Growth Kinetics of *Streptococcus thermophilus* at Subbacteriostatic Penicillin G Concentrations

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ABSTRACT

Streptococcus thermophilus may be subjected to the effects of penicillin G in contaminated milk used for yogurt production. Sensitivity of this microorganism to penicillin G has been conventionally determined by the help of penicillin G-impregnated disks placed on solid media. It was observed that the bacteriostatic penicillin G concentration was much greater in liquid media than in solid media. The conventional disk method may not be appropriate for antibiotic sensitivity determinations if the microorganisms will be used in liquid culture.

A simple mathematical model simulated the growth of *S. thermophilus* in liquid culture. Numerical values of this model's parameters were regarded as the measure of the antibiotic effect on the culture. In penicillin G containing fresh medium, small concentrations of antibiotic decreased the specific growth rate considerably. Increasing the antibiotic concentration caused only slight additional decline. Antibiotic shock, i.e., rapidly introducing penicillin G into an actively growing antibiotic-free culture, stopped growth of the penicillin G-resistant microorganisms, and no death was observed, but a fraction of the microorganisms were killed in the wild culture. Both the wild and the resistant cultures recovered from the shock in a few hours. Addition of penicillin G-resistant microorganisms together with the antibiotic dosage into the wild culture prevented death.

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INTRODUCTION

Penicillin G is often used for treatment of dairy animals; therefore, it can sometimes find its way into milk (7). *Streptococcus thermophilus* is a microorganism of yogurt starter cultures. This microorganism may be subjected to the effect of penicillin G in tainted milk during yogurt production. Sensitivity of *S. thermophilus* to penicillin G was determined previously using solid media (9, 11). In these studies, penicillin G-impregnated disks, placed on the surface of solid media, were incubated and penicillin G diffused into the media. Its concentration was highest near the disk and decreased with distance. Sensitivity of microorganisms to penicillin G were evaluated by considering the antibiotic content of the disk and the size of the inhibitory zone. Reinbold and Reddy (9) and Sozzi and Smiley (11) employed this classical approach and reported that *S. thermophilus* was sensitive to penicillin G.

Only bacteriostatic antibiotic concentrations can be determined conveniently on the solid media. Minimum antibiotic concentration, which is required to prevent growth totally, is referred to as the bacteriostatic antibiotic concentration. Bacteriostatic concentration may not be the same in solid and liquid media of similar composition. Antibiotic concentrations that are lower than the bacteriostatic concentration are referred to as the subbacteriostatic concentrations. Microbial growth may be initiated in liquid media at subbacteriostatic antibiotic concentrations; however, under such a condition, the growth rate might be lowered.

Penicillin G inhibits microorganisms by interfering with the cell wall synthesis (4). It causes lysis and death of gram-positive bacteria (1). Under balanced growth conditions, cell walls are synthesized at the same rate as microbial growth rate. When penicillin G is introduced into a culture its effect will depend on the growth phase and growth rate. The number

of cell wall-synthesizing microorganisms is higher in an actively growing culture, and hence, they are expected to be more susceptible to the penicillin G effect than those growing at a lower rate.

Mathematical models of microbial growth facilitate data analysis. Quantitative measure of inhibition during growth at subbacteriostatic antibiotic concentrations can be evaluated with a mathematical model. A mathematical model can also be employed to describe quantitatively the effect of antibiotic shock on the sensitive culture.

MATERIALS AND METHODS

Streptococcus thermophilus was obtained from Etlik Veterinary Research Institute in Ankara, Turkey. Purity of the microorganisms was assured by isolating a single colony twice, by streak plating, and using these microorganisms throughout the study. Antibiotic-resistant microorganisms were selected from the pure culture using a gradient plate technique (3). Maximum antibiotic concentration was 3 mg/L in the gradient plate. Growth media contained 2% peptone (wt/vol), 1% yeast extract (wt/vol), and 2% lactose (wt/vol). Solid medium contained the same components with addition of 1.5% agar. Antibiotic shock experiments used 300-ml shake flasks with 100 ml of broth at 37°C at a shaking rate of 100 strokes/min. Rapid introduction of antibiotics into an actively growing antibiotic-free culture was referred to as antibiotic shock. Antibiotic shock was applied by rapidly adding penicillin G to a concentration of 3 or 6 mg/L. Penicillin G (Sigma Chemical Co., St. Louis, MO) had a potency of 1170 units/mg. Biomass concentration was determined by measuring optical density at 420 nm with a Model UV-120-2 spectrophotometer (Shimadzu Co., Kyoto, Japan). A calibration curve of dry biomass weight versus optical density was employed to convert optical densities into dry biomass weight (13).

