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Microcalorimetric Measurements of Glucose Metabolism by Marine Bacterium *Vibrio alginolyticus*

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Microcalorimetric measurements of heat production from glucose by *Vibrio alginolyticus* were made to assess the viability of calorimetry as a technique for studying the metabolism of marine bacteria at organic nutrient concentrations found in marine waters. The results show that the metabolism of glucose by this bacterium can be measured by calorimetry at submicromolar concentrations. A linear correlation between glucose concentration and total heat production was observed over a concentration range of 8 mM to 0.35 μM. It is suggested that these data indicate a constant efficiency of metabolism for this bacterium over the wide range of glucose concentrations studied.

The use of calorimetry to study heat production by living organisms is not a new development. As long ago as 1790 calorimetry was used by Lavoisier to study the heat output of small animals (15). The first study reporting quantitative measurement of bacterial heat production was carried out by Dubrunfaut (10), who measured heat production from the fermentation of 3.5 tons (ca. 4.1 t) of sugar. Many early studies were hindered by insufficiently sensitive instrumentation. With the development of modern calorimetric equipment which is commercially available, convenient to use, and sensitive enough to measure bacterial metabolism, the number of studies using this technique has grown. A number of workers have used microcalorimetry to study various aspects of the metabolism of bacteria (3, 4, 6–9). For a more thorough review of biological microcalorimetry, the reader is referred to Beezer (1). Few studies have been carried out with marine bacteria (18) and, to our knowledge, no previous calorimetric studies have attempted to use nutrient levels found in marine environments with pure cultures of marine bacteria. Some experiments at low substrate levels have been performed with terrestrial bacteria (17).

The profile of heat production versus time, or thermogram, of microorganisms has been shown to be sensitive to conditions including culture history, aeration, and growth medium used (2, 12, 21).

Bacterial heat production is composed of several components which may be related by the following equation: $\Delta H_m = \Delta H_c + \Delta H_a + \Delta H_w$, where ΔH_m is the experimentally measured heat production during bacterial growth, ΔH_c is the heat produced by catabolic reactions, ΔH_a is the heat absorbed by anabolic reactions, and ΔH_w is heat associated with other biological processes in the culture (e.g., motility and transport processes). The ΔH_w is generally neglected by bacterial calorimetrists (5). This appears to be justified, since to measure heat production from transport processes, concentrations of both bacteria and substrate must be greatly elevated over those encountered in most bacterial cultures for significant heat production to be measured (16). During fermentative growth the amount of carbon source used for anabolic processes is so small that the ΔH_a is an insignificant contributing factor to the ΔH_m. Under these conditions, then, $\Delta H_m = \Delta H_c$; if the end products of the fermentation are known, this value may be calculated (5, 13). Under aerobic conditions growth efficiencies are greater than under anaerobic conditions (22), and up to 60% of the carbon source may be assimilated by heterotrophic bacteria (20). As a result, ΔH_a is a significant factor, and if an independent technique for determining the fractionation of carbon between biomass and end products is applied in addition to calorimetric measurement, then ΔH_a may be determined experimentally (8).

There have been some suggestions that the growth efficiency of marine bacteria increases at low substrate concentrations (20). If this were the case, the heat production from glucose would be expected to be nonlinear at low substrate concentrations since the energy wasted as heat would decrease as the growth efficiency increased. The data of Lovrien et al. (17) show the opposite effect. Their data show an increase in the molar heat production (ΔH_m per mole of

substrate) as the substrate level decreases. The work was done with *Escherichia coli*, and the result may be expected to differ in the case of a marine bacterium.

In the present study we have examined the applicability of several commercially available calorimetry systems for measuring the heat production of the marine bacterium *Vibrio alginolyticus*. The dependence of heat production on glucose concentration was examined to ascertain whether this technique could be applied at substrate concentrations found in natural waters and to determine the relationship between heat production and substrate concentration.

