Journal of Applied Bacteriology

Edited by

D.E. Stewart-Tull, G.I. Barrow
and R.G. Board

Volume 72, 1992

The bactericidal activity of a methyl and propyl parabens combination: isothermal and non-isothermal studies

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D. GILLILAND, A. LI WAN PO AND E. SCOTT. 1992. The effect of temperature on the kill rate of *Escherichia coli* by methyl and propyl parabens was studied. The kill kinetics was first order. It was shown that the Arrhenius equation provided a good model for describing the relationship between the first order rate constant and the temperature. The activation energy was found to be 274 kJ/mol for exponential phase cells and 168 kJ/mol for stationary phase cells. Exponential phase cells were much more susceptible to the lethal effects of the parabens than were the stationary phase cells. For example, at 34°C stationary phase cells, in chemically defined media, had a kill rate constant of 0·072/h while the corresponding value for exponential phase cells was 0·238/h. In water the rate of kill for exponential phase cells was even faster giving a rate constant of 5·25/h at 34°C. Non-isothermal kinetic testing was not found to be useful for modelling bacterial kill kinetics because we could not achieve the precision required in bacterial enumeration.

INTRODUCTION

In the presence of a bactericidal antimicrobial agent the rate of kill of microbial cells generally increases as the temperature increases (Lynn & Hugo 1983). The effect of temperature is often expressed in terms of a temperature coefficient (Pflug 1972), usually measured as the change in rate constant over a 10°C increase in temperature and referred to as the Q_{10}. Those compounds with high temperature coefficients exhibit greater increases in activity with increasing temperature. However, the temperature coefficient value tends to vary over the temperature range studied, with decreasing values at higher temperature ranges (Karabit *et al.* 1985). The Q_{10} values also vary from one organism to another for the same antimicrobial agent (Karabit *et al.* 1986).

Both isothermal and non-isothermal methods were used to ascertain the effect of temperature on bacterial kill rates.

THEORY

Under standardized conditions bacterial death is often exponential as described by eqn. (1)

$$N = N_0 e^{-k_1 t} \qquad (1)$$

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where N is the number of organisms surviving at time t, N_0 is the initial number of organisms and k_1 is the rate constant describing the kill.

One of the major factors affecting the kill rate of bacteria is the temperature. To describe the effect of temperature many authors have used the Arrhenius equation (eqn. (2))

$$k = A e^{-E_a/(RT)} \qquad (2)$$

where k is the rate constant, R is the gas constant, T is the temperature in degrees Kelvin, A is the pre-exponential constant and E_a is the activation energy.

To test the validity of the Arrhenius equation, adherence of the kill curve to an appropriate rate equation is first established. Most commonly, the first order model (eqn. (1)) is appropriate. If validated, experimental verification of the Arrhenius equation then involves calculation of appropriate rate constants (k) at a number of different temperatures (T) and their statistical evaluation. Generally, the linear form of eqn. (2) is used and adherence to the model is shown by a linear relationship between $\log_e$ (k) and $1/T$.

Many authors have shown that when the range of temperature is wide, marked deviations from linearity are observed when $\log_e(k)$ and $1/T$ are plotted. Models put forward to represent the rate constant–temperature relationship in such cases have included the square root model (Ratkowsky *et al.* 1983) and the Schoolfield model (Schoolfield *et al.* 1981) represented by eqns. (3) and (4), respectively.

$$\sqrt{k} = b(T - T_{min})\{1 - e^{[c(T - T_{max})]}\} \qquad (3)$$

where T is the temperature in degrees Kelvin, T_{min} is the theoretical minimum temperature for growth, T_{max} is the theoretical maximum temperature for growth, b and c are constants and k is the rate constant.

$$\log_e k = A + B/T - \log_e T$$
$$+ \log_e [1 + e^{(F+D/T)} + e^{(G+H/T)}] \qquad (4)$$

where A, B, D, F, G and H are constants, k is the rate constant and T is the temperature in degrees Kelvin.

Data for testing temperature–kill rate relationships are collected as described above (eqns (2)–(4)) and each kill curve is recorded at a constant temperature. Such an experimental set-up is referred to as isothermal testing. An approach which has gained some measure of acceptance in the stability testing of pharmaceutical products is that of non-isothermal testing. In this case temperature is varied continuously during the experiment so that, theoretically, temperature effects on kill rates can be derived from one single experiment. The logic behind the method is as follows.