MICROBIAL GROWTH MODELS

Logarithmic growth prevails when biomass concentration is small, and it is expressed as:

$$\frac{dX}{dt} = \mu_s X \qquad [1]$$

where X, t, and μ_s are biomass concentration, time, and specific growth rate, respectively. At higher biomass concentrations, growth is inhibited by product accumulation, i.e., lactic acid, or due to competition for the nutrient. The logistic equation describes the microbial growth under these conditions:

$$\frac{dX}{dt} = \mu X(1.0 - X/X_{max}) \qquad [2]$$

The parameters μ and X_{max} are the initial specific growth rate and the maximum attainable biomass concentration, respectively. The term $(1 - X/X_{max})$ introduces the inhibitory effect of overcrowding. Lactic acid accumulation is one of the consequences of overcrowding. Equation [2] predicts no growth when biomass concentration X equals X_{max}. Although the logistic equation is new to dairy science literature, it has been previously used in the biochemical engineering research in the United States (5, 6, 12) and elsewhere (8). Its solution, given by Ollis and coworkers (5, 12) is:

$$X = \frac{X_0 e^{\mu t}}{1.0 - (X_0 / X_{max})(1.0 - e^{\mu t})} \qquad [3]$$

Antibiotics inhibit microbial growth. Growth of a microbial culture that corresponds with Equation [2] may undergo a major change when the culture is subjected to an antibiotic shock. Effect of the shock might be introduced into Equation [2] as:

$$\frac{dX}{dt} = \mu\phi X(1.0 - X/X_{max}) \qquad [4]$$

The product $\mu\phi$ can be regarded as the effective specific growth rate after the shock. The parameter ϕ describes the inhibitory effect of the shock numerically. When ϕ is 1, 0, and any negative number represent no effect, complete inhibition with no apparent growth, and death of the microorganisms, respectively.

RESULTS AND DISCUSSION

Growth on Solids Media

Antibiotic-resistant microorganisms were selected from the pure culture with the gradient agar technique (3). At a maximum antibiotic concentration of 3 mg/L a few slow growing, relatively rare, resistant colonies appeared in the gradient plates. No growth was observed in the plates with higher penicillin G concentrations. This result showed that the bacteriostatic penicillin G concentration was about 3 mg/L in the solid medium.

Growth in Penicillin G Containing Liquid Media

Liquid media with 3, 6, 9, 12, and 15 mg/L of penicillin G were inoculated with 3 to 4 mg/L of wild microorganisms. Growth started immediately in all the media, indicating that bacteriostatic penicillin G concentration was greater than 15 mg/L. This was more than five times the bacteriostatic penicillin G concentration on the solid medium. Sensitivity of *S. thermophilus* to penicillin G was conventionally determined by using of penicillin G-impregnated disks on solid media (9, 11). Our results suggest that the conventional disk method may not be appropriate for antibiotic sensitivity determinations if the microorganisms will be used in liquid cultures.

At subbacteriostatic penicillin G concentrations, growth slowed but did not cease. This effect was studied by means of a kinetic model. When inoculum was large, i.e., more than 4 g/L, growth was simulated with the logistic equation (Equation 2). Numerical values of μ and initial biomass concentration X_0 were calculated from the experimental data by following the approach of Weiss and Ollis (12). Numerical values of the parameter μ are plotted versus penicillin G concentration in Figure 1. The parameter μ is the daughter cell generation frequency in the culture at the beginning of fermentation. Its value is not a function of lactic acid accumulation. Figure 1 shows that μ decreased considerably with 3 mg/L, and higher penicillin G concentrations (6 to 15 mg/L) were not more inhibitory. Thus, contamination of liquid cultures of *S. thermophilus* with even small doses of penicillin G might greatly inhibit growth.

