Articles

Kinetics of the Action of Na_2SeO_3 on *Bacillus subtilis* Growth as Studied by Microcalorimetry

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Microcalorimetric bioassay for acute cellular toxicity is based on metabolic heat production from cultured cells. The biological response to toxicants is the inhibition of the heat production rate in cells, and toxicity is expressed as the concentration of toxicant that is 50% effective in this inhibition (IC_{50}). In this paper, the effect of Na_2SeO_3 on *Bacillus subtilis* growth was investigated at 37 ℃ by microcalorimetry. The relationship between growth rate constants (k) and concentration of Na_2SeO_3 (c) shows a logarithmic normal distribution, and IC_{50} is 20.3 μg/mL. All these thermokinetic information is readily obtained by an LKB 2277-204 heat conduction microcalorimeter. Microcalorimetry is a quantitative, inexpensive, and versatile method for toxicology research.

Keywords microcalorimetry, thermochemistry, Na_2SeO_3, *Bacillus subtilis*, thermokinetics

Introduction

In any living system, the various metabolic events occurring within the cells are all of producing heat reactions. Therefore, by monitoring the heat effects with sufficient sensitive calorimeters, the metabolic processes of living cells can be studied. By continuous measurements of the heat effects of the growing cells with a calorimeter, it can be seen that the thermogenic curve reflects time dependence changes in growth patterns. Thus, the calorimeter is a powerful tool for observing living cells, in which the information on their metabolism can be provided. It has recently been demonstrated that calorimetric methods can be used for fundamental studies of bacteria growth.[1,2]

Selenium is one of the necessary trace elements of life, whose function in life science has been widely accepted.[3] Resent research suggests that selenium can protect cell membrane, and its anti-virus and anticancer activity is also very obvious.[4,5] Accordingly, studies of the bioeffect of selenium are very important.

Toxicity of substances can be expressed as LC_{50}, IC_{50} or EC_{50}. The accurate measurement of the effects of Na_2SeO_3 on aquatic systems depends on the reproducibility of acute toxicity tests.[6]

Bioenergetic investigations are important to understand the harmful properties of substances in ecotoxicology.[7] At the same time, microcalorimetry proved to be useful in monitoring cellular metabolism and measuring effect of substances on metabolism.[6,8] Miles and Beezer demonstrated that microcalorimetric studies of bacterial growth reveal temporal details which can not be observed by other techniques.[9] Furthermore, microcalorimetry can also be used to study the metabolism of mitochondria and effects of toxicants on that.[10-14,22,23] From the thermogenic curves, lots of kinetic and thermodynamic informa-

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tion can be obtained. We have studied the effects of toxicants on microbes[15,24] and mitochondrial metabolism,[16] and obtained the considerable kinetic and thermodynamic data.

In this paper, the application of the microcalorimetry which was used to study the effect of Na_2SeO_3 on *Bacillus subtilis* growth at 37 ℃ is briefly described. Heat production in a cell suspension was measured by the thermopile of an LKB 2277-204 heat conduction microcalorimeter. And the inhibition of biochemical reactions in the cells results in a decrease in growth rate constants and heat production.

Experimental

Materials

Bacillus subtilis (CCTCC AB93009) was provided by the Chinese Center for Type Culture Collections, Wuhan University, China. Analytical reagent Na_2SeO_3 was supplied by Zhonglian Reagent Factory, Beijing, China.

Bacillus subtilis was grown on a potato medium, which was prepared as following: 200 g of potato (without peel) was cut into small pieces, cooked for about 40 min and then filtered. After the sediment was discarded, 20 g of glucose was dissolved in the clear solution, and the volume of medium was made to be 1000 mL by distilled water. Then, the medium was sterilized at 120 ℃ for 20 min.

Calorimeter

LKB 2277 Bioactivity Monitor, which is a heat conduction type of microcalorimeter, was used to determine the metabolism of cells. It is designed to monitor continuously a wide variety of processes and complex systems over the temperature range of 20 ℃ to 80 ℃. This system is very sensitive, the detection limit is 0.15 μW and the baseline stability is ± 0.2 μW/d. There are three operating modes for the LKB 2277 Bioactivity Monitor: ampoule mode, flow-through mode and flow-mixed mode. The performance of this instrument and the details of its construction have been previously described.[18]

Preparation of the sample

In this experiment, the solution of Na_2SeO_3 was prepared in sterilized distilled water and prepared freshly every time. At the beginning of the experiment, *Bacillus subtilis* was inoculated in the prepared potato culture medium, initially containing 1×10^6 cells/mL, and the cells used were suspended in the potato culture medium. Then, the fresh Na_2SeO_3 solution was added into the cell suspension.

