸³ In general there are two classes of nonlinear curves; those with a “shoulder” or lag prior to inactivation, and those that exhibit tailing. These two phenomena may be present together, or with other observed kinetics such as biphasic inactivation. A wide variety of complex inactivation kinetics have been reported, and several of these are shown in Figure 2.6. The theoretical basis for assuming logarithmic behavior for bacteria is based on the assumptions that bacterial populations are homogeneous with respect to thermal tolerance, and that inactivation is due to a single critical site per cell.⁸³ Both of these assumptions have been questioned, and thus concerns have been raised regarding the validity of extrapolation of linear inactivation curves.^{84,85}

Stringer et al.⁸² have summarized the possible explanations for nonlinear kinetics into two classes: those due to artifacts and limitations in experimental procedure and those due to normal features of the inactivation process. The first class encompasses such limitations as variability in heating procedure; use of mixed cultures or populations; clumping; protective effect of dead cells; method of enumeration; and poor statistical design. The second class includes such situations as possible multiple hit mechanisms; natural distribution of heat sensitivity; and heat adaptation. These two classes roughly parallel the two concepts reviewed by Cerf⁸⁵ to explain tailing in bacterial survival curves. The first of these (the “mechanistic” approach) also makes the assumption of homogeneity of cell resistance and proposes that thermal destruction follows a process analogous to a chemical reaction. In this approach, deviations from linearity are attributed mainly to artifacts; however, tailing is also related to the mechanism of inactivation or resistance. In the “vitalistic” approach, it is assumed that the cells possess a normal heterogeneity of heat resistance; thus survival curves should be sigmoidal or concave upward.⁸⁵



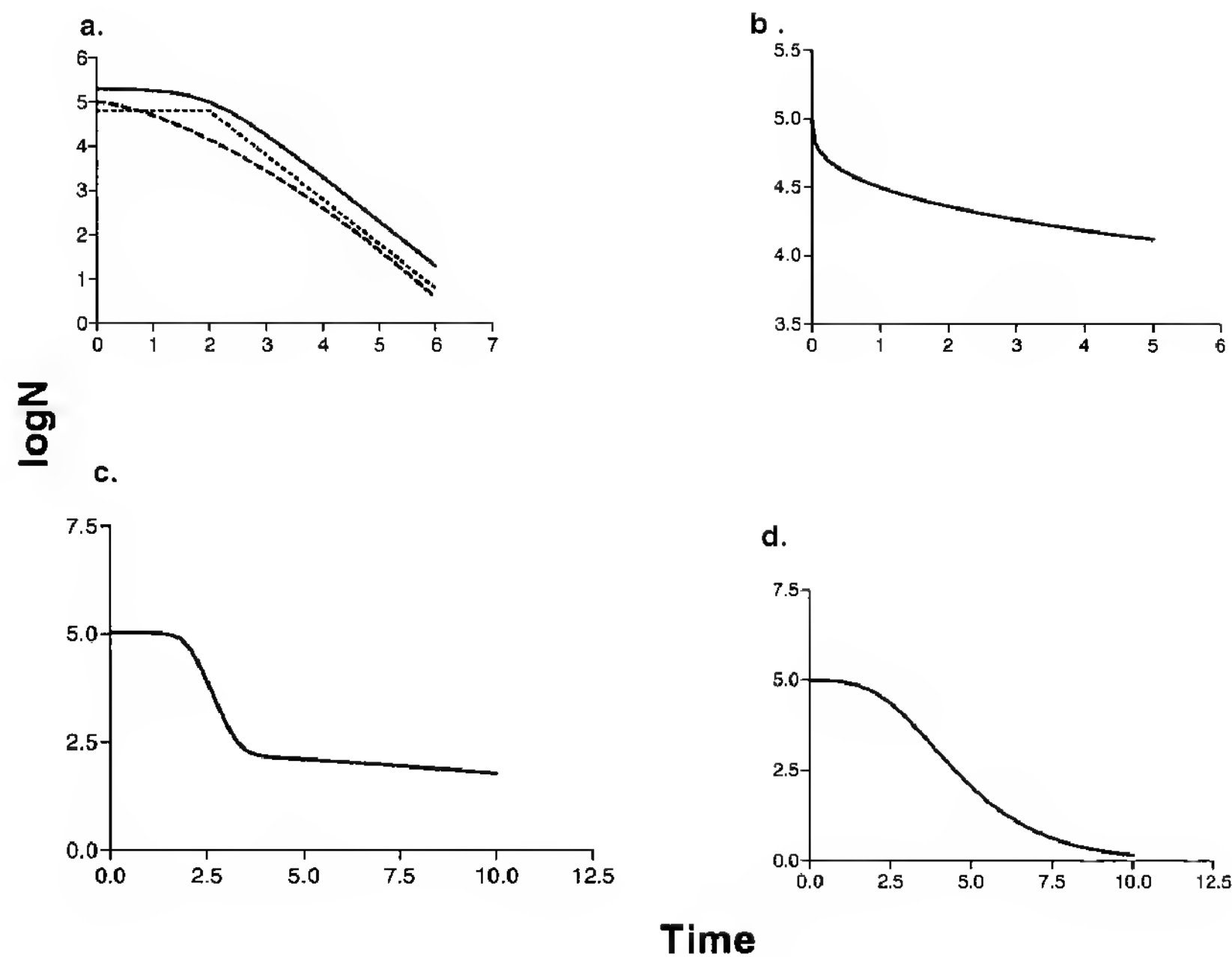
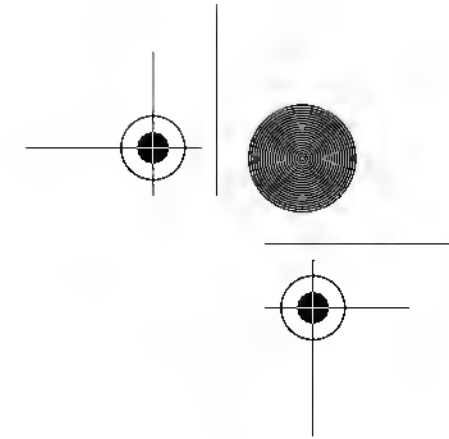
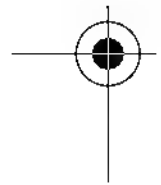
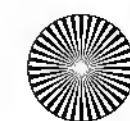
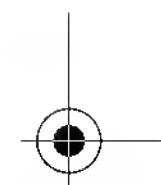
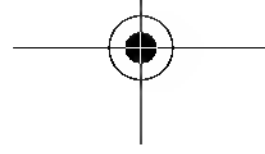
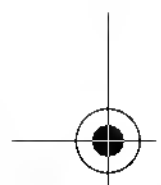
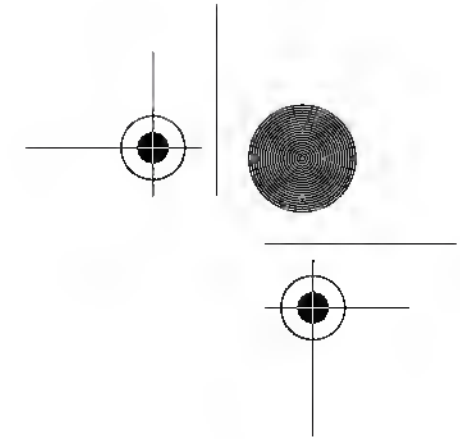
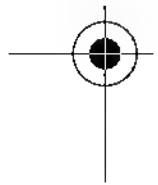


FIGURE 2.6 Examples of thermal death curves: (a) lag or shoulder, with either linear (dotted line), power law where $p > 1$ (broken line), or monophasic logistic (solid line) models; (b) concave with power law where $p < 1$; (c) biphasic logistic; and (d) sigmoidal.

There has been considerable controversy between the two schools of thought, and the literature is divided on the validity of nonlinear survival curves as representing the true state of the cell population. There is certainly evidence that inconsistencies in experimental protocols or the use of incorrect media can lead to artifacts; however, there is little convincing evidence that clumping of cells or the protective effect of dead cells is consistently responsible for nonlinear survivor curves. The current belief is, notwithstanding some contribution by artifacts, that cells do exhibit heterogeneity in thermal sensitivity, and the majority of modeling approaches now make this assumption. There is also inconsistency in actually defining what is meant by an artifact. If one assumes that an artifact in this context is anything that interferes with obtaining a linear death curve, then many of the situations currently classified as artifacts may be natural behavior of cell populations. This is particularly obvious in the study of spore inactivation where standardized suspensions are difficult to obtain, and much effort has been expended to remove artifacts such as genetic variants. The difficulty in obtaining linear kinetics may be a signal that, in most cases, nonlinearity is the norm.

The current theories of microbial inactivation must be revisited in light of recent improved understanding of the effect of heat on microorganisms. We now know that cells do not exist simply as alive or dead, but may also experience various degrees of injury or sublethal damage, which may give rise to apparent nonlinear survival curves.⁸² The induction of heat resistance in food-borne pathogens due to expression of heat shock proteins has been extensively documented in recent years, and may





contribute to apparent nonlinearity, particularly tailing.^{82,86,87} Thus it appears important to model the actual conditions or situations experienced by bacteria in foods rather than relying on simplifications. Survival modeling should also include a more complete understanding of the molecular events underpinning microbial resistance to the environment.

It seems likely that heterogeneity within bacterial populations is responsible in most cases for nonlinear survival curves, and most recent attempts to model survival employ distributions. The use of distributions to account for nonlinearity is not new; log normal distributions had been suggested for this purpose as early as 1942.⁸³ Other distributions such as logistic, gamma, and Weibull have also been suggested; Weibull is the favored approach at the moment (see later). There is no complete agreement on the use of distributions,⁸³ and it is clear that this approach cannot adequately account for changes in heat resistance occurring during heating.

Our lack of understanding of the key physiological aspects of microbial inactivation and the complexities of nonlinear behavior suggest that a truly mechanistic model for thermal inactivation will not be developed in the near future. One approach to quantitating bacterial survival might be the thermal death point concept common to the canning industry. This approach allows one to define the conditions required to achieve a target log reduction, and makes no statement regarding the kinetics of that destruction. This approach has a number of attractive advantages; however, it would still be influenced by such artifacts as changes in heat resistance of a culture and cell injury.⁸³

2.2.3.2 Shoulder/Tail Models

2.2.3.2.1 Linear Approach

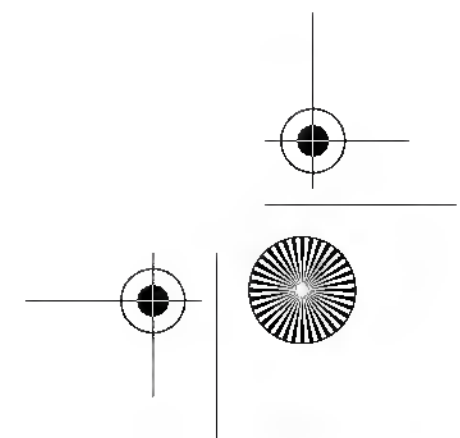
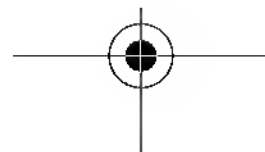
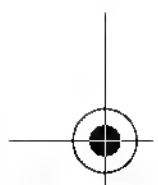
Inactivation curves that deviate from simple exponential often have a lag or shoulder region prior to the exponential inactivation. This shape of inactivation curve is probably the most commonly experienced by researchers. A simple linear model to account for this behavior was developed by Whiting⁸⁸:

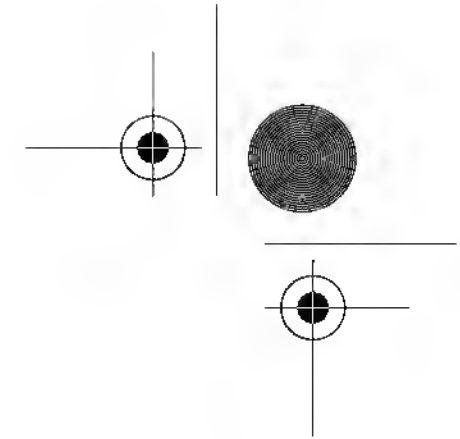
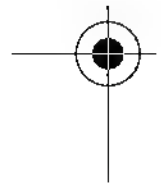
$$\log N = \begin{cases} \log N_0 & \text{when } 0 < t < t_L \\ \log N_0 - \left(\frac{1}{D}\right)(t - t_L) & \text{when } t > t_L \end{cases} \quad (2.23)$$

where t_L is the lag prior to inactivation.