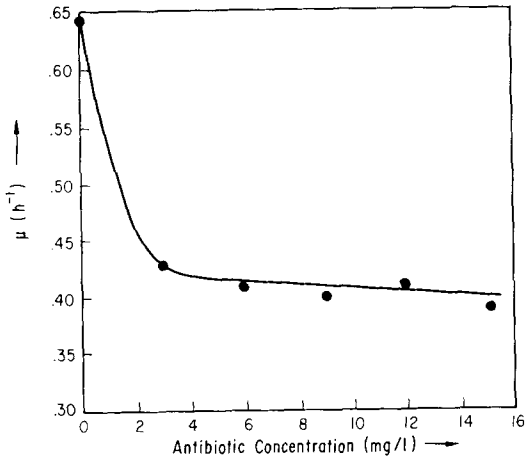

Figure 1. Variation of the initial specific growth rate of the wild microorganisms with subbacteriostatic penicillin G concentration. (Growth curves given in Figure 2.)

Numerical values of X were calculated from Equation [3] by employing previously determined values of X_0 and μ and experimental value of X_{max}. These simulations were plotted against time and compared with the experimental data as shown in Figure 2.

Growth with Penicillin G Shock

In penicillin G shock experiments, initial biomass concentrations of the antibiotic-free cultures were low, i.e., .03 g/L, and the inhibitory effect of overcrowding was negligible. Logarithmic growth prevailed (Equation [1]) with a specific growth rate (μ_s) of .83 h^{-1}. Penicillin G was introduced into these cultures at the beginning of the logistic growth phase when biomass concentrations were about 3 to 4 g/L. These biomass concentrations were close to the initial biomass concentrations in the previous experiments, i.e., those pertaining to growth in penicillin G containing liquid media. No shock was observed in earlier experiments because growth was initiated with subbacteriostatic concentrations of penicillin G while the number of the cell wall-synthesizing microorganisms was small. In the present series of experiments, penicillin G was introduced into the medium while the microorganisms were actively growing; thus, a very large number of growing microorganisms in the active cell wall