Suppose that from an experiment, carried out at constant temperature, the kill curve adheres to eqn (1). A plot of $\log_e (N)$ or $\log_e (N/N_0)$ against time will be linear. If we now increase the temperature during the experimental run, the line will usually curve down as shown in Figs. 1a and 1b. Starting the experiment below the optimum temperature for growth and increasing the temperature during the experiment will slow down the observed kill rate if the temperature effect on growth rate exceeds that on the preservative-induced kill rate. The two effects will counteract each other. In that case the kill plot will show an upward curve.

A curve such as that shown in Fig. 1b can usually be satisfactorily modelled by a low order polynomial equation which can be written as

$$\log_e(N) = f(t) = a_0 + a_1 t + a_2 t^2 + \cdots \qquad (5)$$

The derivative of this equation gives the rate constant k at the prevailing temperature T at time t

$$d [\log_e (N)]/dt = k_1 = a_1 + 2a_2 t + \cdots \qquad (6)$$

Therefore, provided we have the temperature at time t, the corresponding rate constant can be calculated. The appropriate calculations can be easily done using both standard statistical computer packages and non-isothermal specific programs (Li Wan Po *et al.* 1983). More detailed descriptions of non-isothermal stability testing methodology are given elsewhere (Hempenstall *et al.* 1983).

MATERIALS AND METHODS

Preparation of media

The chemically defined medium contained (g/l): Na_2HPO_4, 11·45; KH_2PO_4, 1·4025; $(NH_4)_2 SO_4$, 1·87; $MgSO_4$, 0·187; D-glucose, 0·909; $CaCl_2$ $(2H_2O)$, $1·245 \times 10^{-5}$; $FeSO_4(7H_2O)$, 5×10^{-7}. The pH was adjusted to 6·9 with dilute HCl. All chemicals were of analytical reagent quality.

Preparation of the inoculum of exponential phase cells

Escherichia coli NCIB 8545 was maintained on Tryptone Soya Agar (Oxoid) slopes at 4°C. A loopful of the organism was added to 100 ml of sterile media and grown overnight at 37°C in a shaking waterbath at 100 rev/min. Transfers of organism were made daily for 2 d. On the third transfer the organisms were allowed to grow to an optical density reading of 0·1 at 540 nm (Corning colorimeter 254). This provided cells in the exponential phase of growth. The absorbance value of 0·1 at 540 nm was found to be approximately equal to 1×10^8 cfu/ml. An inoculum of 1×10^9 cfu/ml was prepared by filtering the culture (100 ml), under aseptic conditions, through a 0·45 μm membrane and washing with 100 ml of fresh, pre-warmed media. The organisms were then resuspended in 10 ml of media to give the final inoculum.

Preparation of the inoculum of stationary phase cells

The same procedure was carried out for the preparation of exponential phase cells except that instead of preparing a third transfer the overnight cells were used to prepare the
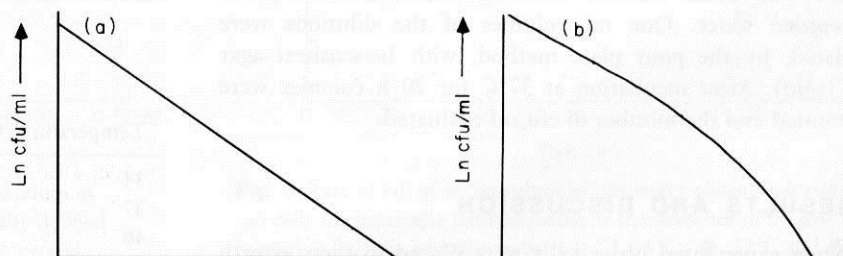

Fig. 1 Theoretical first order kill curve for micro-organisms at (a) constant temperature and (b) when the temperature is increased continuously throughout the

inoculum. The culture was filtered, under aseptic conditions, through a 0·45 μm membrane filter and washed with 100 ml of pre-warmed media. The organisms were then resuspended in 10 ml of media and the density adjusted so that a 1 : 10 dilution gave an absorbance reading, at 540 nm, of approximately 0·1.