An example of the output of this model is the dotted line in [Figure 2.6a](#). The advantage of this model is that linear regression can be used. This simple model has been used effectively to describe the nonthermal inactivation of *L. monocytogenes* as a function of organic acid and nitrite concentrations⁸⁹⁻⁹² and under reduced oxygen.⁹³ A similar two-phase linear model was described for thermal inactivation of *L. monocytogenes* by Bréand et al.⁹⁴

It is quite common for the lag or shoulder region of the survival curve to be highly variable. This makes it difficult to develop secondary models to describe the influence of the environment on the lag. Thus, survival using this model is often described as the time required for a 4-log reduction (T_{4D})^{89,92,95}:





$$t_{4D} = t_L + 4 \cdot D \quad (2.24)$$

2.2.3.2.2 Nonlinear Approach

Complex inactivation kinetics requires the use of nonlinear functions. It should be noted that nonlinearity as it relates to mathematical functions means that the parameters in the equation are nonlinear; the resulting curve may or may not appear linear. Linear regression can be easily performed by most spreadsheet programs; however, nonlinear regression is an iterative process that is supported by more specialized software. These software packages are readily available; thus considerable advances have been made in the development of nonlinear models.

Another of the more common shapes of survival curves is the concave curve, which has no lag, and a single, tailing population (Figure 2.6b). This function is best represented by the power law:

$$\log \frac{N}{N_0} = -\frac{t^p}{D} \quad (2.25)$$

where p is the power. A concave curve is produced when $p < 1$ (Figure 2.6b), and a convex (or shoulder) shape results from $p > 1$ (broken line in Figure 2.6a). A power law function has been used to model curvature in survival curves for *Enterococcus faecium*⁹⁶ and alkaline phosphatase⁹⁷ in milk. Other, seemingly novel, functions that have been derived to fit concave survival curves are really in fact power law functions.^{98,99}

Tailing survival curves can also be represented by the exponentially damped polynomial model. In this model, deviation from simple linear kinetics, experienced while heating *Staphylococcus aureus* in skim milk, was fitted with the nonlinear function¹⁰⁰:

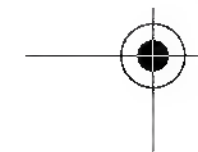
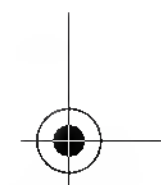
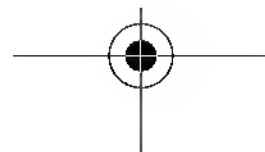
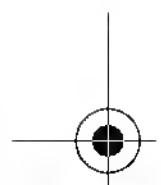
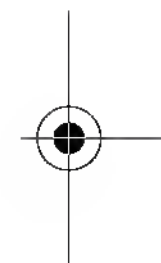
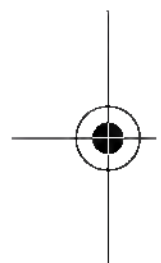
$$\log \frac{N}{N_0} = -kte^{-\lambda t} \quad (2.26)$$

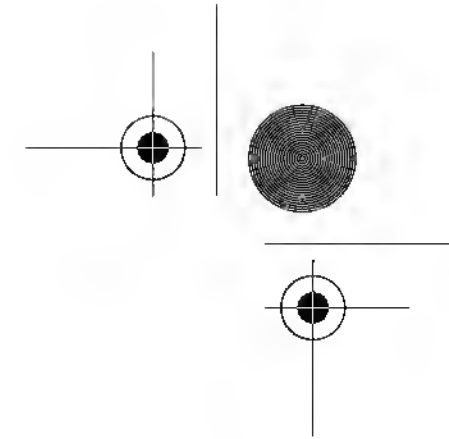
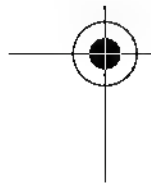
where k is the rate coefficient and λ is the damping coefficient.

As discussed earlier, a logistic equation may be used in growth modeling to modify the simple exponential growth to account for limiting the maximum population size as a result of nutrient limitation. In the same way, a logistic function can be used to account for death being limited by the amount of some stress factor or damage to the cell.¹⁰¹ This “mirror image” of the logistic function is called the Fermi equation, and is used for sigmoidal decay curves, which are symmetric:

$$\log \frac{N}{N_0} = \log \left[\frac{1 + e^{-bt_L}}{1 + e^{b(t-t_L)}} \right] \quad (2.27)$$

where N is the population (cfu ml⁻¹) surviving at time t ; N_0 is the population surviving at time 0; b is the maximum specific death rate; and t_L is the lag phase prior to





inactivation. This equation has been modified to account for situations where one may find both a primary, heat-sensitive population and a secondary more heat-resistant population⁸⁸:

$$\log \frac{N}{N_0} = \log \left[\frac{F(1 + e^{-b_1 t_L})}{(1 + e^{b_1(t-t_L)})} + \frac{(1-F)(1 + e^{-b_2 t_L})}{(1 + e^{b_2(t-t_L)})} \right] \quad (2.28)$$

where b_1 is the maximum specific death rate for the primary population and b_2 is the maximum specific death rate for the secondary population. Traditional D -values may be calculated as $2.3/b$ for each population. Lag phases are not always present, and this can be accounted for by setting the value of t_L to zero. An example of the output of this function is given in Figure 2.6c. The biphasic logistic model has been used to model inactivation of spores of *C. botulinum*,¹⁰² and the nonthermal inactivation of *L. monocytogenes*^{90,92,93} and *S. aureus*.¹⁰³ This model has also been applied to the thermal inactivation of bovine milk catalase¹⁰⁴ and *E. faecium*⁹⁶ during high-temperature short-time (HTST) pasteurization, and inactivation of *E. faecium* during bologna sausage cooking.¹⁰⁵ In situations where a single population exists, F can be set equal to 1 (solid line in Figure 2.6a).

Other variations of the logistic function have been suggested. A four-parameter logistic model was proposed by Cole et al.¹⁰⁶:

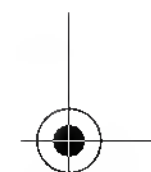
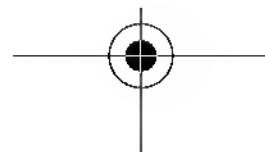
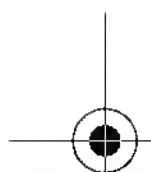
$$y = \alpha + \frac{\omega - \alpha}{1 + e^{\frac{4\sigma(\tau-x)}{\omega-\alpha}}} \quad (2.29)$$

where $y = \log_{10}$ cfu ml⁻¹; $x = \log_{10}$ time; α = upper asymptote; ω = lower asymptote; τ = position of maximum slope; and σ = maximum slope. This model was applied to the survival of *Y. enterocolitica* at suboptimal pH and temperature,¹⁰⁷ and the thermal inactivation of *Salmonella typhimurium*,¹⁰⁸ *C. botulinum*,¹⁰⁹ *Salmonella enteritidis*, and *E. coli*.¹¹⁰

As was shown earlier, the asymmetric Gompertz function has considerable advantages when fitting bacterial growth curves. In keeping with the trend to use mirror images of growth functions to describe inactivation, a reparameterized form of the Gompertz function was suggested by Linton et al.¹¹¹:

$$\log \frac{N}{N_0} = C \exp(-\exp(A + Bt)) - C \exp(-\exp(A)) \quad (2.30)$$

This function has been used to fit nonlinear survival curves of *L. monocytogenes* in buffer¹¹¹ and infant formula.¹¹² An example of the Gompertz function is given in Figure 2.6d. Other applications for the Gompertz equation include the effect of combined high pressure and mild heat on the inactivation of *Escherichia coli* and *S. aureus* in milk and poultry,¹¹³ and the inhibition of Enterobacteriaceae and clostridia during sausage curing.¹¹⁴ In a similar fashion, the mirror image of the



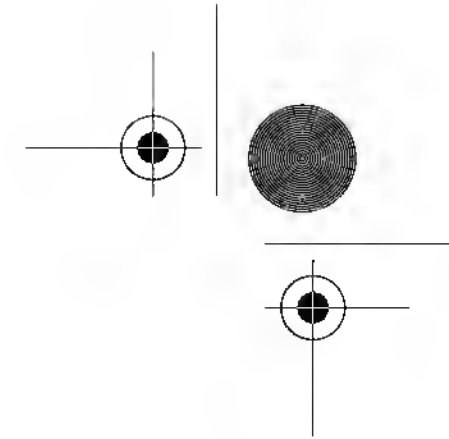
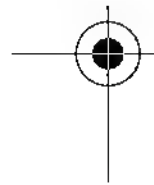


TABLE 2.3
Survival Data from *Pediococcus* sp.
NRRL B2354 at 62°C

Time (min)	Log cfu ml ⁻¹
0	8.4
5	8.3
10	8.1
15	8.1
20	7.7
25	7.3
30	6.9
35	6.4
40	6.2

TABLE 2.4
Parameter Estimates from Fitting the
Logistic and Linear Survival Models to
the Data in Table 2.3

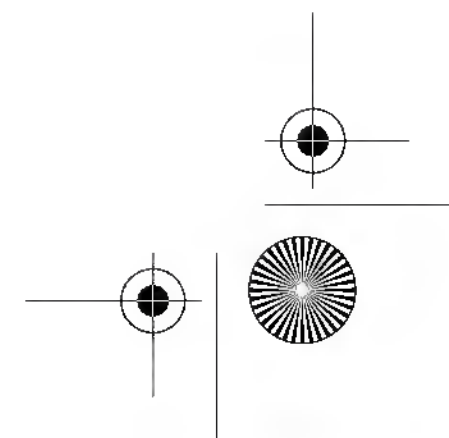
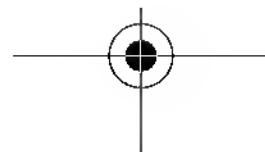
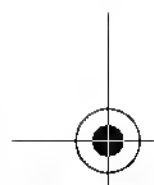
Model	N_0	Lag	D -value	DF	RMSE
Logistic	8.11	12.3	12.0	6	0.089
Linear	8.27	12.8	12.7	6	0.102

Baranyi growth model (see earlier) has been used for fitting nonlinear survival curves for the thermal inactivation of *Brochothrix thermosphacta*¹¹⁵ and *Salmonella enteritidis*.¹¹⁶

2.2.3.2.3 Examples of Model Fitting

It has often proven difficult to accurately fit survival data where a lag exists prior to inactivation. The models we have found most useful in this situation are the single-phase logistic (Equation 2.27) and the two-phase linear (Equation 2.23).

These models were fitted to unpublished data on survival of *Pediococcus* sp. NRRL B2354 heated at 62°C (Table 2.3), using Prism as described above. The results of the fitting are shown in Table 2.4, and in Figure 2.7. The logistic model was slightly better than the linear, with a smaller RMSE. Because of the sharp breakpoint between the shoulder and exponential decay, the D -value for the linear model was slightly larger, while the lag phase was only marginally greater than that in the logistic model. As was found with growth models, there is often little to choose between different models; thus personal preference and experience often dictate which model is generally used. A more complete discussion of model fitting may be found in Chapter 4.



