Microcalorimetric investigation of the toxic action of Cr(VI) on the metabolism of Tetrahymena thermophila BF5 during growth

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Abstract

Tetrahymena thermophila BF5 produce heat by metabolism and movement. Using a TAM air isothermal microcalorimeter, the power–time curves of the metabolism of T. thermophila during growth were obtained and the action on them by the addition of Cr(VI) were studied. The morphological change with Cr(VI) coexisted and biomass change during the process of T. thermophila growth were studied by light microscope. Chromium has been regarded as an essential trace element for life. However, hexavalent chromium is a known carcinogen, mutagen, cytotoxicant and strong oxidizing agent. Cr(VI) of different concentration have different effects on T. thermophila growth with the phenomenon of low dose stimulation (0–3 × 10^{-5} mol L^{-1}) and high dose inhibition (3 × 10^{-5} to 2.4 × 10^{-4} mol L^{-1}). The relationship between the growth rate constant (k) and c is a typical U-shaped curve, which is a characteristic of hormesis. T. thermophila BF5 cannot grow at all when the concentration of Cr(VI) is up to 2.4 × 10^{-4} mol L^{-1}. The microscopic observations agree well with the results obtained by means of microcalorimetry. And T. thermophila BF5 had obviously morphological changes by the addition of Cr(VI).

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Keywords: T. thermophila BF5; Microcalorimetry; Morphological change; Toxicology; Growth metabolism

1. Introduction

Ciliates are widely distributed in different natural environments. Tetrahymena thermophila is a freshwater ciliate belonging to the class of oligohymenophorea, subclass Hymenostomia, order Hymenostomatida, suborder Tetrahymenina (Sauvant et al., 1999). The ciliate T. thermophila is a eukaryotic unicellular microorganism, which has many useful features for experimental toxicological research (Gonzalez et al., 1997). It can be preserved in the laboratory and can be grown to high concentrations in axenic culture. The growth of T. thermophila may indicate the status of aquatic environment. This combination of features has allowed this microorganism to be used as a pharmacological tool in different bioassay techniques to detect toxicants (Schultz et al., 1989; Slabbert and Morgan, 1982). Furthermore, as a reference test species (Protoxkit FTM®, 1998), protozoan T. thermophila shows no correlation with any of the bioassay. This makes the Protoxkit FTM a very valuable bioassay since it probably “covers” a totally different range of sensitivity (Fochtman et al., 2000).

Chromium is a necessary trace element in human body, and plays a special role in sugar and fat metabolism of human body. However, hexavalent chromium is a known carcinogen, mutagen, cytotoxicant and strong oxidizing agent (Flores and Pérez, 1999; Stearns et al., 1995). The Cr(VI) can cross cellular membranes via surface anion transport systems (SO_{4}^{2-} and HPO_{4}^{2-} channels) and is biologically active (Cohen et al., 1983). Chromium is used in numerous industrial applications that are responsible for widespread pollution of soil and groundwater (Chardin et al., 2002). Consequently, the study of the influence of chromium on microorganism is significant for understanding life phenomenon; also it is helpful to solve environment pollution and plays a role in risk assessment. People have studied the function of chromium(VI) in many organism (Sahn and Oeztuerk, 2005; Wise et al., 2004), and chromium is used as a reference compound in Protoxkit FTM.
In recent years, the application of calorimetry in biological chemistry draws more and more attention of researchers (Holzel et al., 1994). Being a universal, integral, non-destructive and highly sensitive tool for many biological investigations, microcalorimetry can provide a lot of kinetic and thermodynamic information (Liu et al., 2000; Xie et al., 1988). *T. thermophila* produce heat by metabolism and movement. If the heat is monitored by microcalorimeter, much useful information, both qualitative and quantitative, may be obtained. The addition of toxic substances results in a change of the heat production.

In this study, the toxic action of potassium dichromate on *T. thermophila* BF5 was investigated by TAM air isothermal microcalorimeter. Simultaneously, haemacytometer was used to determine the population density of *T. thermophila* BF5 cells, and microscope was used to record the morphologic change, so that there is confirmation of the general results obtained by microcalorimetry.

2. Materials and methods

2.1. Instrument

*T. thermophila* BF5 cells were cultivated in constant temperature incubator at 28 °C. A TAM air isothermal microcalorimeter (Thermometric AB, Sweden) was used to determine the metabolic power–time curves of *T. thermophila* BF5 cells. Light microscope (Axioplan 2 imaging and Axioskop 2 universal microscope, Carl Zeiss, HongKong) was used to observe the morphologic change of the *T. thermophila* BF5, and take pictures. The enumerative work was carried through on the haemacytometer (homemade, volume was 100 μL) by microscope (XSP-18B, JiangNan, China).