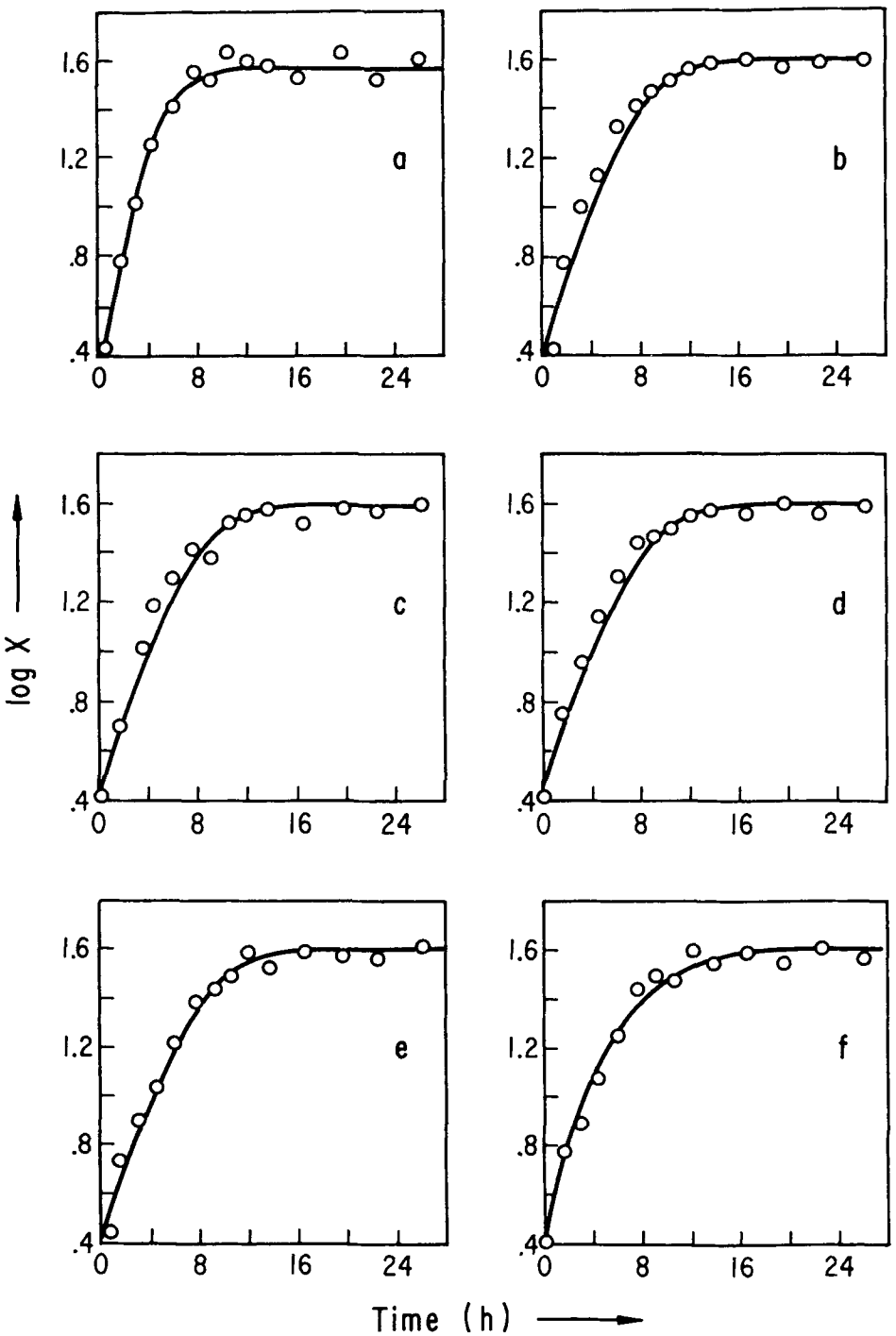

Figure 2. Comparison of the model with the data when growth was started in the media with the following penicillin G concentrations: a) 0 mg/L, b) 3 mg/L, c) 6 mg/L, d) 9 mg/L, e) 12 mg/L, and f) 15 mg/L (— simulation; o data point).

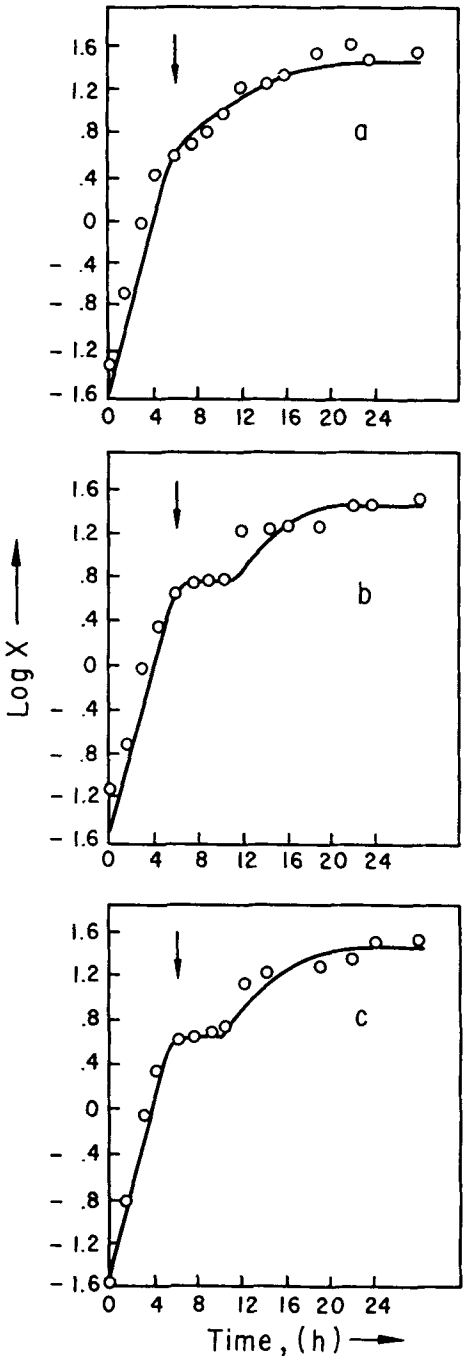

Figure 3. Comparison of the model with experimental data for growth of antibiotic-resistant microorganisms with: a) 1 ml of water addition, b) 3 mg/L antibiotic shock, and c) 6 mg/L antibiotic shock. (o data; ↓ shock; — simulation).