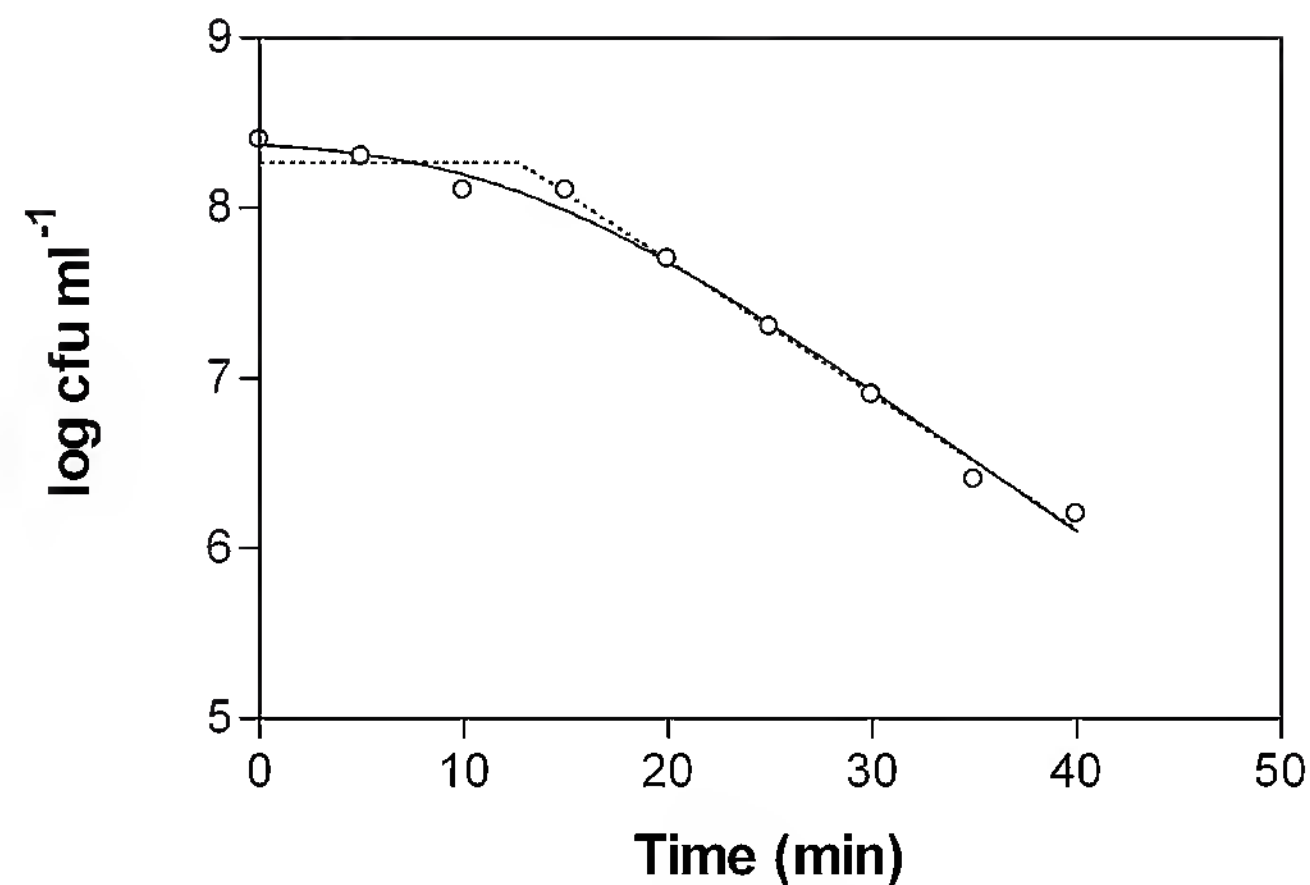
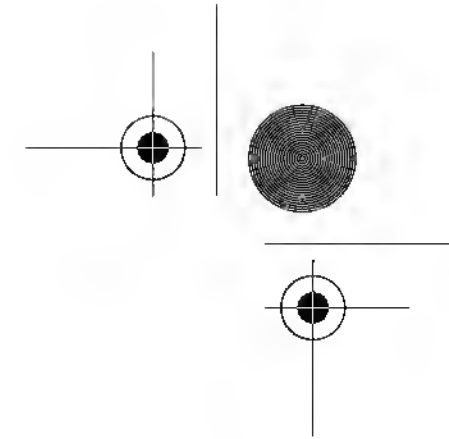
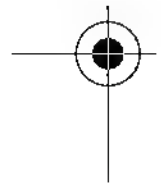
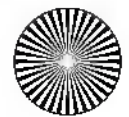


FIGURE 2.7 Example of fitting nonlinear survival data for *Pediococcus* NRRL B2354 using monophasic logistic (solid line) and two-phase linear (broken line) models. (From McKellar, R.C., Modelling the effectiveness of pasteurization, in *Dairy Processing: Maximizing Quality*, Smit, G., Ed., CRC Press Inc./Woodhead Publishing, 2003 pp. 104–129. With permission.)

2.2.4 DISTRIBUTIONS

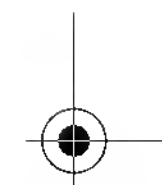
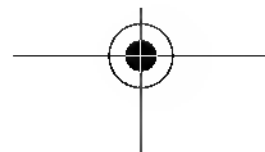
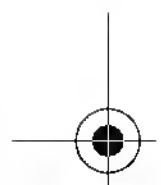
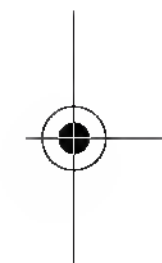
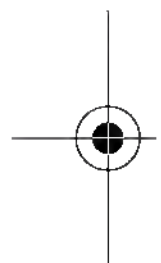
One recent development in the modeling of bacterial survival is the use of distributions. This is based on the assumption that lethal events are probabilistic rather than deterministic. With a large initial population of cells, a continuous function can be used, much like with a chemical reaction (although a chemical reaction appears deterministic only because of the large number of molecules involved). The survival curve for a single cell is a step function, where a cell exists as either alive or dead¹¹⁷:

$$S_i(t) = \begin{cases} 1 & \text{(alive) for } t < t_c \\ 0 & \text{(dead) for } t \geq t_c \end{cases} \quad (2.31)$$

where t_c is the inactivation time. Since all cells would not be expected to die at the same time, values of t_c would follow some sort of distribution. The Weibull distribution is used in engineering to model time to failure, and so it seems appropriate for modeling bacterial inactivation. The distribution of t_c would then follow the probability density function (PDF) for the Weibull (solid line in Figure 2.8):

$$PDF = \frac{\beta}{\alpha} \left(\frac{t}{\alpha}\right)^{\beta-1} e^{-\left(\frac{t}{\alpha}\right)^\beta} \quad (2.32)$$

where α and β are parameters relating to the scale and shape of the distribution, respectively.¹¹⁸ The survival curve is then the cumulative distribution function (CDF) (dotted line in Figure 2.8):



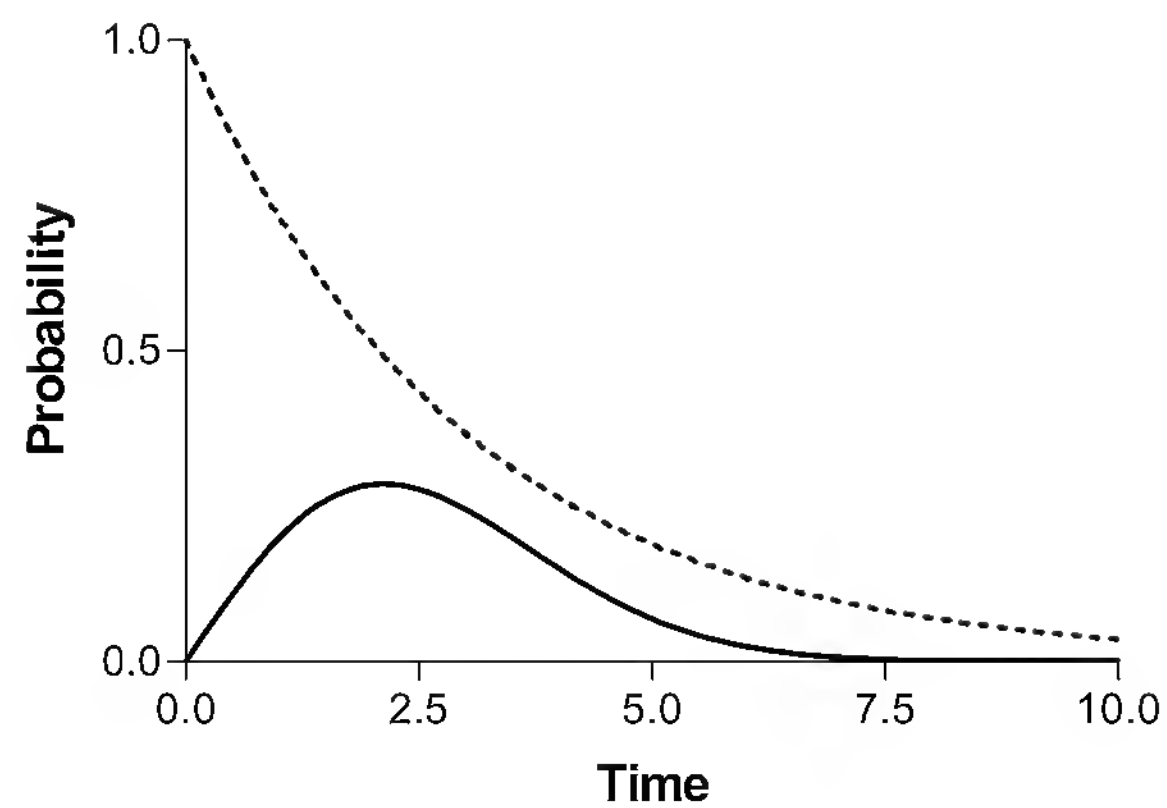
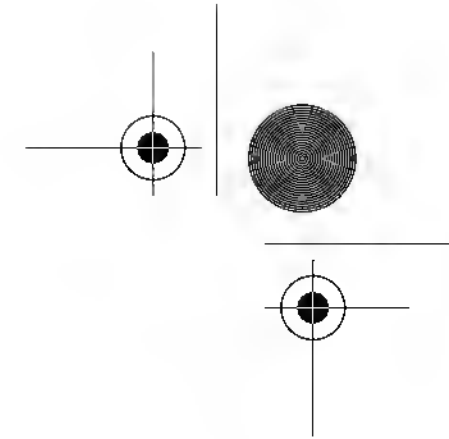
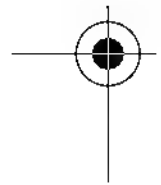


FIGURE 2.8 Probability density (solid line) and cumulative probability distribution (broken line) for the Weibull distribution.

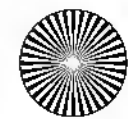
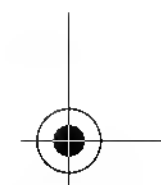
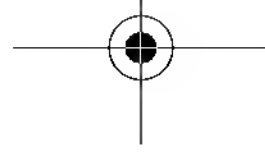
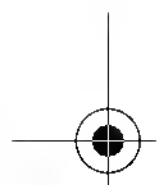
$$CDF = e^{-\left(\frac{t}{\alpha}\right)^{\beta}} \quad (2.33)$$

It can be easily seen that the CDF of the Weibull distribution is essentially a reparameterization of the power law function (Equation 2.25). In the same fashion, the Fermi equation described earlier is the CDF of a log normal PDF.^{21,86}

The Weibull parameter (β) has a very distinct influence on the shape of the survivor curve. When $\beta < 1$, a concave survival curve is obtained, and when $\beta > 1$, the curve is convex. Interestingly, the simple exponential model described earlier is a special case of the Weibull distribution when $\beta = 1$, providing further support for the use of the Weibull distribution as an effective modeling approach for microbial survival. Further, the value of β can have some implications for possible mechanisms of inactivation. When $\beta < 1$, there is an indication that the remaining cells are more resistant to the treatment, while when $\beta > 1$, an accumulation of the lethal effect is observed resulting in increasing rate of destruction with time. The classical D -value from linear survival curves can be related to the 90% percentile of the CDF, which is the time (t_d) required to reduce the number of microorganisms by a factor of 10^{118} :

$$t_d = \alpha(2.303)^{\frac{1}{\beta}} \quad (2.34)$$

There have been a number of recent applications of the Weibull distribution to model survival curves for species of *Bacillus* and *Clostridium* spp.,⁹⁸ *Salmonella*,^{119,120} and *E. coli*.¹²¹ Van Boekel¹¹⁸ has fitted the Weibull distribution to a large number of survival curves obtained from the literature. In almost all cases, the β values were different from 1, indicating that the classical linear model may not be generally applicable. Temperature had a significant effect on the α but not the β parameter. In order to determine if the Weibull distribution is appropriate for a



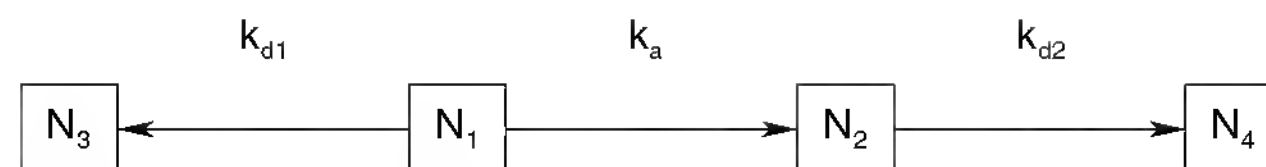
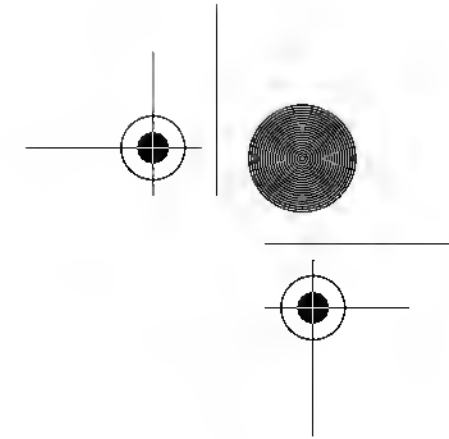
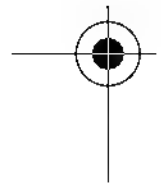


FIGURE 2.9 Model for spore activation and survival.

particular survival curve, a so-called hazard plot^{118,121} of $\ln(-\ln S)$ vs. $\ln t$ should give a straight line. It should also be noted that, when survival curves are modeled using distributions, the presence of a “shoulder” can be attributed to the spread of a distribution being small relative to its mean or mode.¹²²

2.2.5 SPORES

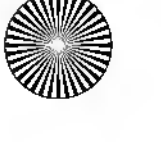
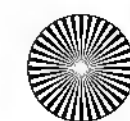
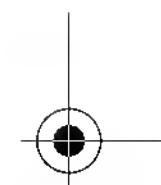
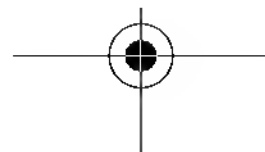
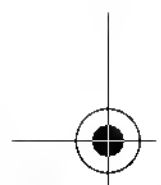
Modeling the inactivation of bacterial spores presents a unique problem. Spore-forming bacteria such as *Bacillus* and *Clostridium* spp. can exist in a dormant (spore) stage that is highly heat resistant. Germination of spores can be achieved by treatment with sublethal heat.¹²³ Because of the extreme heat resistance of some of these microorganisms, activated spore preparations have traditionally been used to establish sterilization protocols in the canning and ultrahigh temperature industries.¹²⁴ As described earlier, the classical view of microbial thermal inactivation ascribes a first-order reaction to the process; however, it has been difficult to consistently achieve simple exponential inactivation with spore preparations. These variations manifest themselves as a shoulder on the decay curve, which has been attributed to activation of spores, and subsequent differences in the heat resistance of dormant and activated spores.¹²⁵ Consistent populations of activated spores are difficult to obtain; thus the shoulder is often ignored, and *D*-values are calculated from the linear portion of the decay curve.