2.2. Materials

*T. thermophila* (BF5, mononuclear) was provided by the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, P.R. China. The culture medium was a solution containing the peptone 1.5 wt.%, glucose 0.1 wt.%, yeast extract 0.5 wt.%, pH 7.2-7.4. It was sterilized in high-pressure steam at 120 °C for 30 min. Lugol’s solution was used to kill cells immediately and it comprised 5 g iodine, 10 g potassium iodide, 5 g sodium acetate, dissolved in 100 mL water. The chemicals used in the experiments were of analytical grade, and doubly-distilled water was used to prepare all solutions.

2.3. Methods

Initially, *T. thermophila* BF5 were cultivated in the incubator at 28 °C. Then, they were inoculated in the prepared 5 mL culture medium in 20 mL glass ampoule, the initial population density was 4.5 × 10^3 cells·mL^-1. K₂Cr₂O₇ with different concentration were added into each glass ampoule. Then, the glass ampoules were sealed with a cap and put into the TAM air isothermal microcalorimeter. A schematic representation of the experimental apparatus has been shown in Fig. 1. The growth process was monitored continuously and its thermogenic curve was obtained. The experiments above were all carried with aseptic technique.

The population density of *T. thermophila* BF5 and the influence of K₂Cr₂O₇ on biomass of *T. thermophila* BF5 were measured with a haemacytometer. First, *T. thermophila* BF5 cells were cultivated as above method. Then, 50 μL sample solution was extracted by using a syringe single, channel pipettor every 6 h into an EP pipe; soon afterwards, adequate Lugol’s solution was added in the sample solution to kill *T. thermophila* BF5 cells. And then the sample solution was diluted at an adequate proportion; 100 μL diluted solution was took out and dropped into haemacytometer to calculate the biomass of the *T. thermophila* BF5.

2.3.1. Thermogenic and biomass curves of *T. thermophila* BF5 during growth at 28 °C

The metabolism of *T. thermophila* BF5 during growth in culture media was studied and the growth thermogenic curve was recorded. The population density was counted by haemacytometer and the growth curve was obtained too. The results are shown in Fig. 2, from which we can see that the metabolic process can be divided into three parts: log phase (AB), stationary phase (BC) and decline phase (CD). The log phase of thermogenic curve agreed well with that of population density. *T. thermophila* BF5 produce heat by metabolism and movement, this indicated that an increase of cell density can result in enhanced produced heat. From Fig. 2, the value of power increased along with the increasing of the cell density of *T. thermophila* BF5. An integral of power versus time obtained the value of heat output (shown in Fig. 2). The experimental condition did not go all the way between microcalorimetry and counting process, this resulted...
Table 1

<table>
<thead>
<tr>
<th>Experiment no. 1</th>
<th>Experiment no. 2</th>
<th>Experiment no. 3</th>
<th>Experiment no. 4</th>
<th>Experiment no. 5</th>
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<th>Mean value</th>
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<td>0.00114</td>
<td>0.00117</td>
<td>0.00122</td>
<td>0.00104</td>
<td>0.00112 ± 0.00007$^b$</td>
</tr>
<tr>
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<td>0.99979</td>
<td>0.9998</td>
<td>0.99944</td>
<td>0.99901</td>
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<tr>
<td>$\bar{k}$ (min$^{-1}$)$^c$</td>
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<td>0.00108</td>
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<td>0.00098</td>
<td>0.00110</td>
<td>0.00110 ± 0.00005$^b$</td>
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<td>$R$</td>
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<td>0.9767</td>
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</table>

$^a$ Rate constant of thermogenic curves.
$^b$ Mean ± S.E.
$^c$ Rate constant of biomass curves.

The rate constant $k$ of $T$. thermophila BF$_5$ during growth in log phase varied along with the concentration of K$_2$Cr$_2$O$_7$ (shown in Fig. 4, some data do not shown in Fig. 3 because of overlapped with each other). From Fig. 4 it can be concluded that stimulation reached a high point when K$_2$Cr$_2$O$_7$ concentration was $1 \times 10^{-5}$ mol L$^{-1}$, and then decreased along with the concentration of K$_2$Cr$_2$O$_7$; inhibition followed exponential function primarily with $R^2 = 0.99507$. When the concentration was less than $3 \times 10^{-6}$ mol L$^{-1}$, the influence by K$_2$Cr$_2$O$_7$ was imperceptible and the thermogenic curve was similitude to the control.

Fig. 4. Relationship between the growth rate constant ($k$) and $C$.

In the lag of biomass after log phase when compared with thermogenic curve.