MATERIALS AND METHODS

Bacterium and medium. The bacterial strain used in these experiments was isolated from Biscayne Bay water and identified as *V. alginolyticus*. The bacteria were grown in a defined minimal growth medium composed of dibasic sodium phosphate (7 g/liter), monobasic potassium phosphate (3 g/liter), sodium chloride (5 g/liter), ammonium chloride (1 g/liter), magnesium sulfate 7-hydrate (10 ml per liter of a 4% [wt/vol] solution), and calcium chloride (2 ml per liter of a 1% [wt/vol] solution). Magnesium sulfate and calcium chloride were autoclaved separately. Glucose solutions were sterilized by filtration. The bacterial culture was maintained on tryptic soy agar (Difco Laboratories) slants at room temperature (25°C). Cultures in liquid medium were started by adding 19.5 ml of minimal medium, 0.5 ml of 0.33 M glucose (smaller volumes added to the medium when experiments were performed in the Tronac calorimeter at reduced glucose concentrations), and a loopful of bacteria from a stock slant to a triple-baffled Nephelo culture flask (Bellco). The cultures were shaken on a rotary shaker (Lab-Line Junior Orbit shaker) at 150 to 200 rpm at room temperature.

Tronac calorimeter. A schematic diagram of the Tronac calorimeter (model 550) is shown in Fig. 1. The isothermal reaction vessel (volume, 25 ml) is contained in a 20-gallon (ca. 66-liter) temperature-regulated water bath during experiments. The water bath was maintained at a temperature of 25.00 ± 0.01°C. The temperature of the contents of the reaction vessel are maintained at a constant temperature (±0.00005°C) by constant cooling from a Peltier cooler and the addition of heat as necessary with an electrical heater. The input from the heater is inversely proportional to the heat production of the reaction in the calorimeter. The contents of the reaction vessel were constantly stirred. For the purpose of bacterial calorimetry an aeration system was built to supply hydrated, temperature-equilibrated gases to the reaction vessel.

In preparation for an experiment, the reaction vessel was treated with Iodophore (Economics Laboratories) and rinsed extensively with distilled water. The stirrer passage was rinsed with water and dried with acetone. The reaction vessel was filled with 70% (by volume in water) ethanol and assembled so that the vessel, thermistors, and stirrer were immersed. The system was allowed to soak in the ethanol solution for 20 min with the stirrer on. The reaction vessel was then rinsed five times with sterile distilled water and assembled, filled with sterile distilled water. Subsequently, the water was poured out and the vessel was assembled and dried with a stream of sterile air. The bacterial culture was grown in liquid medium to an optical density of 0.1 at 520 nm (Bausch & Lomb Spectronic

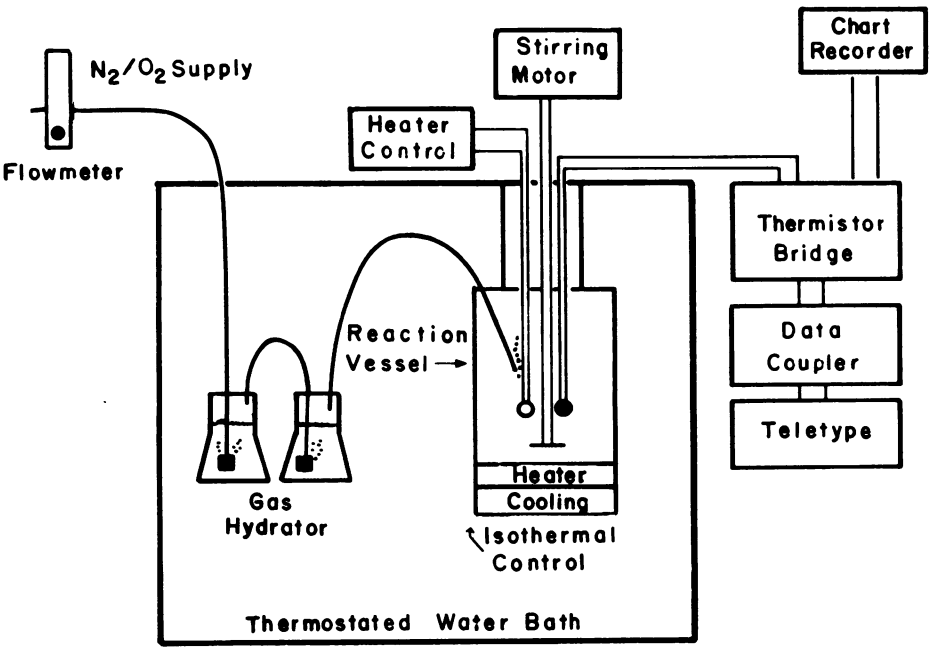

FIG. 1. Schematic diagram of the Tronac isothermal titration calorimeter.