Experimental procedure

Sterilized distilled water, 0.1 mol/L HCl solution, 75% alcohol solution, 0.1 mol/L NaOH solution and sterilized distilled water were pumped through the measuring system to clean and sterilize the flow-cell, respectively.

Once the system was cleaned and sterilized, sterilized distilled water was pumped through the system at a flow rate of 10 mL/h to run the baseline. After a stable baseline had been obtained, the cell suspension, containing *Bacillus subtilis* and Na_2SeO_3, was pumped into the flow-cell by the aid of an LKB-2132 pump at a flow rate of 50 mL/h. When the flow cell was full, the pump was stopped, and the monitor recorded the thermogenic curves of the growth of *Bacillus subtilis* continuously at 37 ℃.

When the pen of the chart recorder had returned to the baseline and stabilized, *Bacillus subtilis* growth had ended. If necessary, further calibration was done after a stable baseline has been obtained.

Results

Thermogenic curves

The growth thermogenic curve of *Bacillus subtilis* in potato medium at 37 ℃ is shown in Fig. 1, and the thermogenic curves of *Bacillus subtilis* growth effected by Na_2SeO_3 are shown in Fig. 2, respectively. According to the thermogenic curves, we can see that *Bacillus subtilis* grow exponentially, and the addition of Na_2SeO_3 has affected the metabolism of *Bacillus subtilis* growth.

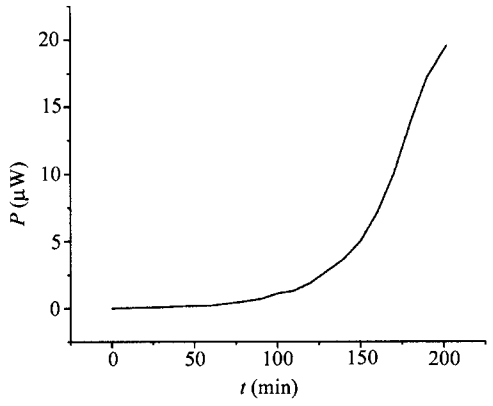

Fig. 1 Thermogenic curve of *Bacillus subtilis* growth in potato medium at 37 ℃.

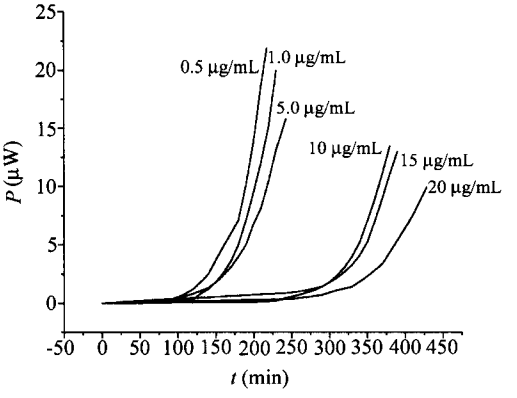

Fig. 2 Thermogenic curves of *Bacillus subtilis* growth effected by Na_2SeO_3 (0.5—20 μg/mL).

Calculation of the growth rate constant of Bacillus subtilis

In the log phase of growth, the cell growth is exponential.[15,18] If the cell number is n_0 at time 0, and n_t at time t,

$$n_t = n_0 \exp(kt) \quad (1)$$

where k is the growth rate constant. If the power output of each cell is w, then

$$n_t w = n_0 w \exp(kt) \quad (2)$$

If the heat output power is P_0 at time 0, and P_t at time t, then

$$P_0 = n_0 w \text{ and } P_t = n_t w, \text{ giving}$$
$$P_t = P_0 \exp(kt) \text{ or } \ln P_t = \ln P_0 + kt \quad (3)$$

The growth thermogenic curves of the log phase correspond to Eq. (3). So, using the data $\ln P_t$ and t taken from the curves to fit a linear equation, the growth rate constant (k) was obtained (Table 1).

Table 1 Thermokinetic values of *Bacillus subtilis* growth at 37 ℃

c (μg/mL)	0	0.5	1	5	10	15	20
k (min^{-1})	0.03180	0.03353	0.03293	0.02821	0.02547	0.02283	0.01604
P_m (μW)[a]	19.6	21.9	20.0	15.8	13.5	13.0	10.1
t_m (min)[b]	202	218	230	243	380	390	430

[a] P_m is the maximum heat output power of log phase; [b] t_m is the time corresponding to P_m.

Relationship between k and concentration of Na_2SeO_3

Fig. 3 shows growth rate constants (k) vs. the corresponding concentration values (c). As shown in Fig. 3, the relationship between k and $\ln c_{Na_2SeO_3}$ is not linear.