TABLE 1. Values of the parameter ϕ and duration of the shock after penicillin G addition to a culture of wild bacteria.

Antibiotic concentration after the shock	Antibiotic resistant microorganism concentration	ϕ	Duration of shock
(mg/L)	(%)		(h)
3	0	−.30	1.5
3	.01	0	3
3	.02	0	2
3	.03	0	5
3	.04	0	2
3	.05	0	2
6	0	−.60	4.5
6	.01	−.43	2
6	.02	−.37	1.5
6	.03	1	. . .
6	.04	0	2
6	.05	0	2

synthesis phase were subjected to the penicillin effect and antibiotic shock was observed. Indications of shock were either death of the microorganisms, as shown by the decline in the growth curves (Figures 4 and 5), or temporary cessation of the microbial growth (Figures 3, 4, and 5).

Effect of antibiotic shock on the resistant culture is shown in Figure 3. Antibiotic shock was caused by introducing small volumes of concentrated antibiotic solution into the medium. Figure 3a shows that the culture was not significantly diluted by addition of equivalent volume of water. Antibiotic shock of 3 or 6 mg/L stopped growth of the microorganisms, but no death was observed (Figure 3b,c).

In Figures 4 and 5, the effect of antibiotic shock of 3 and 6 mg/L on the wild culture is illustrated. Antibiotic shock of 6 mg/L was more effective than the shock of 3 mg/L. Addition of antibiotic-resistant microorganisms together with the shock apparently prevented death of the wild microorganisms as the concentration of the resistant microorganisms increased (Figures 4 and 5). This result indicates that resistant microorganisms might be used in liquid cultures to minimize the effect of contamination by penicillin G.

The highest antibiotic-resistant microorganism concentration was only .05% of the total microbial concentration. When death of wild