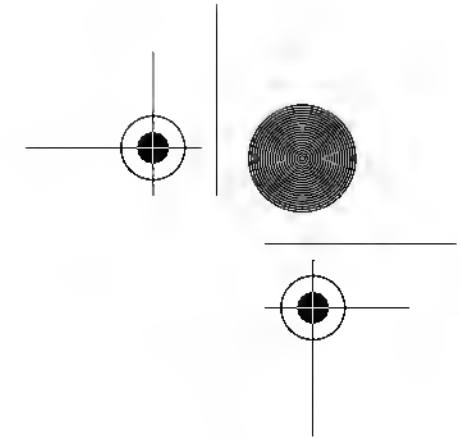
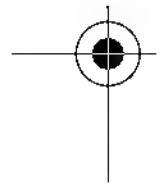
More sophisticated models have been developed to account for the nonlinear aspects of survival curves. These include terms describing the germination of spores prior to inactivation (for descriptions of earlier models, see).^{124–126} Figure 2.9 indicates the process of activation of dormant spores (N_1) into activated (N_2) spores with rate constant of k_a . The activated spores are then inactivated by heat treatment (N_3) at a rate equal to k_{d2} . The model also allows for possible inactivation of dormant spores (N_3) at a rate equal to k_{d1} . All reactions are considered to be independent first-order. The simplest form of this model was described by Shull et al.,¹²⁷ and assumes that only activated spores can be killed ($k_{d1} = 0$) and thus:

$$\frac{dN_1}{dt} = -k_a N_1 \quad (2.35)$$

$$\frac{dN_2}{dt} = k_a N_1 - k_{d2} N_2 \quad (2.36)$$

The model proposed by Rodriguez et al.^{128,129} advances the Shull model by assuming that the dormant spores can also be inactivated:





$$\frac{dN_1}{dt} = -(k_{d2} + k_a)N_1 \quad (2.37)$$

and $k_{d2} = k_{d1}$. Sapru et al.¹²⁴ further extended the model to include a different rate of inactivation for dormant spores ($k_{d1} \neq k_{d2}$). The Sapru model is more general and includes the Shull and Rodriguez models as special cases. This model was proposed for use with *Bacillus stercorophilus* at sterilization temperatures, and an explicit form has been presented¹²⁵:

$$N_1(t) = N_1(0)e^{-(k_a+k_{d1})t} \quad (2.38)$$

$$N_2(t) = N_2(0)e^{-k_{d2}t} + B \cdot N_1(0)(1 - e^{-At})e^{-k_{d2}t} \quad (2.39)$$

with

$$B = \frac{k}{A} \quad (2.40)$$

$$A = k_a + k_{d1} - k_{d2} \quad (2.41)$$

and where $N_1(0)$ and $N_2(0)$ are the number of dormant and activated cells, respectively, at $t = 0$. An example output from the Sapru model is shown in Figure 2.10. With $N_1(0)$ at 1×10^8 and $N_2(0)$ at 1×10^5 , the initial rapid increase in surviving cells is the result of spore activation. This is followed by an exponential decrease

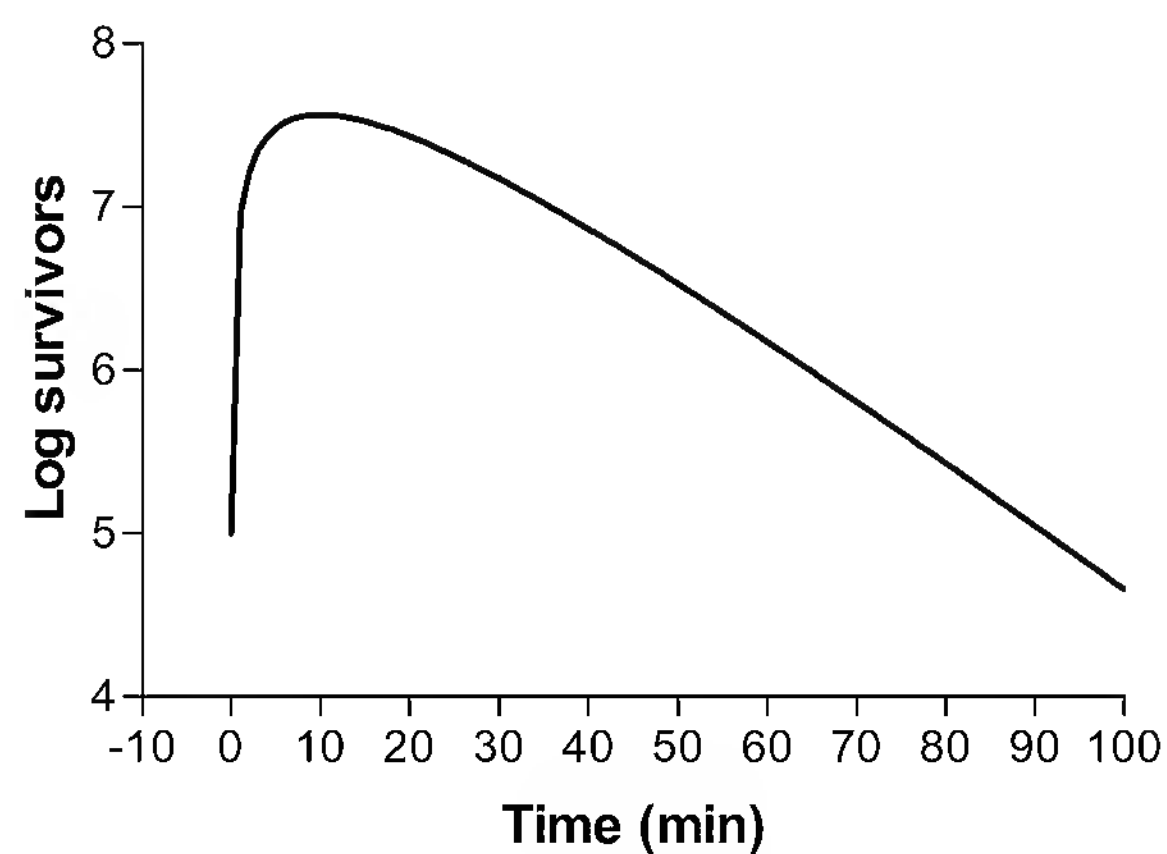
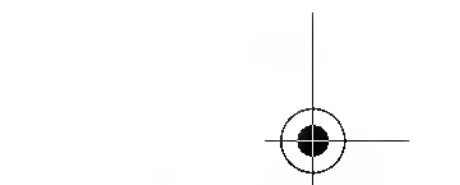
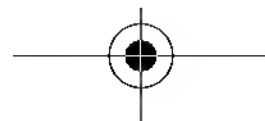
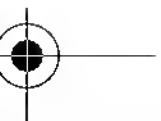
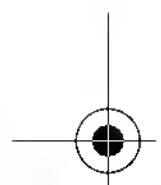
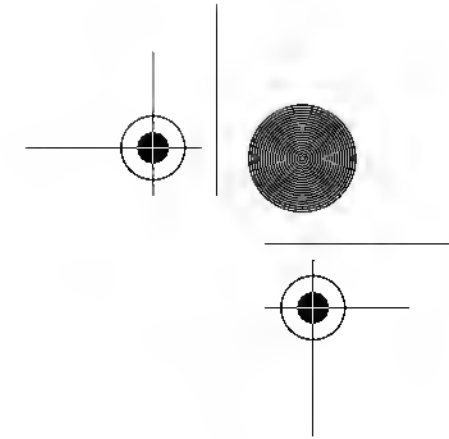
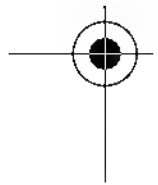


FIGURE 2.10 Output of model for spore activation and survival. (From McKellar, R.C., Modelling the effectiveness of pasteurization, in *Dairy Processing: Maximizing Quality*, Smit, G., Ed., CRC Press Inc./Woodhead Publishing, 2003 pp. 104–129. With permission.)





in surviving cells. This model has been expanded further to include subpopulations of spores having different heat resistances.¹³⁰

2.2.6 PROCESSING MODELS

2.2.6.1 Thermal

Thermal inactivation of microorganisms in static or batch systems is usually described by the D - and z -value concepts as discussed above, with temperature generally held constant. The situation in canning operations or continuous flow systems such as HTST pasteurization, sterilization, and ultrahigh temperature processes is somewhat more complex, due to nonisothermal conditions. In addition, the kinetics of inactivation in continuous systems differs from that in batch systems since in these systems there are additional factors such as pressure and shear forces that can influence microbial survival.¹³¹ As most modern processes are continuous, it is necessary to have information on survival of microorganisms; however, few studies have been published in which laboratory or pilot plant continuous flow systems have been studied.¹³¹

In order to deal with nonisothermal conditions, Bigelow's¹³² model has been the standard for the low-acid canned food industry for many decades. In this approach, the processing time F is determined by integrating the exposure time at various temperatures, $T[t]$, to time at a reference temperature, T_{Ref} ¹³³:

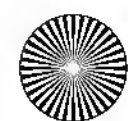
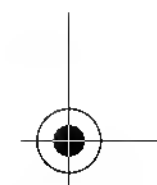
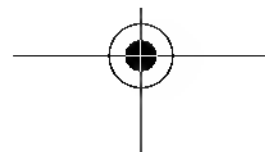
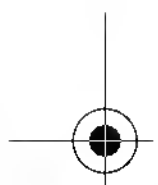
$$F = \int 10^{\frac{T(t)-T_{\text{Ref}}}{z}} dt \quad (2.42)$$

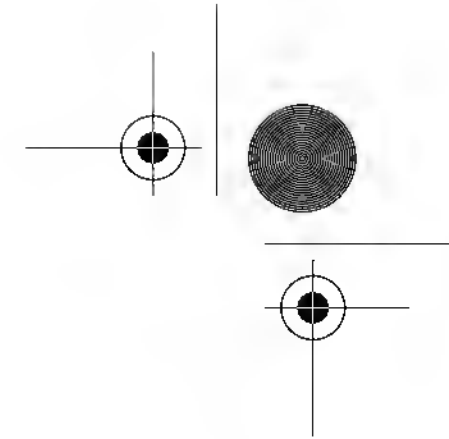
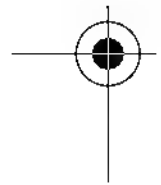
This model is considered to be an approximation of the Arrhenius model, which is valid over a wide range (4 to 160°C) of temperatures¹³³:

$$PE = \frac{1}{t_0} \int_0^t e^{-\left(\frac{E_a}{R}\right)\left(\frac{1}{T} - \frac{1}{T_0}\right)} dt \quad (2.43)$$

where PE = integrated lethal effect, or pasteurization effect; E_a = energy of activation, J mol⁻¹; R = 8.314 J mol⁻¹ K⁻¹; T = temperature, K; T_0 = reference temperature, 345 K; t = time, s; t_0 = reference time, 15 s. The reference temperature (345 K or 72°C) and time (15 s) correspond to the International Dairy Federation standard for pasteurization.¹³⁴

It is often necessary for food processors to demonstrate that the process they wish to use is effective in delivering the required lethal effect for the product and microorganism of concern. The integrated lethal effect is a useful concept, as it allows two or more processes that use different time/temperature combinations to be compared for efficacy against food-borne pathogens; however, there are few data available relating microbial survival to processing conditions. This is of particular concern in the case of pasteurization of milk, where the only accepted test for proper





pasteurization is the alkaline phosphatase (AP) test. The relationship between AP inactivation and survival of food-borne pathogens is largely unknown, as is the response of AP to processing under alternative time/temperature combinations. Thus, modeling of HTST pasteurization of milk was studied extensively by McKellar and coworkers.