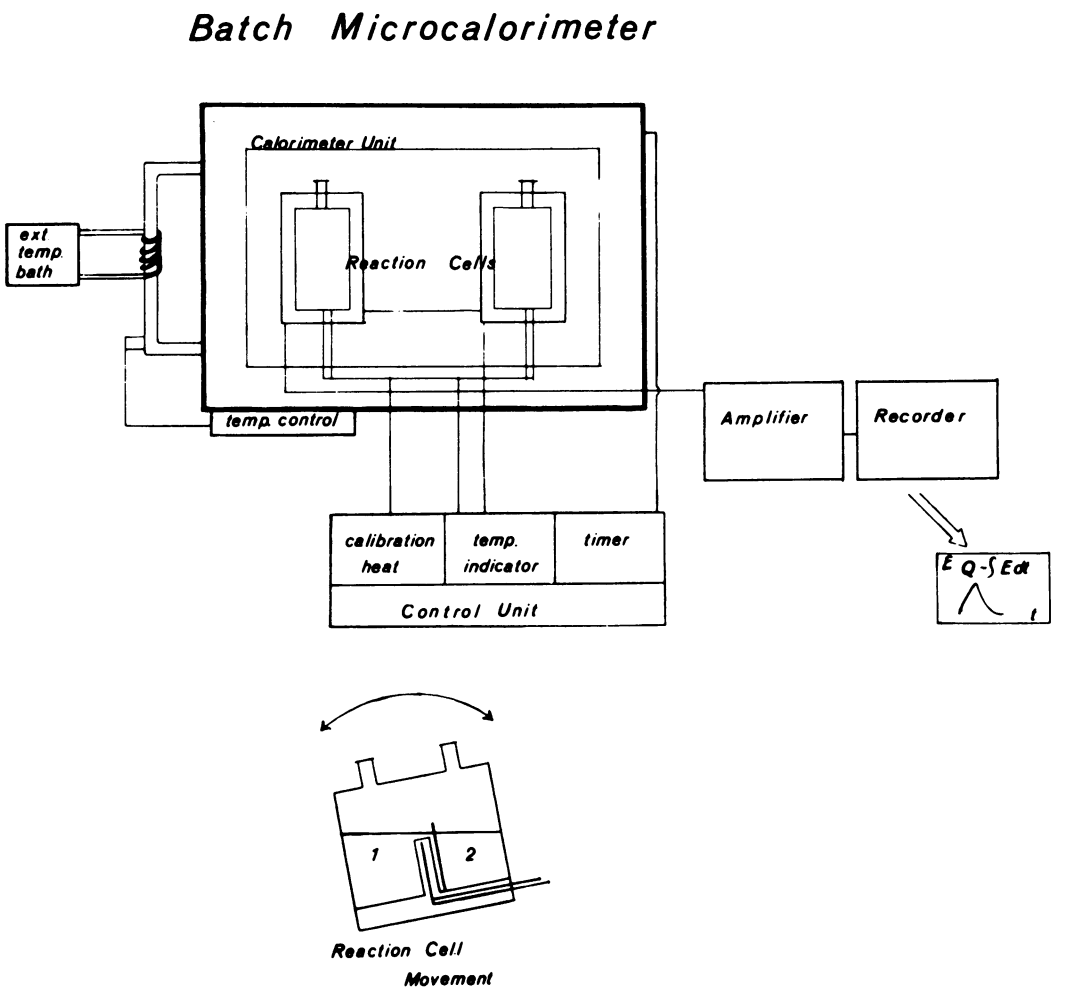

FIG. 2. Schematic diagram of the LKB mixing calorimeter.

20 spectrophotometer) on a rotary shaker and then placed into the reaction vessel. The initial cell concentration, determined by plate counts, was $9 \pm 2 \times 10^7$/ml. The data from the calorimeter were collected on a strip chart recorder (Hewlett-Packard model 7101B) and on paper punch tape. Aeration was achieved by passing a water-saturated, temperature-equilibrated oxygen-nitrogen mixture (50:50; 20 ml/min) through the solution in the reaction vessel with two Teflon tubes. The flow rate was controlled by two SS-2MGD Nupro metering valves. The rate was calibrated with a rotameter (model E29; Air Products).

