A CALORIMETRIC INVESTIGATION OF THE GROWTH OF THE LUMINESCENT BACTERIA *BENECKEA HARVEYI* AND *PHOTOBACTERIUM LEIOGNATHI*

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ABSTRACT Direct calorimetric determinations of the rate of heat production along with simultaneous determinations of the rate of photon emission and the number of viable cells have provided insight into the growth of *Beneckea harveyi* and *Photobacterium leiognathi*. These experiments were performed with a Tronac isothermal microcalorimeter modified with a fiber optic light guide to allow *in situ* detection of light. *Escherichia coli* and a dark variant of *P. leiognathi* were also examined to provide points of reference. It is demonstrated that *B. harveyi* seems to pause in the rate of metabolic heat production at the same point in time that the enzyme luciferase begins to be synthesized. This effect is not removed if *B. harveyi* is grown in conditioned medium. The thermograms for all species are correlated with cell generation time. The heat production per cell indicates that uncrowded cultures produce more heat than older, more crowded cultures, supporting the original observation of Bayne-Jones and Rhees (1929). These observations reopen for examination the suggestion that living systems tend toward a state of minimum metabolism per unit mass.

INTRODUCTION

As part of a thermodynamic investigation of bacterial luminescence involving calorimetric studies of flavin mononucleotide and of in vitro bioluminescence, (Beaudette and Langerman, 1974), (Mangold and Langerman, 1975), (Langerman and Mangold, 1974), we have begun to examine some of the thermal characteristics apparent during growth of luminescent bacteria. Our original goal in these studies was to establish a correlation between metabolic heat production, as observed calorimetrically, and light emission. The observations reported here demonstrate the extreme complexity of such experiments performed in vivo and thus limit definitive conclusions. The observations, however, are sufficiently interesting to warrant detailed investigation.

Bacterial luminescence has been extensively studied. Recent efforts have included a taxonomic study (Reichelt and Baumann, 1973) as well as detailed biochemical investigations of individual species. A wide variety of microbiological studies (Nealson, et al., 1970, Nealson and Markovitz, 1970; Ulitzer and Yashphe, 1975; Cline and Hastings, 1971, 1974*a*,*b*) have elucidated information about cellular control of synthesis and of cofactor requirements, as well as details concerning a variety of mutants.

An equally intensive series of studies concerning the in vitro chemistry (Hastings, 1968; Eberhard and Hastings, 1972; Nicoli and Hastings, 1974) has resulted in the elucidation of stoichiometry, mechanism, and thermodynamic parameters (Langerman and Mangold, 1974; Mangold and Langerman, 1975).

These various studies indicate that in vivo light emission is the result of a two-enzyme system that catalyzes the reactions:

$$H^+ + NADH + FMN \xrightarrow{\text{reductase}} NAD^+ + FMNH_2$$

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + H_2O + RCOOH + h\nu$$

The photon energy is about 60 kcal/einstein^{-1} and depends on the species and the type of aliphatic aldehyde, RCHO.

The use of calorimetry to investigate in vivo metabolic processes has been limited (Forrest, 1969; Johansson, et al., 1975; Belaich and Belaich, 1976*a*,*b*; Poole and Haddock, 1975; Belaich, et al., 1968, Murgier and Belaich, 1971); however, the results obtained are quite interesting. For organisms having a simple degradative pathway for carbohydrates, the energy-limited thermograms were very easy to interpret and were essentially what was predicted from the heat of degradation of the energy substrate. Recent investigations have been directed toward more complex systems. Belaich and Belaich (1976*a*,*b*) have studied the anaerobic growth of *E. coli* and have measured the affinity of the whole cell for various substrates. Poole and Haddock (1975) have examined *E. coli* with genotypically and phenotypically modified electron transport chains and have correlated their observations with the simultaneous determination of oxygen utilization. In our present study, we extend these efforts to luminescent bacteria and report data obtained by simultaneously observing the heat flux in millicalories per second, the light emission intensity, and the number of viable cells.

METHODS

Calorimetry

All calorimetric experiments were performed in a modified Tronac, Inc. (Orem, Utah) 550 isothermal calorimeter. The basic design of the modified isothermal cell is shown in Fig. 1. The major modification of the commercial instrument involved moving the stirrer off-center and installing a 6.4-mm fiber optic light guide in a position to measure the light emission from the solution. The light emission was detected by a simple photometer (Mitchell and Hastings, 1971) and the amplified signal was recorded. Calibration with the light standard of Hastings and Weber (1963) indicated a transmission efficiency of about 3% from the calorimeter to the photometer. A signal proportional to the heat flux, $\dot{q}$, (in millicalories per second), was detected by the calorimeter circuitry and simultaneously recorded on the same chart as the light signal. Periodically, an 0.1-ml sample was removed from the calorimeter, serially diluted, and plated. From this, the number of viable cells in the calorimeter at a given time was obtained. This also allowed the detection of possible contamination. The calorimeter was routinely filled with 15 ml of sterile medium and inoculated with 1 ml of an 8-h growth of the bacteria of interest. All experiments were performed at 25.00 ± 0.01°C. The calorimeter was calibrated electrically.

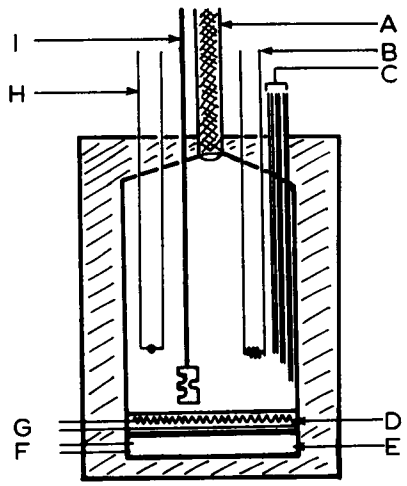

FIGURE 1 Schematic outline of Tronac 550 isothermal calorimeter cell modified with fiber optic light guide. (*A*) Light guide; (*B*) calibration heater; (*C*) sample inlet tubes; (*D*) controlled heater; (*E*) Peltier cooler; (*F, G*) electrical connections; (*H*) control thermistor; (*I*) stirrer.

