Calorimetric Analysis of Microbial Growth: with Special Reference to Quantitative Evaluation of Drug Action

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(Received February 7, 1990; Accepted February 7, 1990)

Our research method for the calorimetric characterization of the biological effects of drugs and other chemicals on metabolic activities of living cells is outlined. The effects of various substances on different microbial systems were studied quantitatively using a calorimeter, and the results were used to plot drug potency curves for each drug. The method was also used to study microbial activity in soil. It was found to be a useful technique for the quantitative characterization of pollutants in ecological systems.

INTRODUCTION

Many investigators have quantitatively studied the biological activities of living cells and tissues by observation of their metabolic heat (1, 2, 10). This method is mainly based on the heat effect being strictly proportional to the metabolic activity. Generally, the magnitude of the calorimetric signal, given in units of power (µW), is the index used to express the biological activity.

However, less attention has been paid to changes with time in the calorimetric signals (the growth thermogram*1), although these changes are a direct reflection of the growth behavior of cells in culture vessels.

It has been long our view that biocalorimetry is most effectively used for the kinetic analysis of microbial cultures. Recently, we developed a highly sensitive multiplex batch calorimeter useful for the detection of small heat effects arising from cellular metabolism, and used it to analyze the growth behavior of microbial cells. The course of heat evolution was observed with microbial cultures grown on different culture media containing antimicrobial drugs and the growth behavior was analyzed in terms of growth kinetics to measure the viable activity of the microbes and the effect of the drugs on them. This enabled us to determine parameters that characterize the drug action and to draw drug potency curves calorimetrically.

The same principle was also adapted to the quantitative estimation of food putrefaction and of environmental pollution. We evaluated the action of food preservatives on actual foodstuffs, and proposed a method to evaluate the effect of pollutants on microbial activity in soil.

MATERIALS AND METHODS

Calorimeter

A multiplex batch calorimeter based on the conduction principle was constructed in this laboratory. The apparatus has 25 calorimetric units, with semiconducting thermopile plates as a sensor, arranged in an aluminum heatsink measuring 350 × 700 × 120mm. The basic structure and its operation are essentially the same as those for six-membered calorimetric units reported elsewhere (8).

Method of Analysis

The heat evolution process $f(t)$ during incu-
bation of microbial cells was obtained from the apparent calorimetric output signal \( g(t) \) at an incubation time of \( t \) from the following relation (3, 9):

\[
f(t) = g(t) + k_1 g(t) \, dt \quad (1)
\]

where \( K \) is the heat leakage modulus (heat conduction constant) of the calorimetric unit decided on the basis of Newton's heat conduction principle (3, 12).

\( f(t) \) is a quantity observable if the calorimetric unit were placed in a hypothetical adiabatic condition; it corresponds to the true thermal process occurring in the calorimetric vessel. The relationships between these two quantities, \( g(t) \) and \( f(t) \), are shown in Fig. 1 for an electrical calibration experiment conducted on one of the calorimetric units and in Fig. 2 for a practical measurement of a fungal culture.

In the electrical calibration experiment, a constant electric power of 330.8\( \mu \)W was applied to a heater mounted on the calorimetric unit. The calorimeter recording \( g(t) \) gave a typical calibration mark and the corresponding \( f(t) \) curve was obtained by computation using eq. (1) as shown in Fig. 1. The \( f(t) \) curve gave a straight line with a slope until the heat power was turned off. Thus the \( f(t) \) curve calculated from the calorimeter recording after correction for the heat leakage closely reflected the temperature change of a calorimetric unit in a hypothetical adiabatic condition. This means that this computation based on eq. (1) is reasonable and can be used in practical measurements.

Figure 2 shows a typical calorimetric recording \( g(t) \) (growth thermogram) observed for the growth of a fungus, Aspergillus oryzae, at 30°C on solid medium with bran. The culture vessel was tightly sealed with a gas-tight cap. When all of the oxygen in the vessel was consumed, the signal rapidly returned to the base line. The corresponding \( f(t) \) curve obtained by eq. (1) is also shown in the figure. This curve is a typical growth curve for microbes. It is interesting that even after the oxygen was consumed (after 40 h of incubation), the \( f(t) \) curve gradually rose, indicating the involvement of heat evolution in minute amounts without oxygen consumption.