LKB mixing calorimeter. A schematic diagram of the LKB mixing calorimetry system (model 10700) is shown in Fig. 2. This calorimetry system detects heat flow from the reaction vessel through thermopiles, which convert temperature changes to voltage, and into a metal heat sink. The calorimeter unit is contained in a constant (±0.01°C)-temperature air bath. There are two reaction cells, each divided into two compartments, with volumes of 2 and 4 ml. The contents of the separated compartments are mixed by rotation of the calorimeter unit (Fig. 2). The voltage outputs from the thermopiles of the two cells are connected in opposition so that the measured signal is the difference between the signals from the two cells. This configuration is referred to as the "twin principle" of this system and allows simultaneous control experiments to be performed as well as helping to eliminate base-line variation due to temperature fluctuations in the constant-temperature air bath.

Bacteria were grown in liquid medium for 18 h (starvation time, ca. 9 h) and harvested by centrifugation (1,200 × *g*, 20 to 30 min). The cells were washed twice in the liquid medium without organic nutrient and suspended at an absorbance of 0.15 (measured in a Bausch & Lomb Spectronic 20) at 520 nm. This suspension contained 8×10^7 viable cells per ml as determined by plate counts.

Glucose was prepared in millimolar concentration ranges in distilled water and then diluted volumetrically 1:1,000 in medium, using a 100-μl Oxford pipette

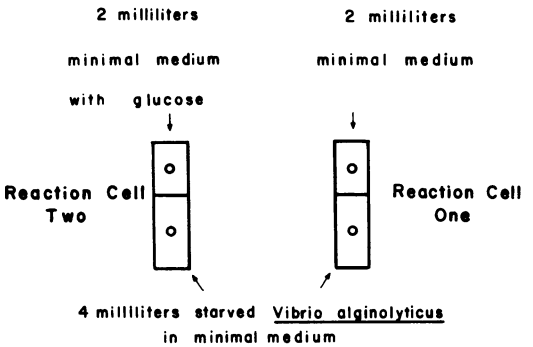

FIG. 3. Experimental setup for solutions in the cells of the mixing calorimeter.

and a 100-ml volumetric flask. This procedure yielded organic nutrients at micromolar concentrations which were then filter sterilized.

Bacterial cell suspension and solutions prepared as described above were placed in the appropriate chamber of the calorimeter (Fig. 3) with a sterile 5-ml syringe. The precision of the procedure for addition of the solutions was checked by weight of water and found to be reproducible to ±0.01 ml or 0.5%. A stable base line was achieved in 1 to 2 h, and then the reactants were mixed. The data from the calorimeter were collected on a Hewlett-Packard model 7101B strip chart recorder or on a Heath-Schlumberger model EU-205-11 recorder. The data were also collected and processed by an automated data system based on a Hewlett-Packard 9830 minicomputer. This system collects data from the Tronac or LKB calorimetry system. Data points were collected from the Tronac system every 100 s and from the LKB every 4.2 s.

After an experiment the reaction cells were flushed with water, rinsed with 0.5 N NaOH–0.1 N HCl–distilled water, soaked for 20 min in 70% ethanol, rinsed in acetone, and dried with air.

Calculation of heat evolution. Both calorimetry systems were calibrated by applying a known current through a heater of known resistance in the calorimetry reaction vessel. The rate of heat production by the calibration heater was calculated from the equation: cal/s = $I^2R/4.184$, where cal is calories, s is seconds, I is the current applied to the heater, R is the heater resistance, and 4.184 is the conversion factor between joules and calories. Voltage output from the calorimeter can be equated to rate of heat production in the reaction vessel by this calibration procedure.

Total heat evolution was calculated by integration of the thermogram. This was achieved with a program that uses a Hewlett-Packard 9830 computer. Digital data collected during the experiments on punch tape were loaded into the computer memory, and integration was performed by using Simpson's rule.

RESULTS

Tronac calorimeter. The results of four experiments in which *V. alginolyticus* was grown in 8.3 mM glucose are shown in Fig. 4. We found that at this glucose concentration the thermogram of the bacterium was reproducible if the inoculum culture concentration and aeration were controlled. Reproducibility of the thermogram was best during the early, exponential phase. When the exponential phase was nearly complete, a decrease in the rate of increase of the heat production rate sometimes occurred. This could have been due to the near exhaustion of oxygen or glucose in the culture. A secondary peak was