The residence times in each section of a pilot-scale HTST pasteurizer and in each of six holding tubes with nominal holding times of 3 to 60 s were calibrated using the standard salt test. Temperatures were taken at the beginning and end of each section using thermocouples. The PE could then be determined for each selected holding time/temperature combination using Equation 2.43. Raw milk at a constant flow rate was allowed to equilibrate at each time/temperature, and a sample was taken at the outflow for analysis. Residual enzyme activity or microbial survivors were matched with the corresponding PE for fitting.⁹⁷

The fitting was accomplished using an iterative procedure in which the $\log_{10}\%$ initial activity or viable counts were regressed on PE, with the value of E_a/R varied to minimize the error sum of squares. Nonlinearity (generally concavity) in the data was modeled using a power transformation (Equation 2.25). The final model was of the form⁹⁷:

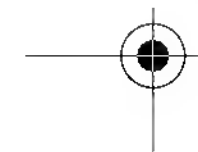
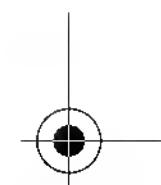
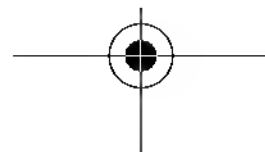
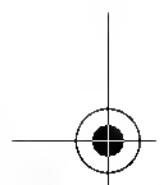
$$\log_{10} \% \text{ initial activity} = a + b \cdot PE^c \quad (2.44)$$

where a = intercept, b = slope, and c = power. Generally, the parameter estimates for at least three trials were pooled, and the model for AP is shown in Table 2.5.

There is also a need to develop models for milk enzymes that might be used to confirm processing at temperatures above or below pasteurization. Higher temperatures ($\geq 75^\circ\text{C}$) are appropriate for processing of more viscous products (such as ice-cream mix), while temperatures below pasteurization (63 to 65°C ; termed subpasteurization or thermization temperatures) are used to extend the storage life of bulk

TABLE 2.5
Model Parameters for Inactivation of Various Milk Enzymes and Food-Borne Pathogens during High-Temperature Short-Time Pasteurization

Target	Trials	Intercept	Slope	Power	E_a/R ($\times 1000$)
Alkaline phosphatase	3	2.05	-4.05	0.50	66.5
γ -Glutamyl transpeptidase	3	2.00	-0.281	0.75	66.5
Lactoperoxidase	3	2.12	-0.10	0.75	59.0
Catalase	3	1.94	-2.65	0.50	82.0
α -L-Fucosidase	3	1.87	-17.6	1.00	39.8
<i>Listeria innocua</i>	5	1.86	-24.9	0.80	59.5
<i>Listeria monocytogenes</i>	3	1.68	-18.4	0.80	48.5
<i>Enterobacter sakazakii</i>	3	2.31	-24.4	0.65	59.5



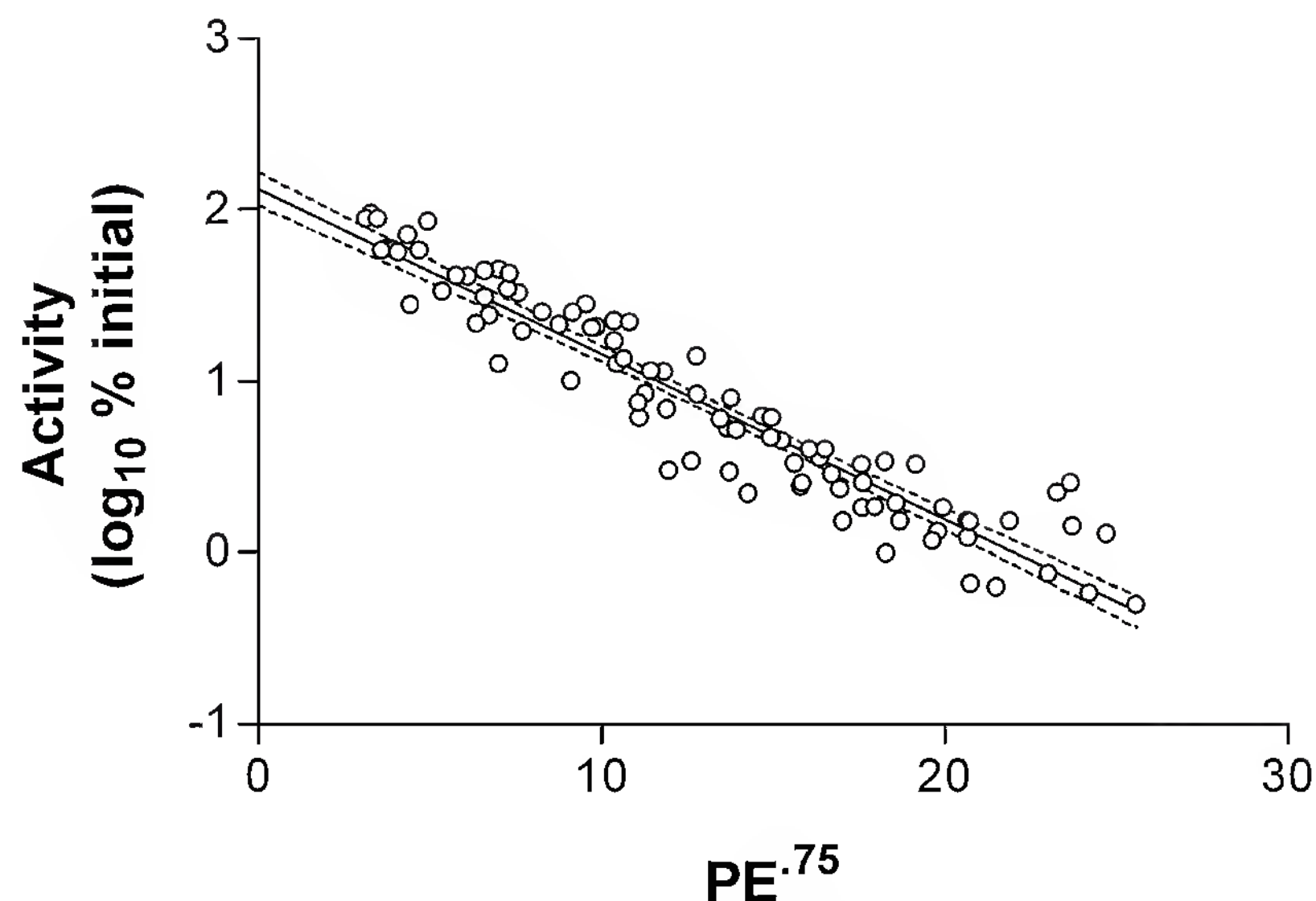
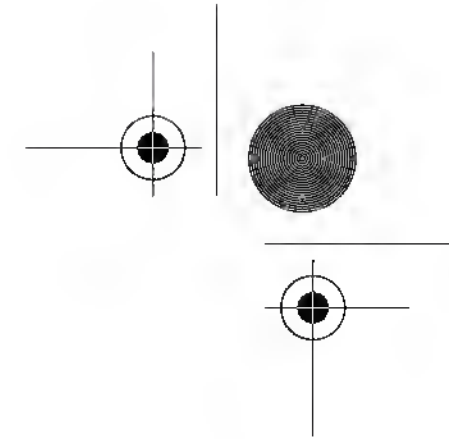
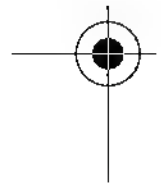
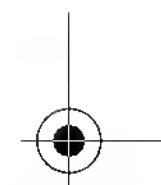
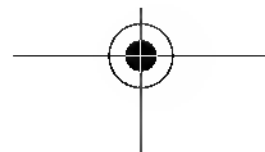
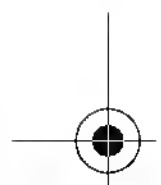


FIGURE 2.11 Linear model relating pasteurization effect (PE) and residual activity of lactoperoxidase during high-temperature short-time (HTST) pasteurization of bovine milk.

milk. Lactoperoxidase (LP) and γ -glutamyl transpeptidase (TP) are two naturally occurring milk enzymes that are inactivated at higher temperatures.¹³⁵ Model parameters for these two enzymes are given in Table 2.5. An example of an inactivation curve for LP is given in Figure 2.11, with the dotted lines representing the 95% confidence limits. There is close agreement among the three trials plotted, a characteristic common for all enzyme models. Models have also been developed for catalase¹⁰⁴ and α -L-fucosidase (FC),¹³⁶ which are appropriate for subpasteurization temperatures (Table 2.5).

Survival models for several food-borne pathogens have also been derived. *Listeria innocua*, a nonpathogen, is often used as a substitute for *L. monocytogenes* in situations (such as food processing environments) where it would be undesirable to introduce pathogens.¹³⁷ A model developed for *L. innocua* (Table 2.5) was shown to underpredict inactivation of *L. monocytogenes*; thus predictions are “fail-safe.”¹³⁸ *Enterococcus faecium*, a nonpathogen, is also used as a model organism for pathogens, particularly in Europe.¹³⁹ The inactivation curve for this microorganism deviated strongly from linearity, and there were large intertrial variations. Thus, a random coefficient model using Equation 2.28 was used to fit the data.⁹⁶ The average $\ln D$ -values for the two populations were 0.825 and 2.856. Models were also generated for *Enterobacter sakazakii*, an “emerging” pathogen found contaminating infant formula.¹⁴⁰ Model parameters compared with those for *L. monocytogenes* (Table 2.5) showed that *E. sakazakii* was more sensitive to pasteurization.

Linear models for milk enzymes were characterized by limited intertrial variability (Figure 2.11). This allowed validation of models using data from other trials that were not used in the construction of the models. In contrast, considerable variation was noted in experiments with microorganisms; thus a different approach



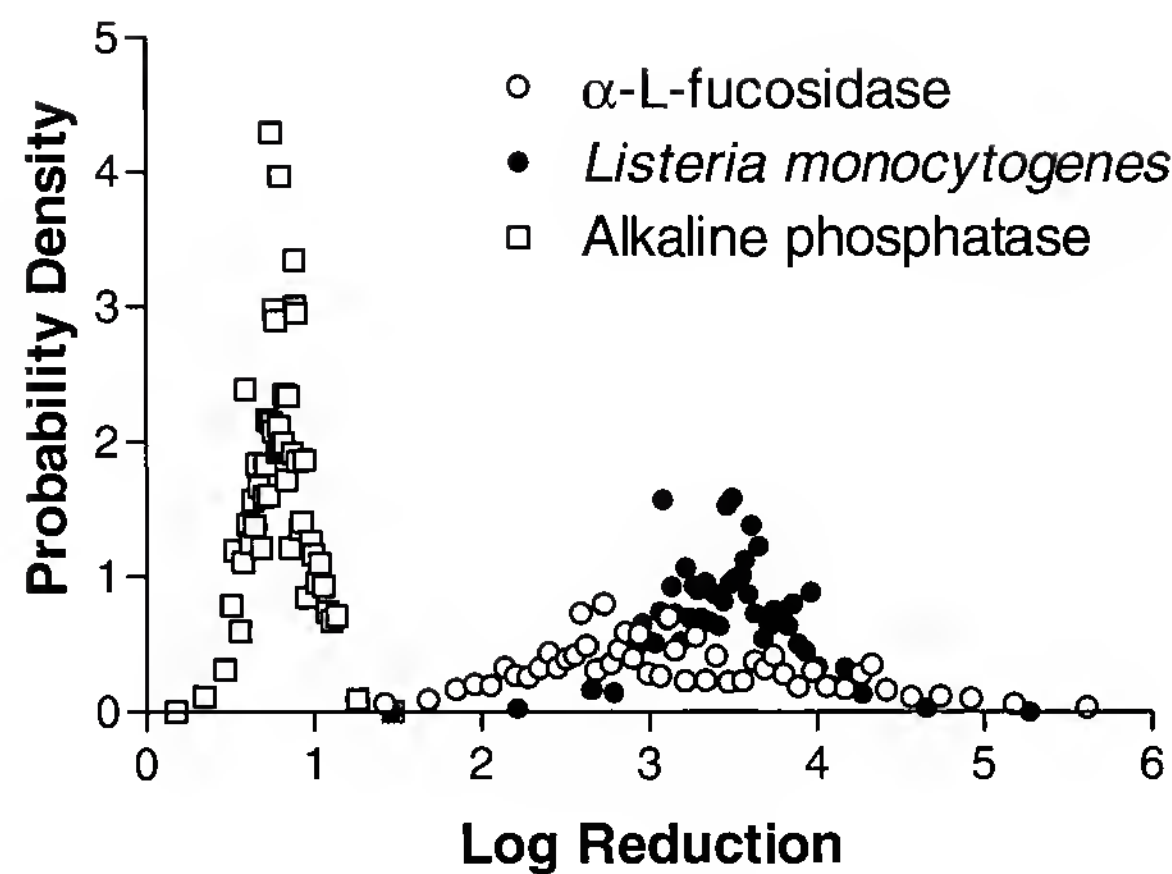
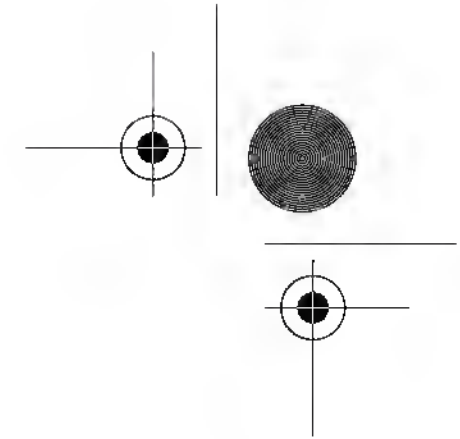


FIGURE 2.12 Probability densities for α -L-fucosidase, *Listeria monocytogenes*, and alkaline phosphatase generated from linear models for high-temperature short-time (HTST) pasteurization using Analytica®, with holding temperature and time of 66°C for 16 s.