**Determination of Growth Rate Constant**

We previously reported that the heat evolution process during the exponential growth of microbes is given by a simple exponential function:

\[
f(t) = AN_0 \exp(\mu t) + BN_0 \quad (2)
\]

where \( \mu \) is the growth rate constant, \( N_0 \) is the total number of viable cells at the start of incubation (the inoculum size), and \( A \) and \( B \) are constants (3, 9).

Using this relation, regression analysis of the \( f(t) \) curve was performed, and the growth rate constant, \( \mu \), was determined as the best-fit value. The dotted line in Fig. 2 is the curve fitted by the regression analysis. The growth rate constant was obtained as

\[
\mu = 5.32 \times 10^{-3} \text{ min}^{-1} \quad (3)
\]

Curve fitting was performed for only a limited incubation period, because growth deviated from the exponential as the oxygen
concentration decreased.

**EXPERIMENTAL RESULTS AND DRUG POTENCY CURVES**

Figure 3(a) shows growth thermograms observed for the growth of *Escherichia coli* at 30°C on bouillon medium containing various concentrations of the antibiotic novobiocin (7). As the concentration of novobiocin increased, the growth thermogram shifted toward a longer incubation period with a slight decrease in the initial slope. This effect of the drug became more evident when the observed growth thermograms were converted to \( f(t) \) curves. The curves obtained by computation with eq. (1) are shown in Fig. 3(b). The change in the growth curve was correlated the concentration of novobiocin. Similar results were also obtained with a culture of *Asp. oryzae* on bran containing different amounts of \( p \)-hydroxybenzoic acid (PHBA). These results are shown in Fig. 4 (11).

In order to characterize this feature of the
drug action more quantitatively, we propose a method of analysis based on the following scheme (5):

\[ V + nS \rightleftharpoons VS_n \rightleftharpoons 2V + P \]

\[ VS_n + mI \rightleftharpoons VI_m \]

where a viable microbial cell V incorporates n moles of nutrient (substrate) S to form an intermediate VS_n which produces a new viable cell, sometimes with the formation of metabolic by-product P. The drug I inhibits the viable activity of the cells by forming nonviable states VI_m and VSnIm, where m is the mean number of molecules of the drug needed to inhibit the replication of the cells.

If we define the substrate constant as 

\[ K_s = [V][S]^n/[VS_n] \]

and the dissociation constant of the drug as 

\[ K_d = [V][I]^m/[VI_m] = [VSn][I]^m/[VSnIm] \],

then the growth rate equation is:

\[ \mu_i = \frac{\mu_m}{(1 + K_s/s^n)(1 + i^n/K_d)} \]

where \( \mu_i \) is the growth rate constant in the presence of a drug at concentration \( i = [I] \) and \( \mu_m \) is the maximum growth rate constant in the absence of the drug and in the presence of excess nutrients.

In actual measurements, the following relationship holds to a good approximation:

\[ K_s \ll S^n \]

Therefore, eq. (5) can be reduced to eq. (7):

\[ \mu_i = \frac{\mu_m}{(1 + i^n/K_d)} \]

The values of \( K_d \) and \( m \) can be calculated by regression analysis on the basis of eq. (7) and a drug potency curve can be obtained by plotting the specific growth activity \( \mu_i/\mu_m \) against the drug concentration \( i \).

The drug potency curve obtained by the above procedure for the action of PHBA on the
growth of *Asp. oryzae* at 30°C on bran is shown in Fig. 5.

Measurements were also made of drug effects on the growth of *Asp. oryzae* at 30°C on bran containing various food preservatives, the drug potency curves were drawn calorimetrically shown in Fig. 6. The drug concentrations are given in logarithmic units so that a wide range is covered.

As understood from eq. (7), the value of \( K_i = K_d^{(1/m)} \) corresponds to the drug concentration at which the growth activity is inhibited by 50%, and the value of \( m \) reflects the cooperative nature of the effects of the drug. Thus the two parameters can be indices of the effective and dynamic concentration range of drug action. These parameters determined by the present calorimetric method are summarized in Table I (5).