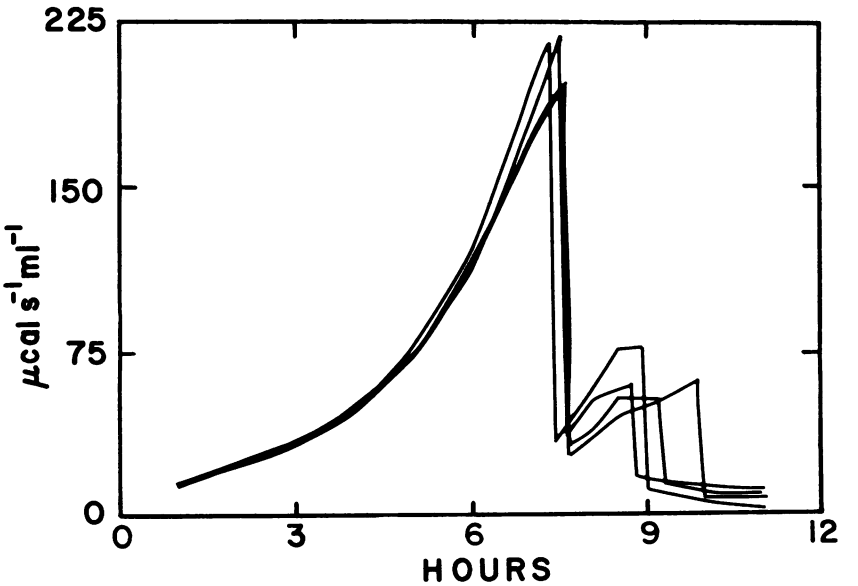

FIG. 4. Repeatability of thermograms generated in the Tronac calorimeter at a glucose concentration of 8 mM.

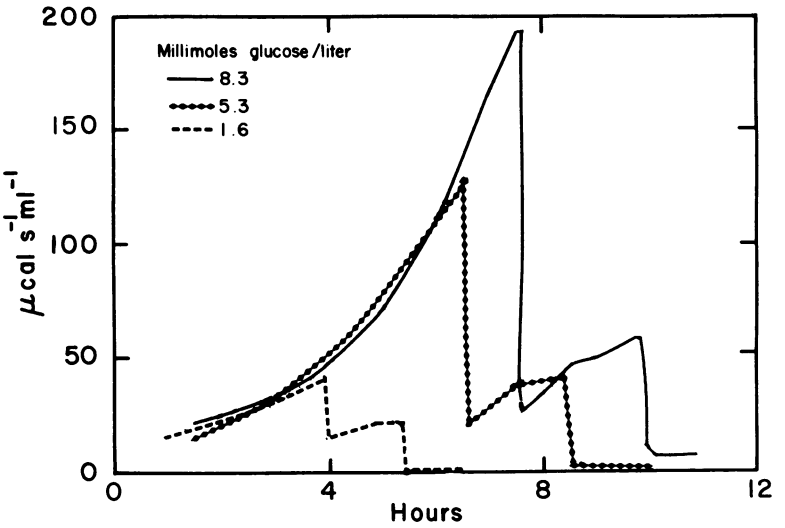

FIG. 5. Thermograms produced by *V. alginolyticus* in the Tronac calorimeter with various glucose concentrations.

always observed in the thermogram. Similar secondary peaks in heat production have been observed by other workers (11, 14) and have been shown to be due to accumulation and degradation of fermentation products in the medium. We examined the effect of lowering the concentration of glucose in the medium on the thermogram of *V. alginolyticus* (Fig. 5). The effect of decreased glucose concentration was simply a linear decrease in the total heat production (Fig. 6). A linear fit of the data (Fig. 6) has a correlation coefficient of 0.99. The rate of increase in the heat production rate was not significantly affected, indicating that the growth rate was unaffected. The lowest concentration of glucose that could be used in the Tronac system was approximately 1 mM.

LKB mixing calorimeter. Experiments performed in the LKB mixing calorimeter yielded thermograms such as the one shown in Fig. 7. Both the rate of heat production and the total heat production are plotted as a function of time. The sharp initial rise in the rate of heat production results from mixing the starved bacteria with medium containing glucose. Since aeration is not possible in this calorimetry system, glucose concentrations had to be low enough that the dissolved oxygen in the medium was sufficient to avoid oxygen limitation. As a result of the low concentrations of glucose, the time required for the thermogram to be completed was much shorter than that for the Tronac system.

