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INFLUENCE OF CYTOTOXIC AGENTS ON THERMOGENESIS IN *Streptococcus faecalis**

T. E. JENSEN, L. D. HANSEN, D. J. EATOUGH, R. D. SAGERS, R. M. IZATT AND J. J. CHRISTENSEN

Center for Thermochemical Studies and Departments of Microbiology, Chemistry and Chemical Engineering, Brigham Young University, Provo, Utah 84602 (U.S.A.)

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ABSTRACT

Changes in the thermogenesis of *S. faecalis* cells resulting from the addition of cyanide, fluoride, penicillin, tetracycline, or human serum have been observed using isothermal calorimetry. The character and extent of the decrease in the thermogenesis rate is reproducible and different for each cytotoxic agent studied. The changes in thermogenesis on addition of tetracycline are quantitatively related to the concentration of antibiotic added to the bacterial culture.

INTRODUCTION

The rate of heat evolution, or thermogenesis, is a direct measurement of the total metabolic vigor of bacterial cells. This property of bacterial metabolism has long been recognized[1] but has been utilized infrequently until recently[2,3]. Microbial thermogenesis has been shown to be reproducible for a given medium and microbe combination. Quantitative calorimetric studies have been reported using different substrates for growing and non-growing yeast or bacterial cells[4–7]. The ability to identify a wide range of bacteria and yeast cell types from the characteristic thermogenesis curve for each genera has been proposed[8–10]. The ability to directly observe the effect of cytotoxic agents on bacterial cells is potentially useful for clinical, pharmacological and immunological studies. Beezer et al.[8] have shown that flow calorimetry could be used to determine the level of microbial infection in urine samples. Flow calorimetry has also been applied to bacterial antibiotic sensitivity and proposed as a clinical tool by Binford et al.[11] but only the qualitative effect of the antibiotic (bacteriocidal, bacteriostatic, no effect) was determined for causative agents of urinary tract infections. In this report, data are presented indicating that the effect of an added antibiotic (tetracycline) on thermogenesis by a test organism (*S. faecalis*) may be used to quantitatively measure the level of the drug present in normal human serum. The technique may offer a rapid bioassay which directly measures drug activity.

*Contribution No. 55.

Isothermal calorimetry[12–14] is a method for measuring directly the rate of endothermic or exothermic heat changes in the calorimeter reaction vessel (growth chamber) while maintaining a constant temperature and therefore, has the advantage of producing a dynamic record (thermogenesis rate vs. time). The calorimetric techniques used previously[4–6,8,9,11] have response characteristics which make it impossible to measure short term (minutes) details of the thermogram. The time response of the instrument used in this study allows the measurement of these details[12–14]. The isothermal calorimetric technique applied to the study of the effect of cytotoxic agents on bacterial cells has been found to be significantly faster (<1 h compared to 18–36 h per sample) than present indirect techniques, i.e., dilution plate count, and requires only small amounts (100–250 μl) of cytotoxic samples. The calorimetric technique also compares favorably with newly developed agar diffusion[15], disk-plate[16], and photometric[17] techniques in rapidity of measurement and potentially is applicable to the study of any cytotoxin–cellular system. More significantly, for tetracycline the kinetic and total effects on the rate of heat evolution by the metabolizing cells are a reproducible function of the concentration of the cytotoxic agent added. Thus, calorimetry may potentially be used to test the cytotoxic sensitivity of bacterial or other cellular systems[11], to identify microbe strains[8–10] or, as stressed in this report, to measure levels of cytotoxic agents in serum or other samples.

We have used isothermal calorimetry to study the effect of the cytotoxic materials KCN, NaF, penicillin 'G', tetracycline hydrochloride and normal human serum on metabolizing *S. faecalis* cells. (Disk sensitivity tests using twelve antibiotics with the test organism showed tetracycline and penicillin were the most bacteriocidal). The effect of changes in concentration of the cytotoxic agent and of the introduction of human serum have been studied for tetracycline hydrochloride. The results indicate that isothermal calorimetry should be a useful research tool to: (a) study the reponse of bacterial (or other) cells to cytotoxic materials; (b) determine concentrations of cytotoxic substances, i.e., serum antibiotic concentrations; (c) study cytotoxic serum factors; and (d) study other cellular processes that exhibit a change in the rate of heat produced by growing cells.