Analysis of the values of growth rate constants (k) and the corresponding concentration values (c) shows a logarithmic normal distribution (shown in Fig. 3), as described in References [15,19]. Thus,

$$k = B_1 \cdot \exp[-B_2(c + B_3)^2] \quad (4)$$

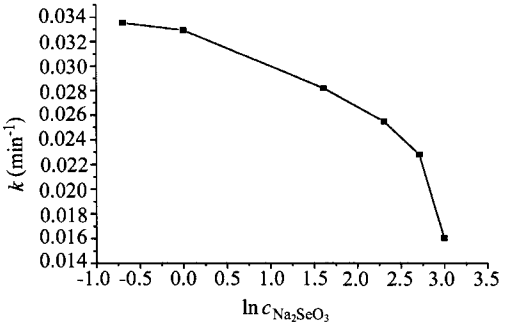

Fig. 3 Relationship between k and $\ln c$.

This model can be adjusted conveniently to this inhibitor, where k is the growth rate constant, B_1, B_2

and B_3 are constants, c is the concentration of Na_2SeO_3. Eq. (4) can be rewritten as:

$$\ln k = \ln B_1 - B_2(c + B_3)^2 \quad (5)$$

Using a least square method, the values of B_1, B_2 and B_3 are 0.03789 min^{-1}, -0.8336×10^{-3} $(mL/\mu g)^2$ and 12.0 $\mu g/mL$, respectively. The correlation coefficient R is 0.99927, which is very high. From all these calculated results, we can obtain relationship between k and concentration of Na_2SeO_3 (0—20 $\mu g/mL$) as:

$$k = 0.03789 \exp[-0.8336 \times 10^{-3}(c + 12.0)^2] \quad (6)$$

Inhibitory ratios and half inhibitory concentrations

High concentrations of Na_2SeO_3 will inhibit *Bacillus subtilis* growth, and the growth rate constant will decrease. So, the inhibitory ratio (I) can be defined as:

$$I = [(k_0 - k_c)/k_0] \times 100\% \quad (7)$$

where k_0 is the rate constant for *Bacillus subtilis* growth without inhibitor, and k_c is the rate constant for *Bacillus subtilis* growth inhibited by an inhibitor with a concentration of c.

When the inhibitory ratio (I) is 50%, the corresponding half-inhibitory concentration of the inhibitor can be represented as IC_{50} which can be regarded as the inhibiting concentration causing a 50% decrease of the *Bacillus subtilis* growth rate constant. Data for I are shown in Table 2. Fig. 4 shows the relationship between I (%) and c (Na_2SeO_3). From Fig. 4 and Table 2, we can obtain directly that IC_{50} is about 20.5 $\mu g/mL$. We can also calculate IC_{50} using Eq. (6), in which $k = k_0/2$, and the calculated IC_{50} is 20.3 $\mu g/mL$. The values of IC_{50} obtained by these two methods are the same, showing that the action model [Eq. (6)] is correct. This is very significant in toxicology research and should be studied further.

Table 2 Rate constants (k) and inhibitory ratios (I) for the growth of *Bacillus subtilis* at 37 ℃

c ($\mu g/mL$)	0	0.5	1	5	10	15	20
k (min^{-1})	0.03180	0.03353	0.03293	0.02821	0.02547	0.02283	0.01604
I (%)	—	-5.4	-3.6	11.3	19.9	28.2	49.6

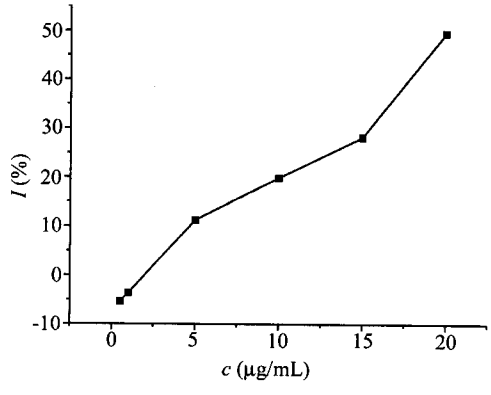

Fig. 4 Relationship between I and c.

Relationship of P_m and k

From Table 1, with the decreasing of the growth rate constant of *Bacillus subtilis*, the culture time increased, while P_m decreased. If linear regression was made between P_m and k, a relationship between P_m and k was obtained as:

$$P_m = 0.3628 + 609.017k \quad R = 0.98686$$

It is shown in Fig. 5.