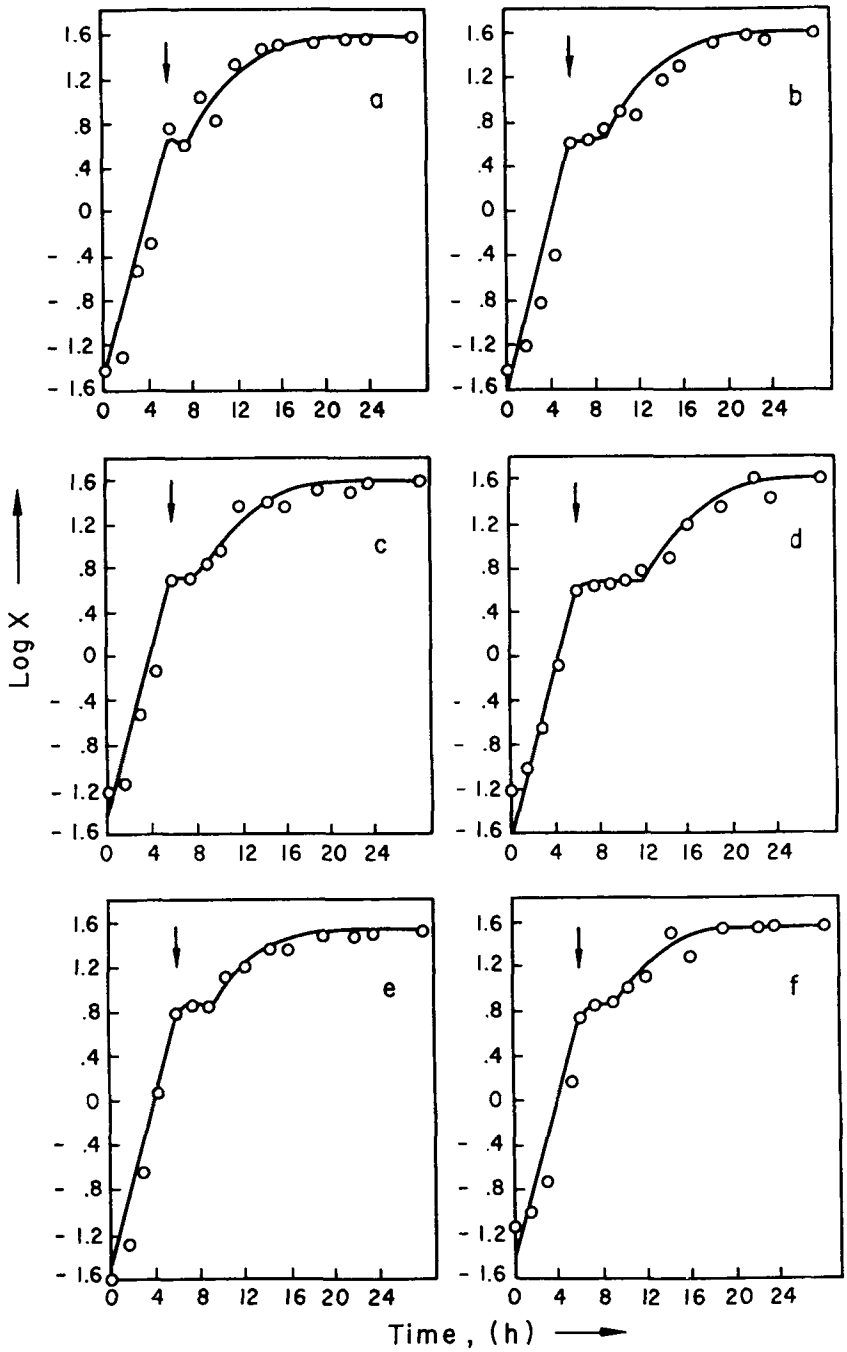

Figure 4. Comparison of the model with the experimental data when wild microorganisms were cultivated with 3 mg/L of antibiotic shock and antibiotic-resistant microorganism addition. Antibiotic-resistant microorganisms made up the following percentages of the wild microorganisms: a) 0%, b) .01%, c) .02%, d) .03%, e) .04%, and f) .05% (o experimental data; — simulation; ↓ shock).