MATERIALS AND METHODS

Streptococcus faecalis (BYU Lab Strains) cells were grown in rich growth medium (10 g of tryptone, 5 g of yeast extract, 5 g of K_2HPO_4, 5 g of NaCl, 10 g of glucose per liter). Cells were harvested from log growth culture (6 liters) by centrifugation for 10 min at 5000 × *g*. The packed cells were resuspended in sterile distilled water and centrifuged again for 5 min. This washing procedure was repeated three times. The final cell pellet was resuspended in twice its volume of phosphate buffered saline (5 g of K_2HPO_4, 5 g of NaCl per liter, pH = 7.2). This resting cell stock was stored at 0°C.

All calorimetric studies were made in a Tronac isothermal calorimeter. Details of equipment operation and data analysis have been described[13,18]. The test growth culture consisted of 24 ml of the growth medium described above to which was added

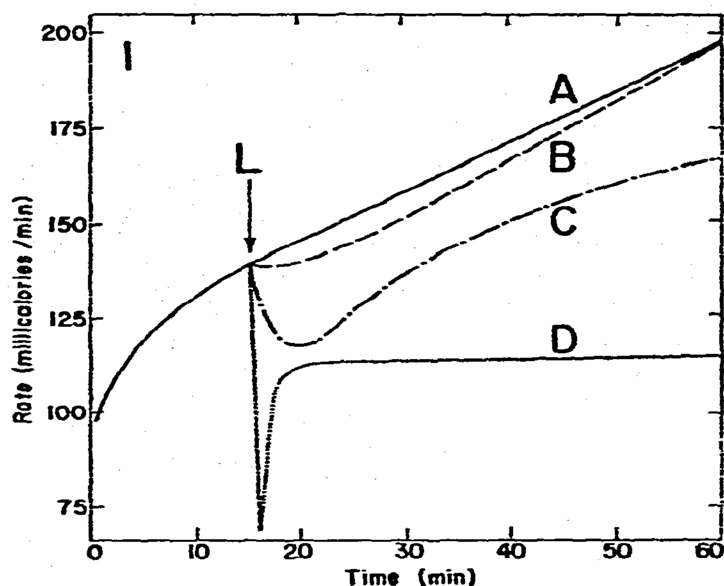

Fig. 1. Plot of rate of heat production vs. time for the addition of samples to *S. faecalis* (25 ml of 4% DMSO broth), A (———) the standard curve. The arrows indicate the injection of: B (– – –) 250 μl of 4% DMSO broth, C (—·—·) 250 μl of 0.60 *M* NaF, D (······) 250 μl of 0.63 *M* KCN.

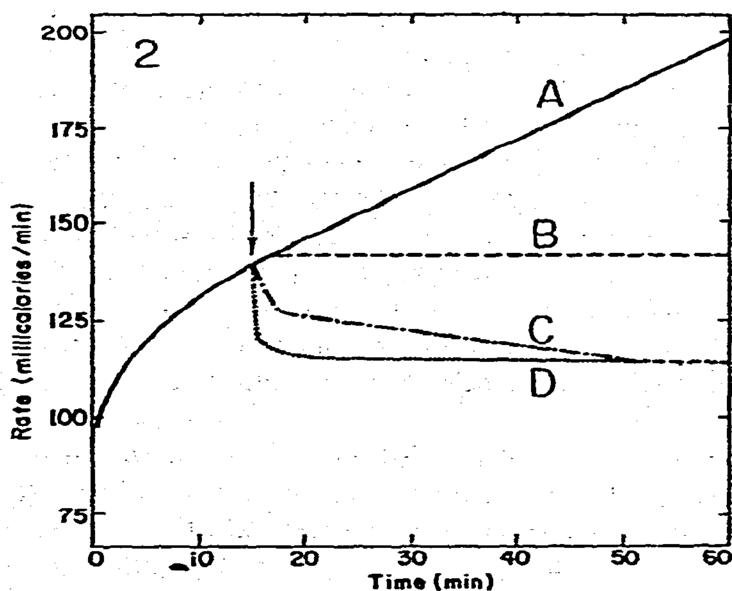

Fig. 2. Plot of rate of heat production vs. time for the addition of samples to *S. faecalis* (25 ml of 4% DMSO broth). A (——) the standard curve. The arrow indicates the injection of: B (— — —) 100 μl of 12.6 μg Penicillin 'G' per μl, C (—·—·) 100 μl of 1.2 μg tetracycline HCl per μl, and D ······) 100 μl 11.8 μg of tetracycline HCl per μl.