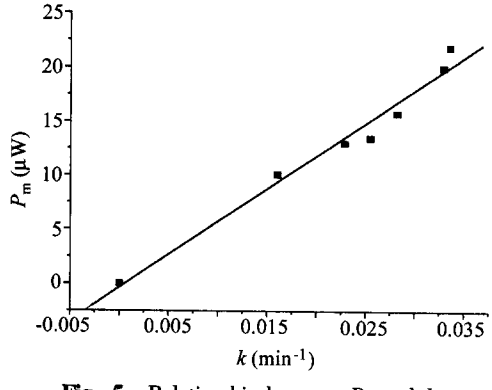

Fig. 5 Relationship between P_m and k.

Relationship of P_m and c

From the data in Table 1, if the linear regression between P_m and c was made, the linear relationship be-

tween P_m and c, $P_m = 20.1446 - 0.5265c$, $R = -0.95416$ was obtained. It is shown in Fig. 6.

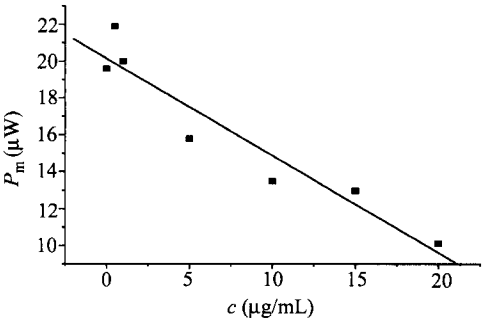

Fig. 6 Relationship between P_m and c.

Relationship of t_m and c

With the addition of Na_2SeO_3, the growth thermogenic curves become aboard, and the maximum time (t_m) of the log phase become longer. From the data in Table 1, if we fit linear relationship between t_m and c, the relationship between t_m and c, $t_m = 211.95 - 11.83c$, $R = -0.96589$ was obtained. It is shown in Fig. 7.

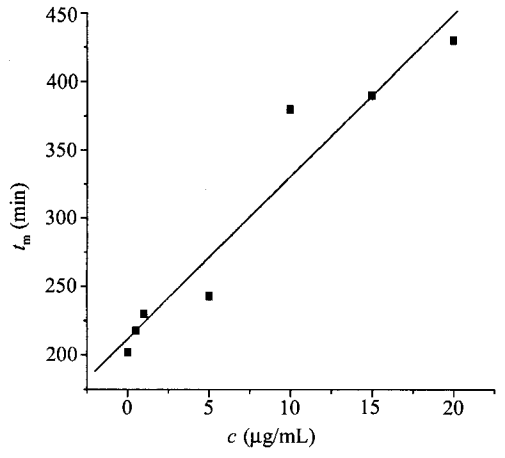

Fig. 7 Relationship between t_m and c.

Discussion

According to the thermogenic curves and thermokinetic values, it can be seen that low concentration of Na_2SeO_3 has stimulating action on *Bacillus subtilis* growth, however, high concentration of Na_2SeO_3 has inhibitory action on *Bacillus subtilis* growth. Furthermore, the percent of inhibition of *Bacillus subtilis* growth increased with the increase of concentration of Na_2SeO_3, and the growth of *Bacillus subtilis* was inhibited completely in the present of Na_2SeO_3 of 40 μg/mL.

Se is one of required trace element for biology. In low concentration, selenium is good for cellular mobility and beneficial to the synthesis of DNA and RNA of cells. Therefore, selenium is important to preserving the regular growth and biological activity of the cells. However, in high concentration, Se has inhibitory effect on microorganism. All the cellular levels, maybe Na_2SeO_3 combined with sulfhydryl groups on membrane proteins, resulting in cross-linking and inactivation. As a result, this changes permeability of cell membrane and disrupts transport of nutrients and waste across the membrane. Some studies showed a correlation between toxicity and sulfhydryl affinity, suggesting that the cross-linking of membrane proteins is a major factor in the toxic effects of toxicants.[20]

Microcalorimetry is suitable for toxicological tests with a high degree of reproducibility. It not only provides thermodynamic information, but also provides kinetic information. Calorimetry can enhance the accuracy of the determination of the physiological activity of the cultures. The calorimetric determination of toxicity results in lower as well as higher values of standard data within the range of variances of toxicity determinations by different laboratory using one standard method.[25]

Calorimetry has been proved to be a useful tool for measuring the energy flow in natural samples. It is adaptable to toxicity studies in any type of cells. The advantage of calorimetry is that it measures the total heat energy flow, and calorimetry in this respect is its nonspecificity. By combining calorimetry and other specific methods, several different and important goals may be reached in studying the energy flow in natural environments.[21]

New methods and approaches are needed in toxicity studies and for the development of toxicity test systems. Several of the presentations indicate the potential of the applicability of calorimetry combined with other methods to determine the influence of toxicants.

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