We examined the heat production from glu-

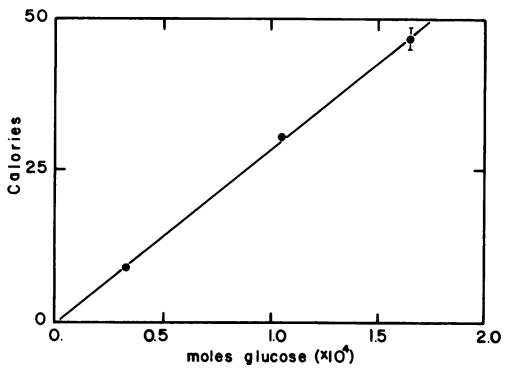

FIG. 6. Effect of glucose concentration on heat produced from glucose by *V. alginolyticus* in the Tronac calorimeter.

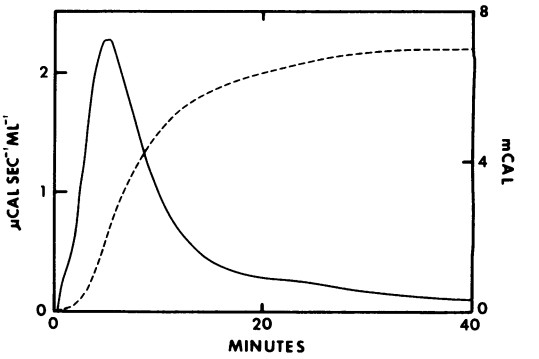

FIG. 7. Thermogram (solid line) and integration of the thermogram (dashed line) of *V. alginolyticus*. This experiment was performed in the LKB mixing calorimeter with 5×10^{-6} M glucose.

cose in this calorimeter from 7×10^{-6} to 3.5×10^{-7} M. A linear correlation between glucose present in the calorimeter and heat production was observed (Fig. 8). A linear fit of the combined data from both calorimeters has a correlation coefficient of 1.00. The data are graphically represented on a log-log plot (Fig. 9) because of the large range of data. The fit was done with nontransformed data.

DISCUSSION

These results show that calorimetry is a method that can be usefully applied to the study of marine bacteria at substrate concentrations equivalent to those that may be found in natural waters. Glucose concentrations reported in seawater range from undetectable ($<10^{-8}$ M) to approximately 1 μM (19, 23). The experiment with the mixing calorimeter is useful as a model for the response of marine bacteria to sudden increases in substrate concentrations.

The linear correlation between the amount of glucose present in the calorimeter and heat production indicates that, within the error of measurement, the loss of heat per mole of glucose utilized by this bacterium is independent of glucose concentration over the range studied. The heat generated per mole of glucose determined from the linear fit of the data is −320.3 kcal (−1,340 kJ)/mol. This corresponds to 48% of the heat of combustion of glucose (−670 kcal (−2,803 kJ)/mol). The remaining 52% is captured through biosynthetic reactions or left as residual in the medium. It must again be pointed out that a direct correlation between this "energetic efficiency" and carbon partitioning between biomass and respiratory end products cannot be assumed since the metabolic heat production under aerobic conditions is the sum of exothermic catabolic reactions and endothermic anabolic reactions occurring simultaneously. We have performed [$U-^{14}$C]glucose tracer studies with this bacterium in the same medium and found that 47 ± 5% of the glucose carbon is respired and 48 ± 5% is assimilated (A. S. Gordon, Ph.D. thesis, University of Miami, Coral Gables, Fla., 1982). It appears, then, that in this case the energetic efficiency and efficiency measured in terms of carbon partitioning between biomass and end products are indistinguishable within the experimental error.

Although the total heat produced per mole of glucose is the same, the shapes of the thermograms produced in the two calorimetry systems are distinctly different. This difference may be attributed to variation in the physiological state of the organisms when they are placed in the calorimeter, to the large difference in glucose concentration used in the two systems, and to differences in oxygen availability to the organisms. In experiments in which millimolar glucose concentrations were used, cells were introduced into the calorimeter in exponential growth phase and continued to grow until the glucose supply was exhausted. The thermogram, thus, exhibits an exponential increase until a maximum rate of heat production is reached. The glucose concentration controls the maximal rate of heat production and the duration of time of the exponential growth in the calorimeter (Fig. 5). Secondary peaks seen in the thermograms at millimolar glucose concentrations are similar to those observed in previous studies (11, 14). Such peaks were shown to be caused by accumulation and subsequent degradation of fermentation products in the medium. Production of fermentation products may occur in microzones of anaerobiosis in the reaction vessel due to imperfect aeration. When micromolar glucose concen-