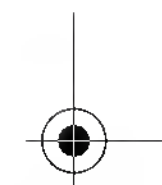
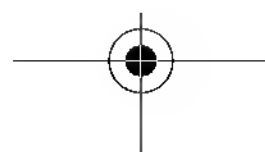
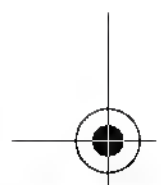
was taken. Model parameters were incorporated into risk analysis software (@RISK, Palisade Corporation, Newfield, NY) as normal distributions, with means taken from Table 2.5 and standard deviations taken from the intertrial variations. When simulations were performed (1500 iterations), outcomes (log reduction in this case) were expressed as distributions.

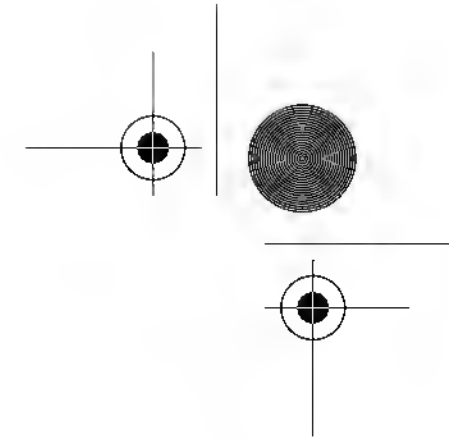
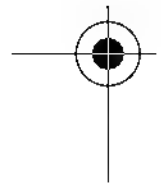
Simulated log reductions were generated for AP, FC, and *L. monocytogenes* using a holding time of 65°C/15 s (thermization), and the probability density functions are shown in Figure 2.12. These conditions resulted in a narrow band of probabilities for AP, with greater predicted range for both FC and *L. monocytogenes*. AP is not completely inactivated, while FC (a potential indicator of thermization) experiences a >2 log reduction in most iterations. The mean log reduction of *L. monocytogenes* under these conditions is >3.

Models that can predict the probability of achieving a desired level of safety are an important addition to risk assessment models, which are still largely qualitative and based primarily on expert opinion (see Chapter 6 for a more complete discussion on expert systems). The pasteurization models described above have been incorporated into the risk analysis software Analytica® (Lumina Decision Systems, Los Gatos, CA), a commonly used software for building risk assessment models for the food industry. These models are now being incorporated into the USDA's Pathogen Modeling Program (available from <http://www.arserrc.gov/mfs/pathogen.htm>).

2.2.6.2 Alternative Technologies

Thermal treatment has been the traditional method for processing of many foods; however, with the increased consumer demand for fresh, less processed foods, new technologies have evolved. Some of these are based on temperature, such as microwave, radio frequency (RF), and ohmic heating, while others depend on other forms of microbial inactivation, such as high pressure (HP), pulse electric field (PEF),





pulsed or ultraviolet light, and ultrasound. In 1998, the U.S. Food and Drug Administration commissioned the Institute of Food Technologists to provide scientific review and analysis of issues in food safety, food processing, and human health. The first of these reports, entitled “Kinetics of Microbial Inactivation for Alternative Food Processing Technologies,” was released in 2000¹⁴¹ and is available at the following web site: <http://vm.cfsan.fda.gov/~comm/ift-toc.html>. Since this report comprehensively reviews the scientific literature and makes recommendations for future research, it is beyond the scope of this chapter to reproduce this body of work. Instead, several key areas will be highlighted.

Many novel thermal technologies base their antimicrobial effect on temperature; thus inactivation of microorganisms can be modeled using the traditional calculations for D -value and z -value (see earlier). Processes that depend on other mechanisms of inactivation such as HP and PEF require modified equations with different parameters. For example, HP effects on microbial population can be modeled using a function similar to the traditional D -value¹⁴²:

$$\log\left(\frac{D}{D_R}\right) = -\frac{(P - P_R)}{z_R} \quad (2.45)$$

where D_R = the decimal reduction time at a reference pressure P_R and z_R is the pressure required for a 1-log reduction in D -value. An alternative model has been proposed by Weemaes et al.¹⁴³:

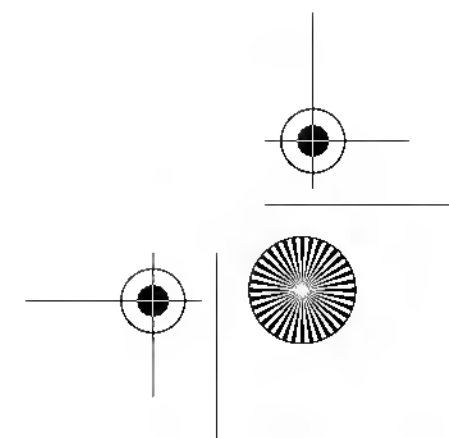
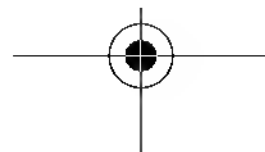
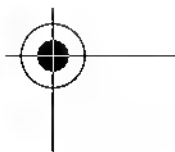
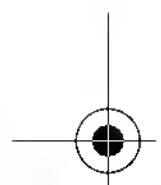
$$\ln(k) = \ln(k_R) - \left(\frac{V(P - P_R)}{RT_A}\right) \quad (2.46)$$

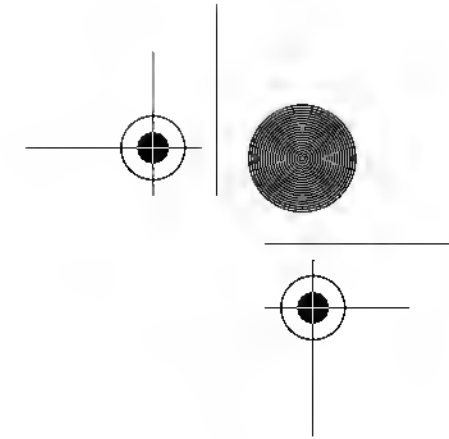
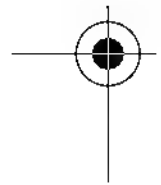
where k_R is the reaction rate constant, and P_R the reference pressure, V the activation volume constant, P the pressure, and T_A the absolute temperature. With PEF processing, a model describing the influence of the electric field intensity on reduction of microbial population can be described, which is similar to those used for thermal and pressure processing:

$$\log\left(\frac{D}{D_R}\right) = -\frac{(E - E_R)}{z_E} \quad (2.47)$$

where D_R is the decimal reduction time at a reference field intensity E_R , and the electric field coefficient z_E is the increase in the electric field intensity E required to reduce the D -value by 1-log. An alternative model based on the Fermi equation was proposed by Peleg¹⁴⁴:

$$\frac{N}{N_0} = \frac{1}{1 + e^{\frac{E - E_d}{K}}} \quad (2.48)$$





where E_d is the electric field intensity when the microbial population has been reduced to 50%, and K is a coefficient based on the slope of the survivor curve. A similar model was proposed by Hülshager et al.¹⁴⁵:

$$\frac{N}{N_0} = \left(\frac{t}{t_c} \right)^{-\frac{E-E_c}{K}} \quad (2.49)$$

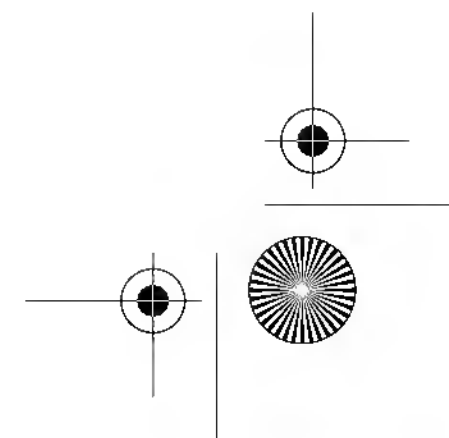
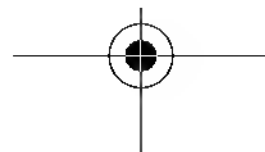
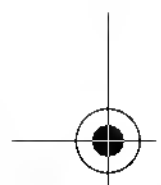
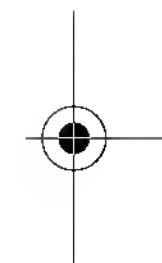
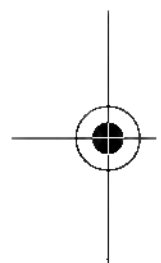
where t is the treatment time, t_c the minimum treatment time for inactivation, E_c the minimum field strength for inactivation, and K is a specific rate constant. This function is similar to Equation 2.48 except that it also accounts for exposure time at a given electric field intensity.

The Institute of Food Technologies report has raised a number of relevant issues that would benefit from some discussion here. Kinetic parameters for microbial populations exposed to thermal treatment are well documented and provide a good basis upon which to develop models for alternative thermal processes. The nonthermal models described above assume that microbial inactivation is a first-order reaction; however, as mentioned earlier, there is little direct evidence supporting this view. It will be necessary to further evaluate the adequacy of linear survival models, and to hopefully develop a universal model applicable to both thermal and non-thermal processes. In addition, experimental protocols have been found to be inadequate to provide statistically reliable parameters for microbial reduction resulting from exposure to alternative technologies. This is particularly a problem with high pressure processing, where data are needed at different pressures with control of temperature and product. The inactivation mechanism for thermal destruction of microbes is generally well known, and evidence for additional independent mechanisms with processes such as ohmic heating is still lacking. Further work is needed to elucidate the mechanism of inhibition with alternative treatments such as PEF and HP, and to assess possible synergistic effects between alternative technologies and temperature.

2.2.7 INJURY/REPAIR MODELS

Almost without exception, available models for microbial growth and death have been developed using fully viable, unstressed cells; thus the resulting models represent the idealized scenario. It is well known that bacterial cells exposed to some form of sublethal stress require an adaptation or recovery period prior to growth; however, mathematical models do not incorporate the influence of stress. This was emphasized in a study designed to model the evolution of a log phase in *L. monocytogenes*, induced by acid, alkaline, and osmotic shocks.¹⁴⁶ When lag-phase cells (which are more sensitive to environmental stress than stationary-phase cells) were exposed to changes in pH or increased levels of NaCl, the subsequent generation times predicted by commercially available software were shorter than the observed experimental generation times.

The physiological events that account for microbial injury and repair are poorly understood; thus there have been very few attempts to apply mathematical models