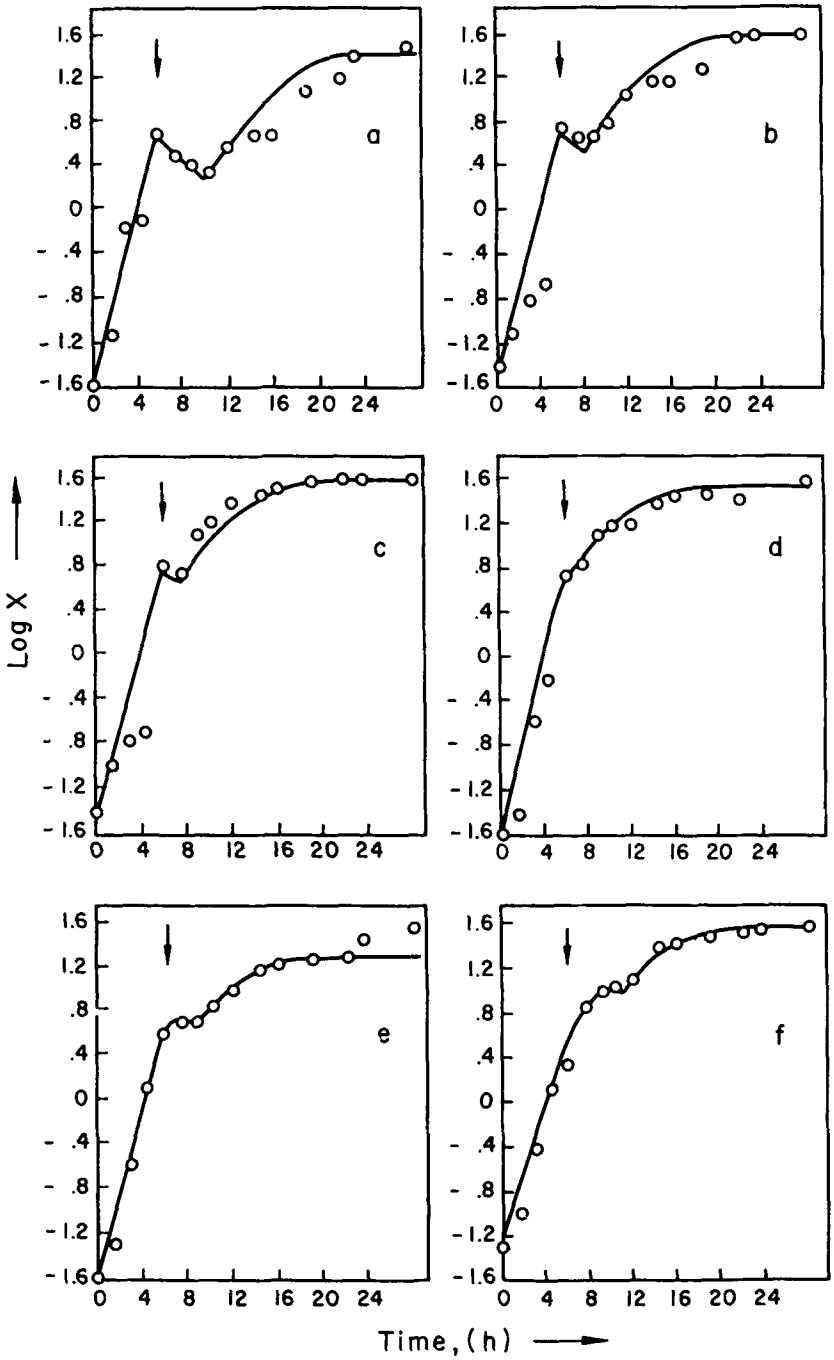

Figure 5. Comparison of the model with the experimental data when wild microorganisms were cultivated with 6 mg/L of antibiotic shock and antibiotic-resistant microorganism addition. Antibiotic-resistant microorganisms made up the following percentages of the wild microorganisms: a) 0%, b) .01%, c) .02%, d) .03%, d) .04%, and e) .05% (o experimental data; — simulation; ↓ shock).

microorganisms was prevented by addition of resistant microorganisms, a period of nongrowth was observed (Figures 4 and 5). Length of this period could not be correlated with the concentration of the resistant microorganisms. Reasons were not identified for recovery of *S. thermophilus* from penicillin G shock. Some microorganisms are resistant to penicillin G due to the production of penicillin-cleaving enzyme β-lactamase, which hydrolyzes β-lactam rings and converts them into penicilloic acids. Penicilloic acid is not active as an antibiotic (1). Inducible β-lactamases are a major factor for development of antibiotic resistance in gram-negative bacteria (10). The recovery mechanism of *S. thermophilus* from the penicillin G effect and the role of resistant microorganisms in bringing about such a recovery needs to be studied further.

In Figures 3 to 5, Equation [1] simulated the data in the exponential phase. Antibiotic shock was introduced at the beginning of the logistic phase. Equation [4] simulated the logistic phase. A constant value was assigned to ϕ (Table 1), and Equation [4] was solved with Euler integration with 1-h increments (2). The value of ϕ remained constant until the effect of shock was totally overcome by the microorganisms, and then it became 1. The shock value of ϕ and length of the shock were considered as the numerical measure of the antibiotic shock. Values of parameter μ were taken from Figure 1 and considered the antibiotic concentration after the shock. In Table 1, values of ϕ and length of the shock showed that when .02% or less antibiotic-resistant microorganisms were introduced into the culture, antibiotic shock was more severe with 6 than with 3 mg/L of penicillin G. At .04 and .05% resistant microorganism concentrations, the same shock was experienced in both media.

CONCLUSIONS

Subbacteriostatic penicillin G concentration was higher in liquid than in solid media. When using subbacteriostatic penicillin G concentrations, initial specific growth rates (as defined by the logistic equation) decreased with increasing antibiotic concentration. Decrease of the specific growth rate was high with 3 mg/L of penicillin G. Additional decrease in specific growth rate with increased penicillin G concentration was smaller. Penicillin G shock killed a fraction of the wild microorganisms. It did not kill but stopped growth of the resistant microorganisms. Both the resistant and the wild cultures recovered from the shock in a few hours. Penicillin G-resistant microorganisms exerted a protective effect on the wild culture. A modified logistic equation was employed to simulate growth with penicillin G shock. The modified logistic equation helped in the numerical expression of antibiotic shock.

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