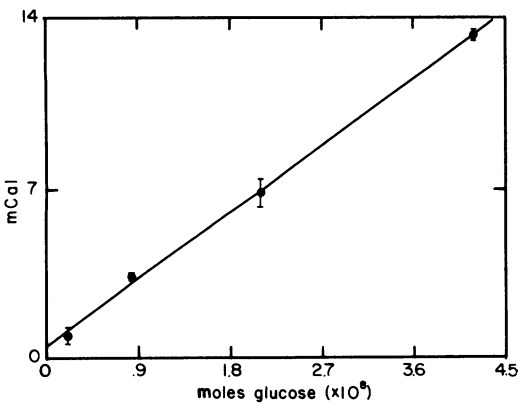

FIG. 8. Total heat produced by *V. alginolyticus* as a function of glucose concentration in the LKB mixing calorimeter.

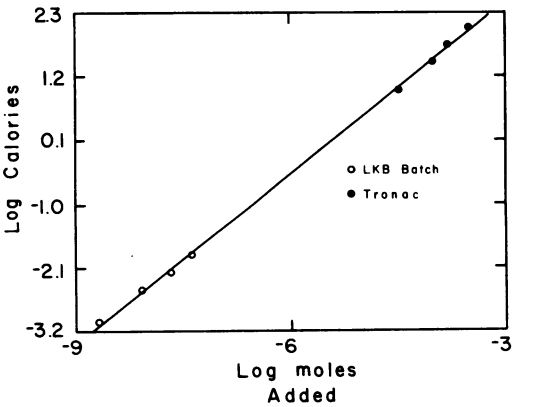

FIG. 9. Plot of heat production by *V. alginolyticus* in both the Tronac and LKB calorimeters as a function of the amount of glucose in the calorimeter.

trations were used, cells were introduced into the calorimeter in stationary phase. When the starved cells are mixed with glucose, there is an abrupt rise in the rate of heat production which is not attributable to bacterial multiplication since the onset of multiplication could not occur in this short time. Doubling time for this bacterium in the medium used is ca. 1.5 h. No growth could be detected by plate counts or turbidimetric measurement after experiments in this system because of the small amount of glucose added and the relatively large number of bacteria. Aeration of the calorimeter was not necessary due to the low glucose concentration. No anaerobic microzone formation would be expected in this system since dissolved oxygen was sufficient for glucose oxidation. The lack of a secondary peak is therefore not unexpected. We have, however, observed secondary peaks in the mixing calorimeter at much higher glucose concentrations (3.5×10^{-5} M) or when inorganic nutrient concentrations were greatly reduced (14; A. S. Gordon, F. J. Millero, and S. M. Gerchakov, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, N79, p. 186).

A direct relationship between initial glucose concentration and the maximum rate of heat production is apparent from the experiments performed at millimolar glucose concentrations in the Tronac calorimeter [(~25 μcal/s per ml)/(initial millimoles/liter)]. This ratio is approximately 20-fold greater in the experiments performed in the LKB mixing calorimeter at micromolar glucose concentrations. There is no a priori reason to expect that this ratio should be the same for both systems considering the differences between them. In the mixing calorimeter the peak heat production occurs almost immediately after mixing and would therefore correspond to a near maximum in glucose concentration in the reaction cell. In contrast, the maximum rate of heat production in the experiments performed in the Tronac calorimeter occurs just before glucose depletion. The ratio of these peak heights to the initial glucose concentration would not be expected to be comparable between the two systems, although the ratio should be reasonably constant within the individual systems as the glucose concentration is varied, if all other parameters remain constant.

The results of the present study are not consistent with the results of Lovrien et al. (17), who found that the heat per mole of glucose oxidized by *E. coli* increased with decreasing concentration. This discrepancy may be because *E. coli* is not as well adapted to life in nutrient-poor situations as is this marine bacterium.

The apparent constancy of the growth efficiency of this bacterium at the lowest substrate concentrations studied does not support the suggestion by some workers (20) that the growth efficiency of marine bacteria increases at low substrate concentrations. It is possible that these efficiency shifts occur at substrate levels lower than were used in the present study or that such shifts are limited to bacteria other than the one used in this study.

ACKNOWLEDGMENTS

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