In Fig. 7, the drug potency curve obtained calorimetrically for the effects of the three antibiotics, streptomycin, tetracycline and chloramphenicol, on *E. coli* growth is shown. The parameters \( m, K_d, \) and \( K_i \) are also summarized in Table I (5). The changes in the growth thermograms in the presence of certain drugs (penicillin G, ampicillin and polymyxin B) could not be explained quantitatively by the model of non-competitive inhibition defined by scheme (4). These drugs are known to inhibit the biosynthesis of bacterial cell walls. With all three antibiotics, it was found that the growth thermograms shifted toward a longer incubation period, but the growth rate constant remained unchanged, even when one of the drugs was present in the incubation medium. This characteristic feature may be explained by the drug reducing the effective inoculum size because of its specific binding to the cell to inhibit cell division. On the basis of a scheme in which the decrease in the effective inoculum size was proportional to the \( m_N \)-th power of the drug concentration, the observed changes in growth thermograms were analyzed to obtain the drug potency curve. The results are shown in Fig. 8 (6).
Table 1  Inhibitory parameters of some drugs on the growth activity of microbes measured by the calorimetric method

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Microbes</th>
<th>$m$</th>
<th>$K_d$ $(\text{mol dm}^{-3})^m$</th>
<th>$K_i$ $(\text{mol dm}^{-3})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ethyl alcohol</td>
<td><em>Asp. oryzae</em></td>
<td>3.3</td>
<td>0.815</td>
<td>0.94</td>
</tr>
<tr>
<td>$p$-hydroxybenzoate</td>
<td><em>Asp. oryzae</em></td>
<td>1.9</td>
<td>0.102</td>
<td>0.300</td>
</tr>
<tr>
<td>sodium benzoate</td>
<td><em>Asp. oryzae</em></td>
<td>2.1</td>
<td>0.00278</td>
<td>0.0607</td>
</tr>
<tr>
<td>calcium propionate</td>
<td><em>Asp. oryzae</em></td>
<td>1.4</td>
<td>0.0113</td>
<td>0.0408</td>
</tr>
<tr>
<td>potassium sorbate</td>
<td><em>Asp. oryzae</em></td>
<td>1.2</td>
<td>0.00964</td>
<td>0.0209</td>
</tr>
<tr>
<td>chloramphenicol</td>
<td><em>E. coli</em></td>
<td>1.3</td>
<td>$1.46 \times 10^{-8}$</td>
<td>$0.94 \times 10^{-6}$</td>
</tr>
<tr>
<td>tetracycline</td>
<td><em>E. coli</em></td>
<td>0.7</td>
<td>$3.49 \times 10^{-5}$</td>
<td>$0.43 \times 10^{-6}$</td>
</tr>
<tr>
<td>streptomycin</td>
<td><em>E. coli</em></td>
<td>1.2</td>
<td>$8.69 \times 10^{-9}$</td>
<td>$0.19 \times 10^{-6}$</td>
</tr>
</tbody>
</table>
Calorimetric Analysis of Microbial Growth

Figure 7

Drug potency curves for the action of (a) streptomycin, (b) tetracycline and (c) chloramphenicol on *E. coli* growth drawn calorimetrically.

Figure 8

Drug potency curves for the action of (a) penicillin G, (b) ampicillin and (c) polymyxin B on *E. coli* growth drawn calorimetrically.

Figure 9 shows the results of experiments on microbial degradation of glucose in soil containing various amounts of chromium as a model pollutant (4). The specific degradation rate of glucose was determined by the same procedure and plotted against the chromium concentration with the results shown in Fig. 10. Our calorimetric method is also useful for the quantitative characterization of pollution in ecological systems.

The actual biochemical processes in living cells are more complicated than what we have assumed here, and the parameters defined above have limited biological significance in themselves, but the drug potency curves drawn are obtained by fitting of the experimental data. Therefore, we believe that they are completely reliable. The method described here will be useful in the quantitative characterization of the biological effects of various drugs and foreign substances on living cells.

ACKNOWLEDGMENT

The author wishes to express his thanks to his colleagues and the students in his groups, especially Mrs. S. Harada, Mr. S. Itoh, Miss R. Kaneda, Mr. A. Katarao, Mr. T. Kawabata, Mr. T. Kimura, Mrs S. Kitamura, Miss K. Kohno, Mr. K. Matsuoka, Mr. M. Mitani, Mr. S.C. Moon, Mr. T. Mune, Mr. H. Okuno, Miss Y. Sagara, Mr. H. Saneshige, Dr. H. Yamano and Mr. N. Yamato for their contributions to the experimental work during the last 12 years.
Fig. 9 Microbial degradation thermograms of glucose in soil containing various amounts of potassium dichromate at 30°C. Chromium concentrations / ppm are: (a) 0, (b) 25, (c) 50, (d) 75, (e) 100, (f) 125, (g) 150 and (h) 200 on the element base \( \text{(Cr}^{6+} \text{)} \).

Fig. 10 Effect of chromium concentration on microbial degradation of glucose in soil at 30°C.

REFERENCES