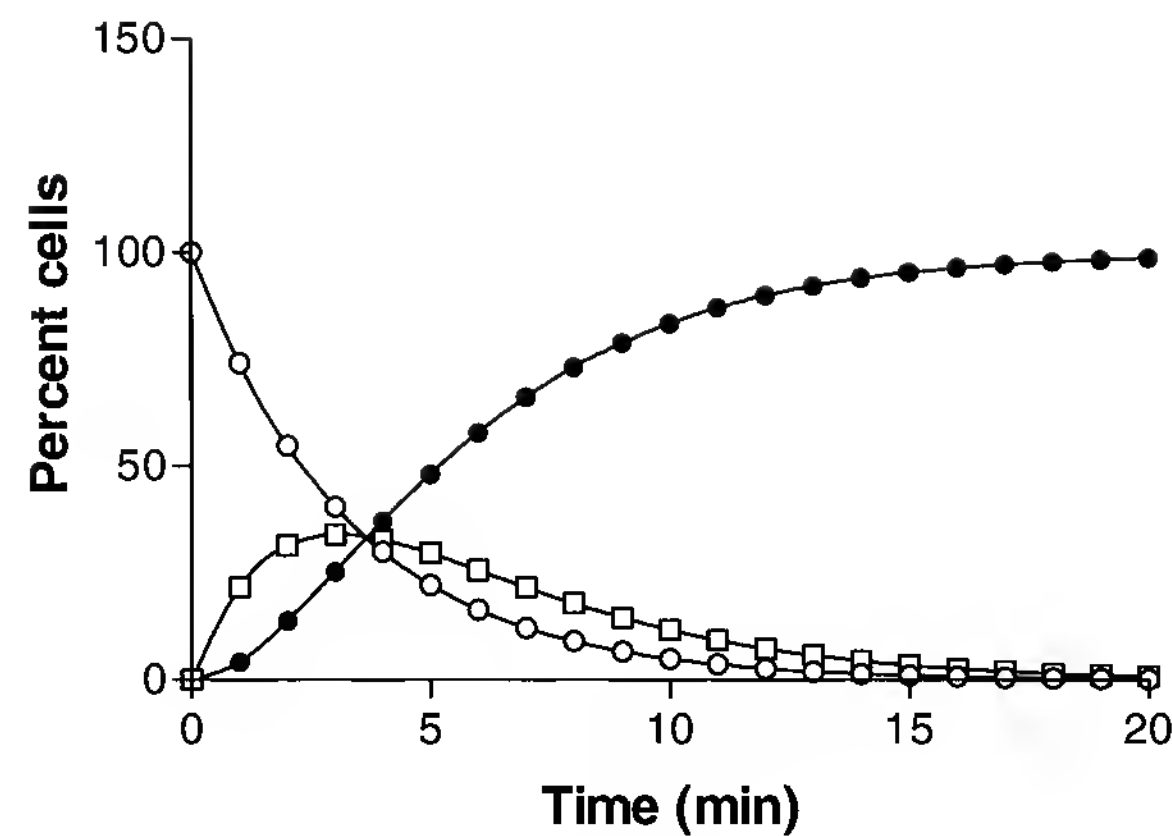
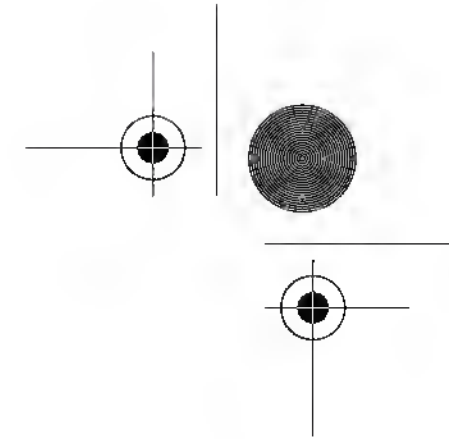
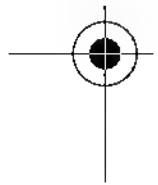


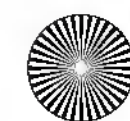
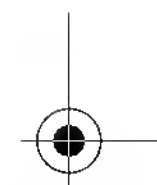
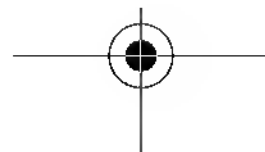
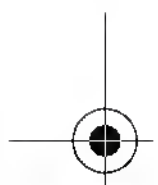
FIGURE 2.13 Change in populations of uninjured (○), injured (□), and dead (●) cells during sublethal heating according to Equation 2.50.

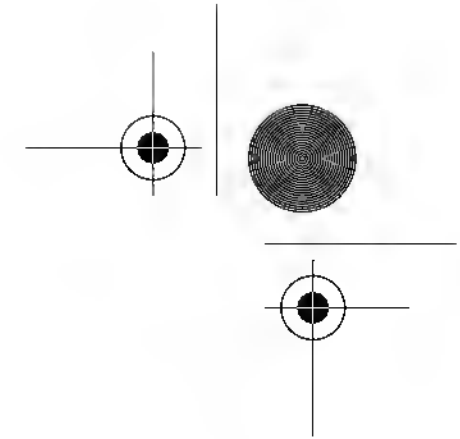
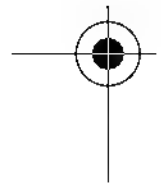
to the phenomena of bacterial cell injury and resuscitation. The models that do exist are of two general types: those that aim to quantitate the extent of injury with increased exposure to stress, and those that attempt to predict the time required for repair and recovery of viability.

Several attempts have been made to model the extension of the lag phase in response to stress. A model to describe the relationship between lag prior to growth and stress duration was proposed by Bréand et al.¹⁴⁷ This model was developed to reflect the observation that the lag increased with increasing stress duration, and then decreased to a minimum lag at longer stress times. The empirical model described the influence of stress on the lag with a linear function, followed by a logistic decrease. Cheroutre-Vialette and Lebert¹⁴⁸ proposed the use of a recurrent neural network to model the changes in lag phase and growth rate experienced by *L. monocytogenes* exposed to osmotic and pH shock. Lambert and van der Ouderaa¹⁴⁹ compared the relative ability of the Bioscreen (see Chapter 1) and viable counts to quantitate the inactivation of microorganisms by disinfection. They proposed a simple first-order inactivation reaction with accumulation of injured cells prior to complete loss of viability:



where A_1 are the uninjured cells, A_2 the injured cells, and P are the dead cells. k_1 and k_2 are rate constants for injury and death, respectively. Populations of viable, injured, and dead cells were simulated based on the data of Lambert and van der Ouderaa,¹⁴⁹ and are shown in Figure 2.13. These responses were confirmed using image analysis of colony sizes on agar plates; viable and injured cells could be distinguished on the basis of size. Colony size was also used to quantitate cells of *L. monocytogenes* that had been injured by exposure to heat or starvation.¹⁵⁰ The colony size distribution was normal for uninjured cells, but demonstrated a right-





hand skew with injured cells. Percent sublethal injury could be related to colony area using a linear function. More recent studies using the flow cytometer to measure the distribution of the lag times of individual cells of *Lactobacillus plantarum* also showed a deviation from normality with heat-treated cells. The extreme value distribution was found to be the best function for fitting both injured and uninjured cells:

$$F(x) = 1 - \exp\left(-\exp\left[\frac{x-a}{b}\right]\right) \quad (2.51)$$

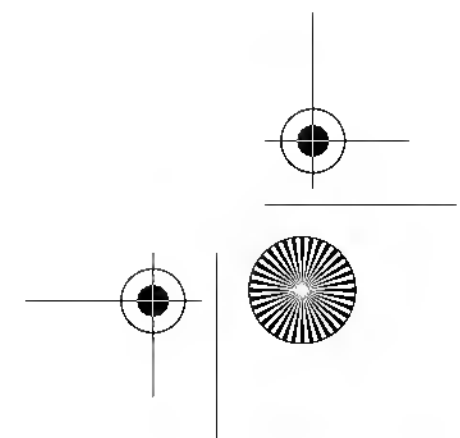
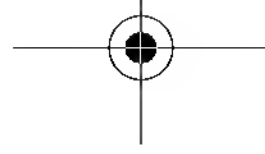
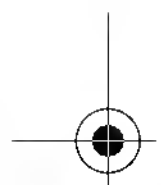
where a and b are unknown parameters.

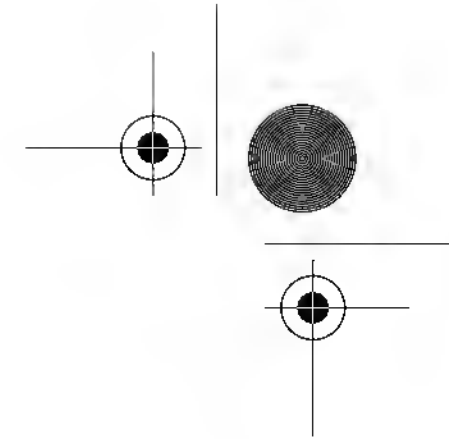
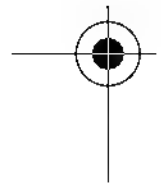
There are few studies that aim to model the recovery of cells from injury. Injured cells can be differentiated on the basis of increased sensitivity to selective media (e.g., 5% NaCl), and it is thus possible to develop models to predict the time required for cells to repair damage due to stress. This process is complicated by our lack of information on the true nature of injury in bacterial cells, and the mechanism by which cells recover. The two-compartment kinetic model developed by Hills and Mackey to describe bacterial growth⁴ was revised and extended to account for cell injury and resuscitation.⁵ In the revised model, there are rate constants for injury (R_i) and resuscitation (R_r), and parameters to describe the decrease (a) and increase (b) of the injury and resuscitation curves.⁵ This model was used to fit data from the resuscitation of *L. monocytogenes* after exposure to sublethal heat.¹⁵¹ It was shown that resuscitation could best be described with a reduced model with the parameter for increasing rate of recovery (b_r) eliminated. A quadratic regression model was subsequently derived that expressed the lag as a function of temperature and the initial number of injured cells.¹⁵¹

2.2.8 COMBINED GROWTH/DEATH MODELS

There have been a limited number of attempts to combine growth and death functions into single models. These are often simply combinations of functions such as the Gompertz or logistic with their mirror images. For example, a two-term model describing the behavior of *Lactobacillus* spp. during the ripening of fermented sausage incorporated a Gompertz function for both growth and death.¹⁵² In a similar fashion, the logistic function and its mirror image, the Fermi equation, have been combined.^{21,101} The latter model has been expanded to include a proposed distribution of cell resistances to stress, resulting in a death model that varies in shape.²¹ The Baranyi model for growth was also combined with its mirror image to describe growth and death for *Brochothrix thermosphacta*.¹¹⁵ In this model, a smoothing function was included to account for the transition between growth and death phases.

Other combined functions have used simple exponential growth and decline.^{68,153,154} In one of these models,⁶⁸ the lag phase preceding growth was handled by a first-order step that represented spore germination, repair, or adaptation. Jones et al.¹⁵³ described the adaptation of cells to growth as a transition between cells in two states, immature and mature. This model reduced to a simple balance between growth and death, with variations in division and mortality rates being described by empirical functions.¹⁵³

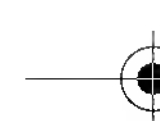
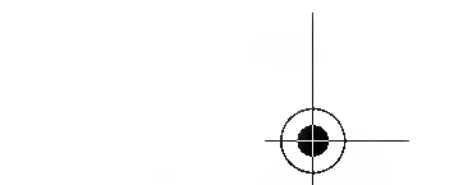
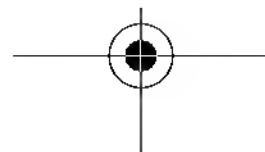
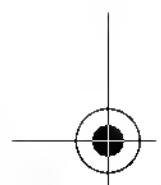
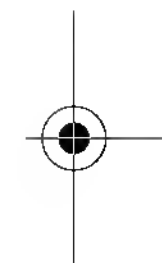
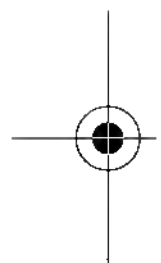


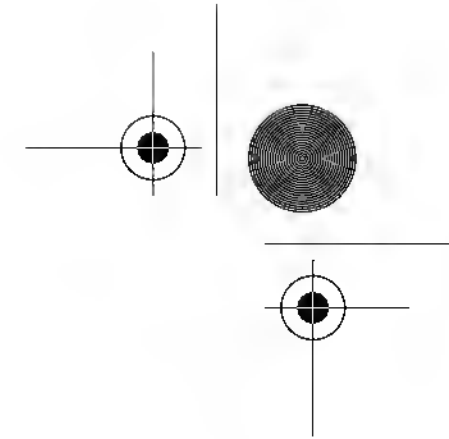
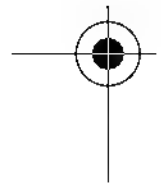


It is questionable if expressing death as a mirror image of growth is valid. There is little direct evidence that the lag phases preceding growth or death are due to similar physiological phenomena, although a convincing theoretical argument has been offered in support of this hypothesis.⁶⁵ It seems likely, however, that the stationary phase of growth and the “tailing” phase of inactivation are the result of different physiological processes. Models that address growth and death as different processes, and attempt to describe the response of microbes to their environment in terms of transitions between states, would seem to be the most useful for future development.^{68,153}