3.3. Growth of $T$. thermophila BF$_5$ with Cr(VI) coexisted at 28°C

Low concentration of K$_2$Cr$_2$O$_7$ stimulated the growth of $T$. thermophila BF$_5$, otherwise relative high concentration of K$_2$Cr$_2$O$_7$ inhibited $T$. thermophila BF$_5$ growth (shown in Fig. 3). $T$. thermophila BF$_5$ growth was inhibited completely when concentration of K$_2$Cr$_2$O$_7$ reached $2.4 \times 10^{-4}$ mol L$^{-1}$.
High concentrations of K₂Cr₂O₇ would inhibit growth with the rate constant decreasing. So, the inhibitory ratio (I) can be defined as:

\[ I(\%) = \frac{k_0 - k_C}{k_0} \times 100 \]  

(4)

where \( k_0 \) is the rate constant of the control and \( k_C \) is the rate constant for \( T. \) thermophila BF5 growth inhibited by an inhibitor with a concentration of \( C \). When the inhibitory ratio \( I \) is 50%, the corresponding concentration of inhibitor is called the half inhibitory concentration \( IC_{50} \) (according to Protoskit™). IC₅₀ can be regarded as the inhibiting concentration of causing a 50% decrease of the growth rate constant. From the data in Table 2, we can obtain directly that IC₅₀ is about 8.2 × 10⁻⁸ mol L⁻¹. According to protoxkit test, the value of 24 h EC₅₀ was 23.4 mg L⁻¹ or 7.95 × 10⁻⁵ mol L⁻¹ (Fochtman et al., 2000). The result of microcalorimetry substantially agreed with that obtained by Protoskit™.

The values of heat output during log phase (\( Q_{\text{heat}} \)) of the cell growth and period of growth (\( Q_{\text{total}} \)) were shown in Table 2. When stimulation increases in the range of 3 × 10⁻⁸ to 1 × 10⁻⁵ mol L⁻¹, \( Q_{\text{heat}} \) reduces with increasing concentration of K₂Cr₂O₇. In the range of 1 × 10⁻⁵ to 1.6 × 10⁻⁴ mol L⁻¹, stimulation decreased and translated into inhibition, furthermore \( Q_{\text{heat}} \) increased with the increase of the concentration of K₂Cr₂O₇. Heat output was an integral of power versus time, and enhanced inhibition resulted in the lag of log phase, so \( Q_{\text{heat}} \) increased along with the increasing of K₂Cr₂O₇ concentration during the linear range. On the other hand, K₂Cr₂O₇ inhibited cells multiplication rather than killed all the cells, and \( Q_{\text{heat}} \) increased along with the concentration of K₂Cr₂O₇ increasing.

3.4. Microscopic observations

As confirmation of the calorimetric experiments, a microscopic method was used to follow the growth of \( T. \) thermophila BF5. Without Cr(VI) in the culture medium, \( T. \) thermophila cells were active and swimming here and there. When dripped down a little high concentration K₂Cr₂O₇ (2.5 × 10⁻⁴ mol L⁻¹) solution into the medium, it could be seen that \( T. \) thermophila BF5, circumrotated in the same position and behaved like “floundering”. Through the microscope, it can be seen that cells died and ruptured rapidly, shown in Fig. 5.

The cells density through the growth was calculated by haemacytometer at an interval of 6 h, the results were shown in Fig. 6. From Fig. 6, it can be concluded that cells multiplication were stimulated by the addition of low concentration of K₂Cr₂O₇, otherwise relative high concentration of K₂Cr₂O₇ inhibited that. The rate constant was obtained, and the results were shown in Table 3. The half inhibitory concentration was higher than the result that obtained by calorimetric experiments.

The morphological change of \( T. \) thermophila BF5 cells influenced by Cr(VI) was obtained (shown in Fig. 7). Cells proliferated more slowly in the culture medium by the addition of K₂Cr₂O₇ when the concentration was higher than 2.5 × 10⁻⁴ mol L⁻¹, and cells profile became round otherwise cells cultured in normal medium were pyriform.

4. Discussion

Bioenergetic investigations which should be most important in the field of the assessment of harmful properties of substances in ecotoxicology are closely related to the applicability of the

<table>
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<tr>
<th>C (mol L⁻¹)</th>
<th>A (min⁻¹)</th>
<th>R²</th>
<th>I (%)</th>
<th>IC₅₀ (mol L⁻¹)</th>
<th>( Q_{\text{heat}} ) (J)</th>
<th>( Q_{\text{total}} ) (J)</th>
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<tr>
<td>0</td>
<td>0.00112</td>
<td>0.99985</td>
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<td>2.4 × 10⁻⁴</td>
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<td>–</td>
<td>–</td>
<td>2.4 × 10⁻⁴</td>
<td>75.00</td>
<td>–</td>
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Twenty-four hours EC₅₀ by Protoskit™: 7.95 × 10⁻⁵ mol L⁻¹ (datum from Fochtman et al., 2000).