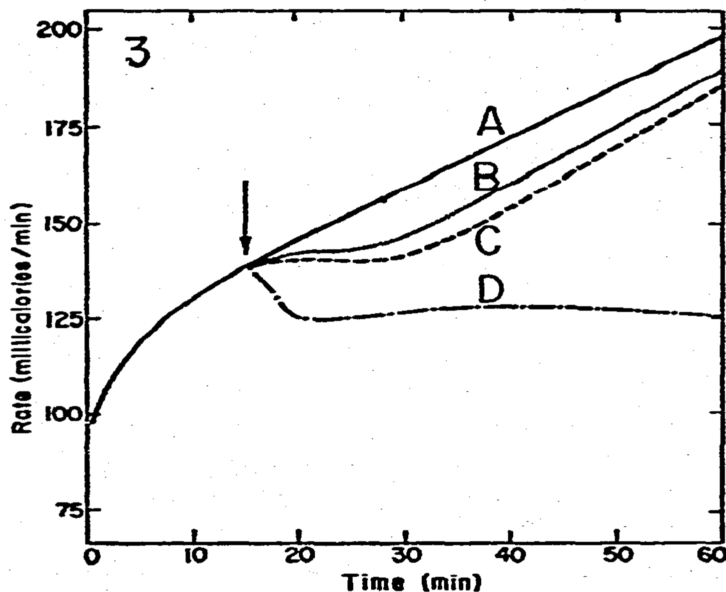

Fig. 3. Plot of rate of heat production vs. time for the addition of samples to *S. faecalis* (25 ml of 4% DMSO broth). A (——) the standard curve. The arrow indicates the injection of: B (— — —) 200 μl of heat inactivated human serum, C(—·—·) 200 μl of normal human serum and D (......) 200 μl of 0.28 μg of tetracycline HCl per μl normal human serum.

1 ml of dimethylsulfoxide (DMSO), and 0.1 ml of the resting cell suspension. After mixing of the growth culture, about 15 min were required to achieve temperature equilibration of the reaction vessel at 37°C. An additional 15 min were required to establish a stable linear increase in the thermogenesis rate.

Over a two-week period, samples of the test growth culture produced the same thermogenesis rate curve (designated curve A in Figs. 1–3). Following attainment of linearity (designated by L in Fig. 1), the standard curve remained linear for at least a 90-min period. The addition of DMSO (4% by volume) to the rich growth medium decreased the slope of the linear portion of the curve by 5%. However, with added DMSO, the transition to the linear region of the curve is more apparent and this region remains linear for at least twice as long as in the absence of DMSO, thus allowing changes in cell metabolism caused by the addition of cytotoxic agents to be more readily detected as deviations from the straight line portion of the standard curve.

In a typical determination, a small volume (100–250 μl) of a solution of the cytotoxic agent [KCN and NaF (reagent grade); Penicillin 'G', as the potassium salt, ~1400 units per mg, Nutritional Biochemicals; and tetracycline HCl (intravenous), Lederle Laboratories] in the medium or serum was added to the test growth culture (25 ml) in the calorimeter reaction vessel after the straight line portion of the standard curve had been reached. Introduction of small volumes (250 μl) of DMSO growth medium (curve B, Fig. 1) to the growth environment did not have a significant effect on the standard curve.

RESULTS

The changes in the thermogenesis rate curve resulting from addition of F^- (curve C) and CN^- (curve D) are compared to the standard curve in Fig. 1. In Fig. 2 the results of addition of penicillin 'G' (curve B) and two concentrations of tetracycline HCl (curves C and D) to the test broth are compared to the standard curve. Curves for addition of normal human serum (curve C), heated human serum (56 °C for 30 min) (curve B), and human serum plus tetracycline HCl (curve D) are shown in Fig. 3. The uncertainty in the rate of heat production, (dQ/dt), given in Figs. 1–3 is ± 1 mcal min^{-1} or approximately 1–2% of the measured value. In each case, the response of the test growth culture to the added cytotoxic agent or serum sample is rapid and reproducible. The overall response of the cells to an added cytotoxic agent was different for each agent used.

DISCUSSION

Addition of cyanide ion to the test culture resulted in a sharp edcrease in the rate of heat evolution followed by a rapid return to an almost constant rate of heat output. This interesting response indicates that the presence of substrates for functional enzymes not affected by cyanide ion results in a continued constant rate of heat evolution at approximately 95% of the level seen before addition of cyanide ion. Fluoride ion had relatively little effect on these cells. After the initial response, the rate of heat production parallels the standard curve, indicating the bacterial system is able, at least partially, to compensate for the effects of fluoride at the concentration used.