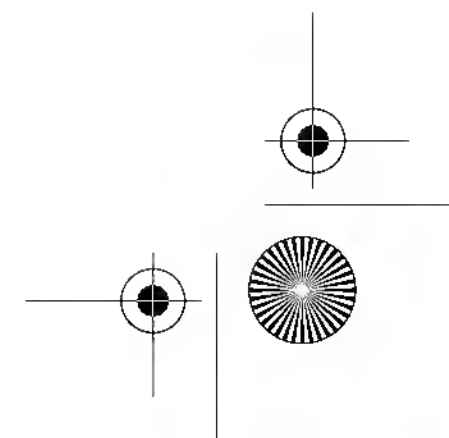
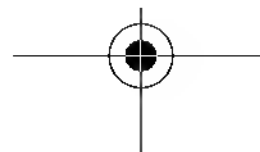
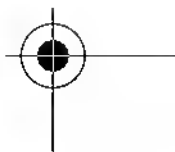
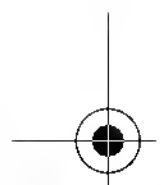
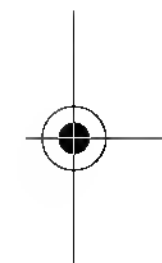
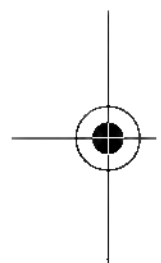
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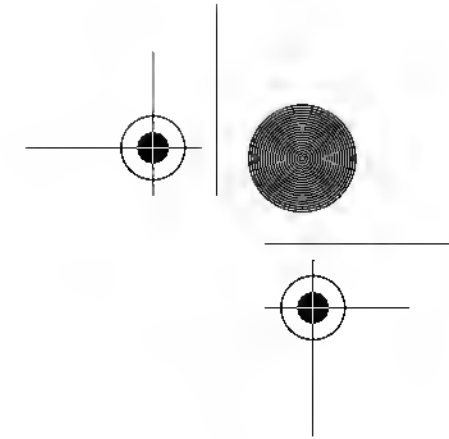
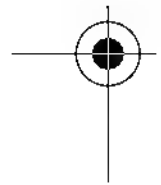
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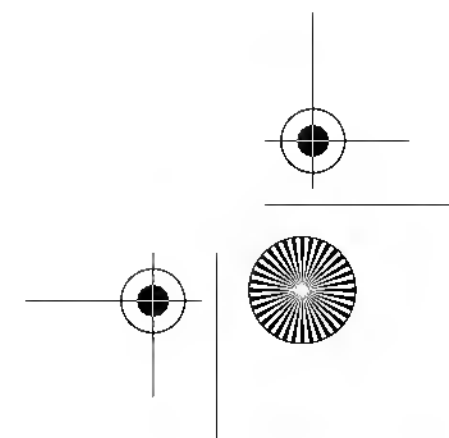
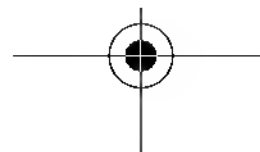
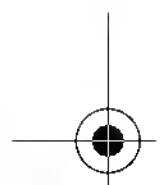
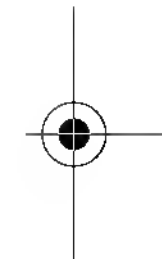
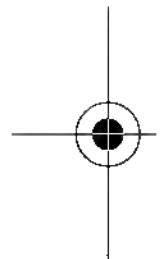


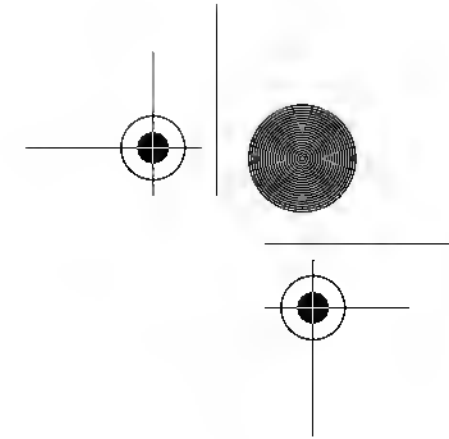
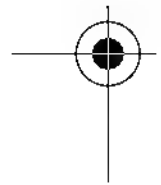
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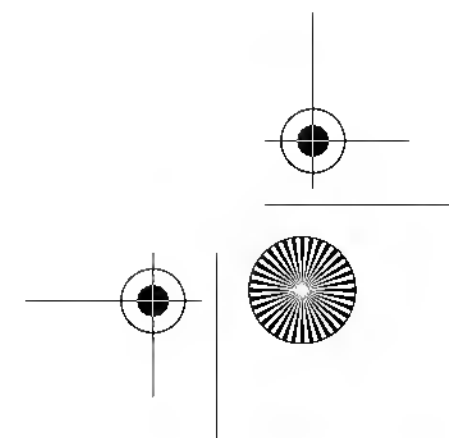
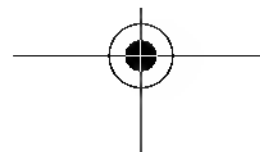
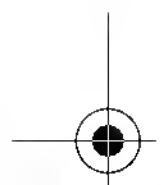
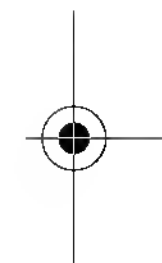
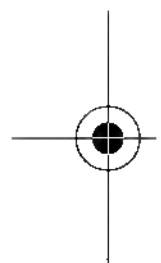


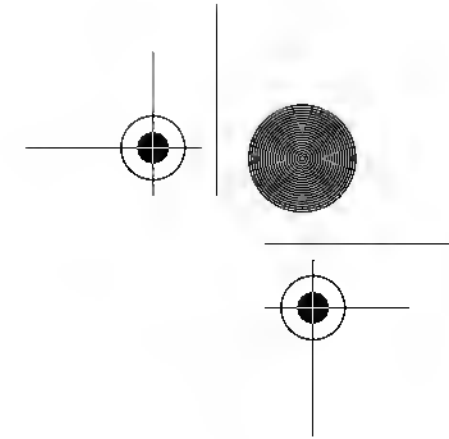
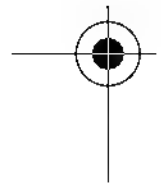
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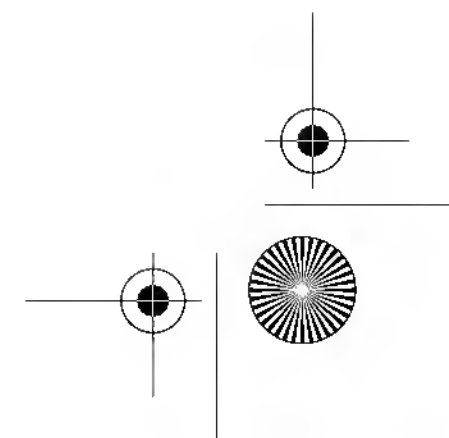
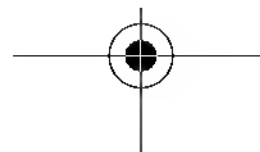
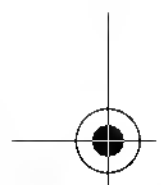
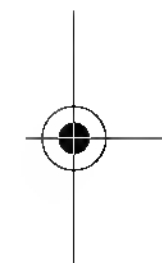
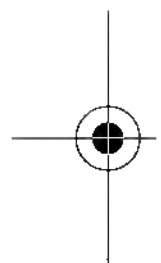


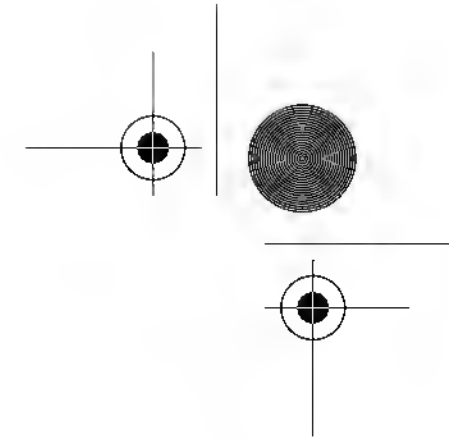
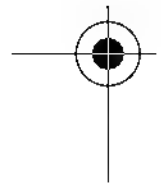
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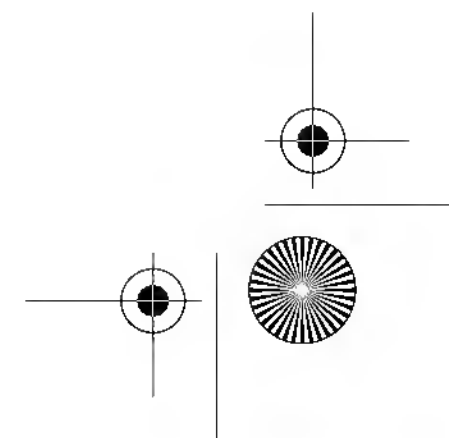
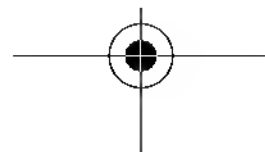
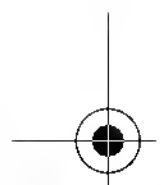
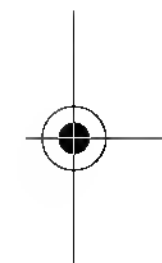
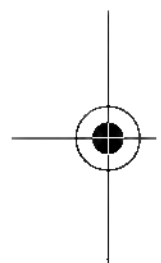


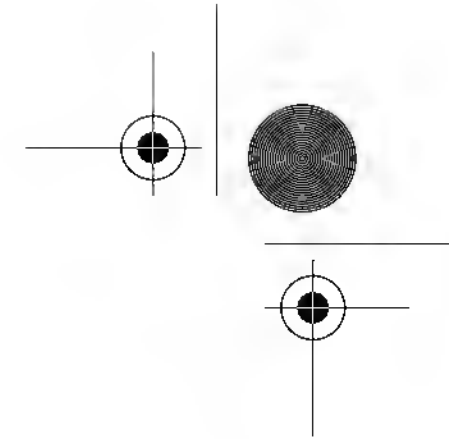
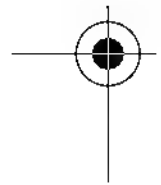
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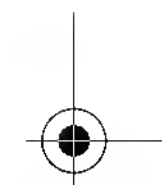
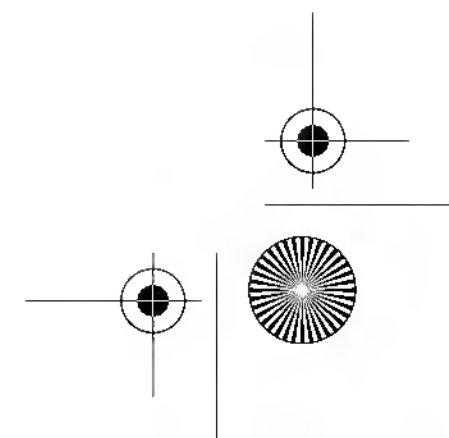
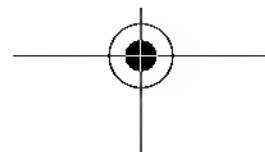
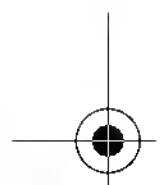
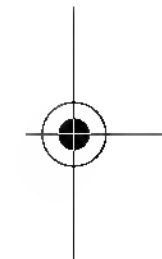
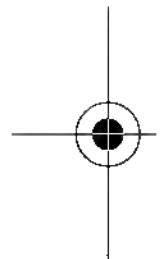


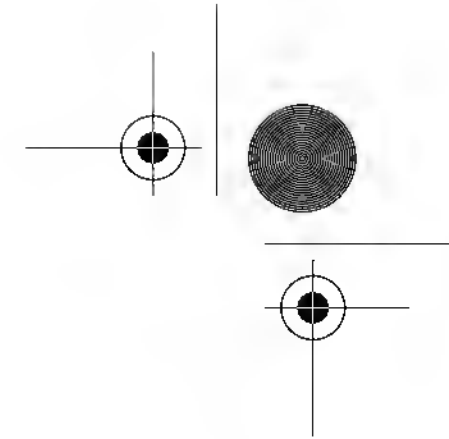
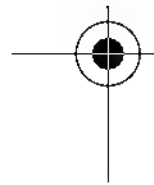
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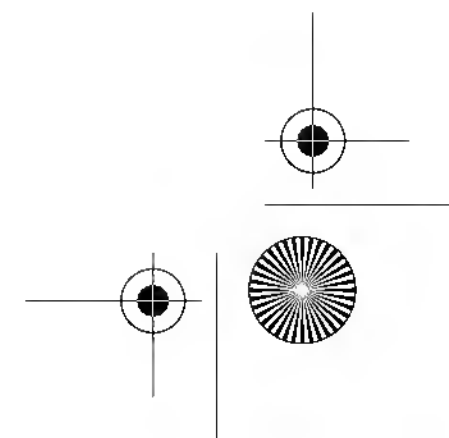
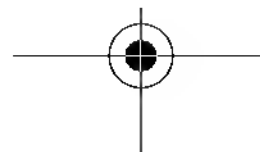
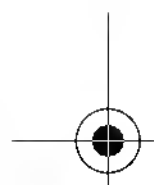
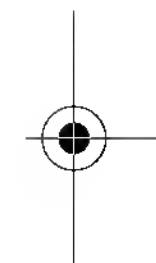
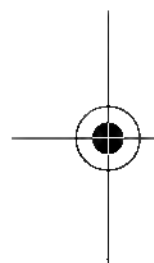


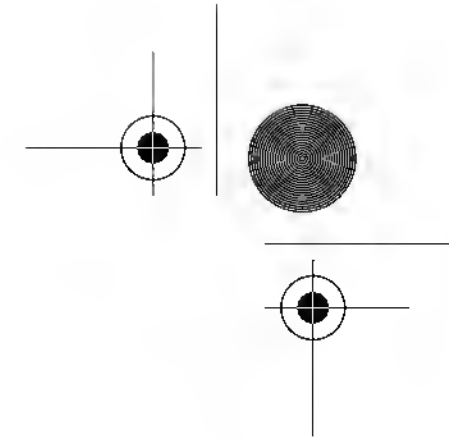
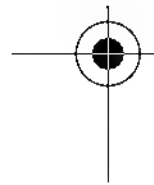
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