- Correlation coefficient.
- Average of four times experiments.
- Average of three times experiments.
Fig. 5. Rupture of *T. thermophila* BF5 cell with K$_2$Cr$_2$O$_7$ dripped down in the culture medium. (a) K$_2$Cr$_2$O$_7$ free. (b)–(f) Process of rupture when K$_2$Cr$_2$O$_7$ ($2.5 \times 10^{-4}$ mol L$^{-1}$) was added, initial cells density was about $4.5 \times 10^3$ cells mL$^{-1}$, persisted about 10 min.

Fig. 6. Growth curves of *T. thermophila* BF5 influenced by different concentration K$_2$Cr$_2$O$_7$ at 28$\degree$C.

Direct calorimetry in biology because there is scarcely another method to analyze metabolic activities possessing such a general validity as the calorimetry (Weppen and Schuller, 1984). In this paper, the toxic action of Cr(VI) on *T. thermophila* BF5 was studied by microcalorimetric method. This ciliate is widely distributed in freshwater of natural environment. Studying the cytotoxicity of the metals in industrial effluent to ciliated protozoa is important for two principal reasons. One is ecotoxicological, to help in assessing the impact of metal pollution on organisms of very different complexity and position in aquatic food webs. The other is to develop alternative monitoring strategies to whole animal tests (Dayeh et al., 2005).

Comparing the experimental results of calorimetry and biomass determination, a little difference can be found (the generation time is 618.9 and 630.1 min, respectively). This owed to the distinction of two methods. *T. thermophila* BF5 was intact in the glass ampoule during the determination by use of calorimetry, on the other hand, biomass determination inevitably influenced the growth of *T. thermophila* BF5. *T. thermophila* BF5 is a kind of aerobic microbe. During the operation, the culture medium needed shaking up to ensure the biomass was well-proportioned, this leaded to more oxygen could be utilized by *T. thermophila* BF5. Under this condition, *T. thermophila* BF5 grew well and had longer generation time. And the value of IC$_{50}$ of the toxic action of Cr(VI) on *T. thermophila* BF5 was different too ($8.2 \times 10^{-5}$ and $1.4 \times 10^{-4}$ mol L$^{-1}$, respectively).

The non-conventional tests performed were Toxkits$^\text{TM}$ developed by scientists of Ghent University, Belgium. And Protoxkit$^\text{TM}$ was used in toxic test on protozoans (Pauli and Berger, 2000; Dmitruk and Dolejido, 2000; Fochtman et al., 2000). According to Protoxkit$^\text{TM}$, EC$_{50}$ was calculated by 24 h turbidity under the action of toxicant in different concentration. In
Fig. 7. Morphological change of *T. thermophila* BF5 cells affected of Cr(VI). The concentration of K2Cr2O7 (mol L−1): (a) 0; (b) 4 × 10⁻⁵; (c) 1 × 10⁻⁴; (d) 2 × 10⁻⁴. Cells were cultured at 28 °C in the glass ampoule for 2.5 d.

In this paper, IC₅₀ was calculated by rate constant during growth, through which we could investigate the action of toxicant on *T. thermophila* BF5 throughout cells growth in given volume of culture medium. The values of two methods were approached to each other (7.95 × 10⁻⁵ and 8.2 × 10⁻⁵ mol L⁻¹, respectively).

From Fig. 3 and Table 2, it can be concluded that Cr(VI) observably changed the growth of *T. thermophila* BF5 with the phenomenon of low dose stimulation and high dose inhibition. This is termed hormesis (Phillip et al., 2000). Hormesis aroused more and more interests of researchers but the mechanism is not well understood (Calabrese, 2005; Stebbing, 1998; Calabrese and Blain, 2005). This paper offered a calorimetric method for studied the hormesis. The relationship between the growth rate constant (k) and C (shown in Fig. 4) is a typical U-shaped curve, which is a characteristic of hormesis.

The heat output of log phase has dose-relationship with the concentration of Cr(VI). Low dose stimulated the growth of *T. thermophila* BF5, and heat output reached the high point more quickly, this was contrary to the relationship between the growth rate constant (k) and C. However, the total heat output has no obvious relationship with the concentration of Cr(VI). Q_total possible rested with the amount of culture medium that *T. thermophila* BF5 utilized for metabolism. Therefore, addition of Cr(VI) in the culture medium inhibited the growth of *T. thermophila* BF5, but this ciliate maybe adjusted to the new condition and utilized the reductive Cr. This hypothesis could explain the slight increase of Q_total with the increasing concentration of Cr(VI) and need farther confirmation. Microscopic observations shown that the growth of *T. thermophila* BF5 inhibited by Cr(VI) and cells have morphological changes, but *T. thermophila* BF5 did not stopped proliferation.

Acknowledgements

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