Preparation of test solutions

The appropriate weight of the methyl and propyl esters of *p*–hydroxybenzoic acid (Sigma) were added to 1 l of medium, or water, and placed in a sonic bath for up to 4 h to aid solubilization. The solution was filter-sterilized and 100 ml of test solution dispensed into 250 ml flasks. Media without any parabens were employed as controls. Before inoculation, the test solutions were maintained at their respective test temperatures in a shaking waterbath for at least 18 h.

Test procedure

In the experiments involving kill at various constant temperatures the test solutions were maintained at each temperature in separate shaking waterbaths (Grant SS40-D). In experiments involving a gradual increase in temperature over time the test solution was maintained in a Grant W14 waterbath connected to a Grant temperature programmer PZ1. Since the Grant W14 waterbath is not a shaking waterbath, small sterile, magnetic teflon-coated fleas were included in the test solutions and were stirred on an Inspin 2 (Baird and Tatlock). The temperature programmer was set to increase in temperature at the rate of 1°C/h, commencing at 34°C and rising to 42°C. This was closely monitored by a built-in thermometer and the inclusion of a thermometer in a control flask which was present in the waterbath.

Measurement of microbial numbers

After inoculation of the test solutions viable counts of microbial numbers were made at regular intervals. At each time interval a 1 ml sample was removed from the test solution and serial 10-fold dilutions were made in 0·1% peptone water. One ml volumes of the dilutions were plated, by the pour plate method, with Isosensitest agar (Oxoid). After incubation at 37°C for 20 h colonies were counted and the number of cfu/ml evaluated.

RESULTS AND DISCUSSION

When exponential phase cells were placed in their growth medium the growth could be satisfactorily described by eqn
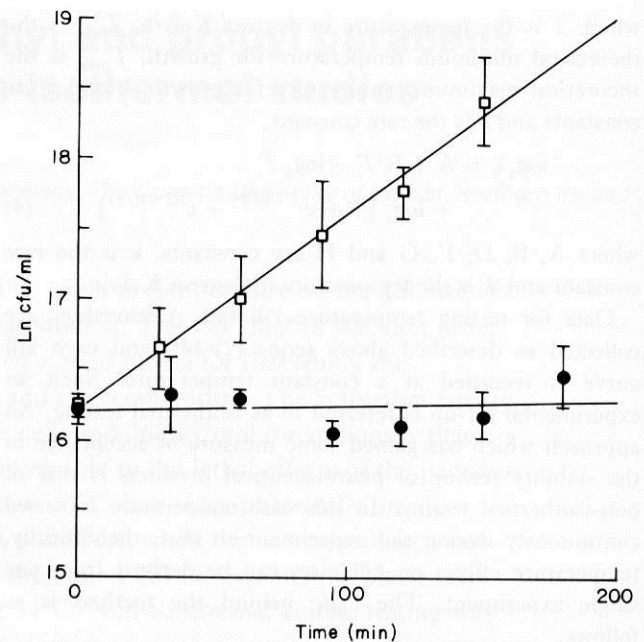

Fig. 2 Comparison of the rate of growth of exponential phase *Escherichia coli* cells in □, chemically defined media and ●, water. (Error bars are s.e. for 3 days' results.)

(1) and a positive rate constant was obtained (Fig. 2). As expected, when the growth medium was substituted with water, no growth took place (Fig. 2). With 0·12% w/v methyl paraben and 0·012% w/v propyl paraben added to the growth medium bacterial kill was observed.

The kill curve could be satisfactorily modelled by first order kinetics (Fig. 3) as shown by the linear semi-logarithmic plot of the number of surviving organisms against time. Figure 3 also shows that the rate of kill increased as the temperature increased. Table 1 lists the kill rate constants corresponding to the four different temperatures studied.

Table 1 The effect of temperature on the kill rate constants for inocula prepared from exponential and stationary phase *Escherichia coli* cells in chemically defined media in the presence of 0·12% w/v methyl paraben and 0·012% w/v propyl paraben

Temperature(°C)	Rate constant/h (mean ±s.e.)	
	Exponential phase cells	Stationary phase cells
34	−0·243 ± 0·026	−0·072 ± 0·016
37	−0·510 ± 0·008	−0·141 ± 0·027
40	−1·546 ± 0·173	−0·243 ± 0·026
42	−3·442 ± 0·097	

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