When penicillin 'G' was added to the test culture the rate of heat produced became constant at about the same rate as that existing when addition occurred. This constant rate is above that of the cyanide poisoned cells. The difference could indicate that the initial effect of penicillin on cell wall synthesis does not result in significant changes in the majority of enzyme systems which affect bacterial metabolism. The ability to recognize and distinguish between such differences could provide a useful method to study the extent of the cytotoxic influence of various agents on cells by directly following the heat output response of those cells.

In the case of tetracycline HCl, the difference in antibiotic concentration of the material added results in curves which differ from each other and from the standard curve. Tetracycline at a final concentration of 47 ng μl^{-1} of 4% DMSO broth produces the same final response as CN^-. The final baseline is, however, approached in two steps, the first a rapid reaction which is over in less than 1 min (the response time of the instrument used) and the second, a slower response requiring an additional 4.8 min for the attainment of a constant rate of heat output in the bacterial system. At a lower final concentration, 4.8 ng μl^{-1} tetracycline, the end result is the same, however, the approach to the final rate of heat production is slower. Again, two distinct effects are seen, the first lasting 2.7 min and the second, 40 min. The initial

response of the cells to a final concentration of 2.3 ng/μl^{-1} tetracycline, added as 0.2 ml of 284 ng/μl^{-1} tetracycline in human serum, was similar to that seen for tetracycline in broth, with a total time of 4.8 min for the response. Any second response to tetracycline in the presence of serum was masked by the effects of the serum (see Fig. 3, curve D) along with the attainment of the non-linear portion of the standard curve.

The action of tetracycline on *S. faecalis* involves at least two distinct steps, both of which appear to involve zero-order kinetics. These steps could be related to transport and/or protein inhibitor binding. The $t_{1/2}$ values for the two events, summarized in Table 1, appear to be related to the tetracycline concentrations within the error limits of the measurements. The large uncertainties in the smaller $t_{1/2}$ values result from limitations in the time response of the instrument. Measurement of $t_{1/2}$ values could be improved by using a calorimeter with a faster response time, such as a conventional isoperibol instrument[13,14]. These $t_{1/2}$ values could serve as the basis for rapid, quantitative analysis for tetracycline or extended for use with other antibiotics of more significant clinical interest.

TABLE 1

CALCULATED $t_{1/2}$ VALUES OBTAINED FROM THE DATA IN FIGS. 2 AND 3 FOR THE TWO STEP RESPONSE OF *S. faecalis* TO TETRACYCLINE AT THE INDICATED FINAL CONCENTRATION

[*Tetracycline*] (*ng/μl*$^{-1}$)	$t_{1/2}$^{a} (*min*)	$t_{1/2}$^{b} (*min*)
47.0	0.5±0.6	2.9±0.6
4.8	1.4±0.6	21.0±2.0
2.3[c]	2.4±0.6	

[a] First region — fast response. [b] Second region — slow response. [c] Sample in normal human serum.

Addition of normal serum to the test culture causes a reduction in the rate of heat production by the bacterial cells, but the reduction is only temporary. The titer of cytotoxic serum factors is proportional to the displacement of the normal serum curve from the standard curve. The return of the slope of the curve associated with addition of normal human serum to a slope approximately parallel to that of the standard curve would result from the continued normal metabolism of the unaffected bacterial cells.

Heated serum had less of an inhibitory effect than normal serum on the heat production of the bacterial cells. This was possibly due to the inactivation of heat-labile cytotoxic factors. It would not be expected that normal serum complement would be responsible for the difference observed between the effect of normal and heated serum since complement does not have a significant bacteriocidal effect on Gram positive cells. Thus, the difference between the bacterial heat production response to normal and heated serum is probably due to non-complement heat labile serum factor(s) such as lysozyme and/or β-lysin.

It should be emphasized that even at a serum dilution factor of 1:126, calorimetry directly yields information about the response of bacterial cells to heat stable and heat labile cytoxic agents in serum. Use of a standard viable cell plate count method at such a serum dilution would result in data that are indirect and not likely to be statistically significant.

When serum with tetracycline present was added to the test culture a marked response in the rate of heat production was observed. The observed change is a composite result of the action of the serum factors and antibiotic on the cells. However, the initial response to tetracycline is still clearly seen, indicating tetracycline may be quantitatively assayed in serum samples using the described calorimetric response. The fast response of bacterial cells to cytotoxic agents and the pattern and extent of change in the thermogenesis curve should be considered as useful tools for clinical studies and for basic research in cellular metabolism.

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