Air at the same chemical potential as the calorimetric solution was continuously bubbled into the calorimetric vessel by a small air pump. Control experiments using medium alone demonstrated the reproducibility and lack of interference of the bubbling method.

Species

The strains of bacteria examined included *Beneckea harveyii* (formerly MAV), *B. harveyi,* strain MB-20, *Photobacterium leiognathi* (strain S_1), a noncharacterized dark variant of *P. leiognathi* isolated in this laboratory, and *E. coli* B. The photobacteria were growh in a complete sea water medium (Nealson, et al., 1970) while the *E. coli* was grown in Difco nutrient broth (Difco Laboratories, Detroit, Mich.).

RESULTS AND DISCUSSION

The results of our observations are presented in Fig. 2–7. These are graphs of heat flux and light flux (for the luminous bacteria) and viable population as a function of time. The initial experiments dealt with *B. harveyi,* and provided an indication of the complexity of the processes being examined. This species of bacteria has been demonstrated to have a complex growth pattern that involves conditioning of the medium by the growing cells (autoinduction) (Nealson, et al., 1970) as indicated in Fig. 2. At the point when the luciferase begins to be expressed, as indicated by the increased light flux, a noticable, reproducible decrease in heat flux occurs (dashed vertical line). This occurs concurrent with the first doubling of the cell population and thus suggests that it is due to endergonic anabolic metabolism. To investigate these observations further, conditioned medium was prepared by allowing *B. harveyi* to grow in complete medium up to the point of induction of luciferase, removing cells by centrifugation, sterilizing the medium with a Millipore filter (Millipore Corp., Bedford, Mass.), and then re-

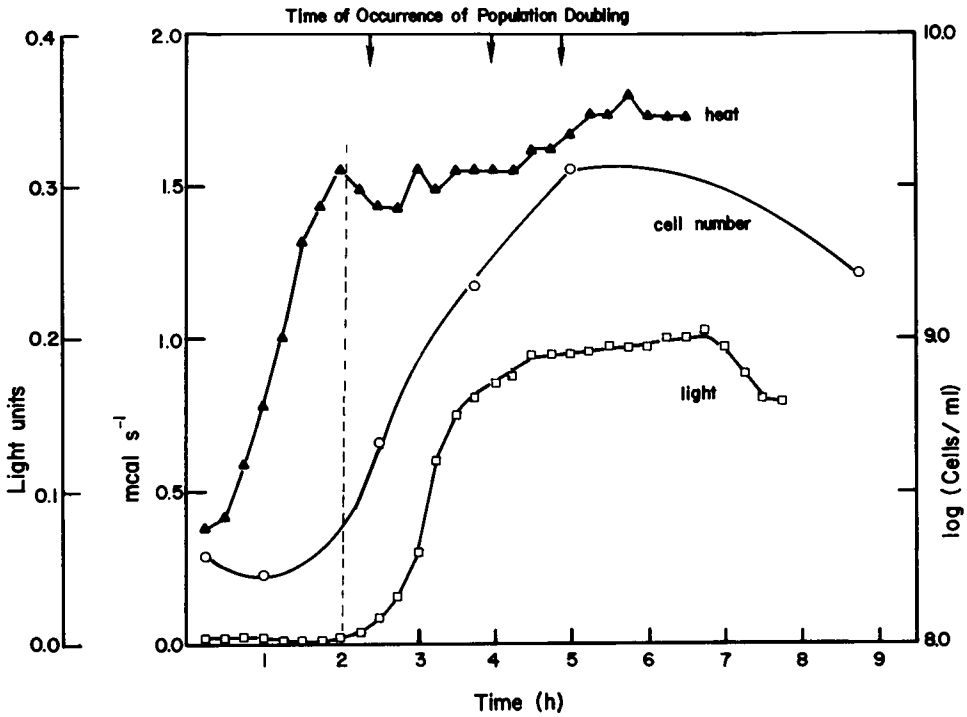

FIGURE 2 Thermogram combining light production, expressed in light units (1 light unit = 1.44×10^{12} photons·s^{-1}), and viable cells (per milliliter) for *B. harveyi* in complete medium. The scale at the top indicates the point in time when each population doubling occurred during each experiment.

inoculating with fresh *B. harveyi*. These data are reported in Fig. 3. It is quite apparent that all of the characteristics of the thermogram are altered in conditioned medium. Both the time-course and the absolute magnitude of the curves change. Under these conditions a decrease in heat flux is still noticable concurrent with the second cell-doubling period. Thus, we may suggest that this particular effect is related to a "poising" of a significant fraction of the culture for cell division.

B. harveyi, strain MB-20, also studied in the calorimeter (Fig. 4), produces large quantities of luciferase and in the environment of the calorimeter demonstrated a very short doubling time. The thermogram does not indicate any apparent "poising" of the culture at times corresponding to the early doublings.

E. coli B was examined under aerobic conditions to provide reference data for further experiments. These data are presented in Fig. 5. The thermogram is quite complex, but the large excursions in heat flux have been previously attributed to changes in the energy source utilized by the bacteria (Belaich, et al., 1968; Murgier and Belaich, 1971).

To investigate luminescent bacteria further by calorimetric observation, a brighter strain of bacteria was obtained. This species, *Photobacterium leiognathi* (a slant of

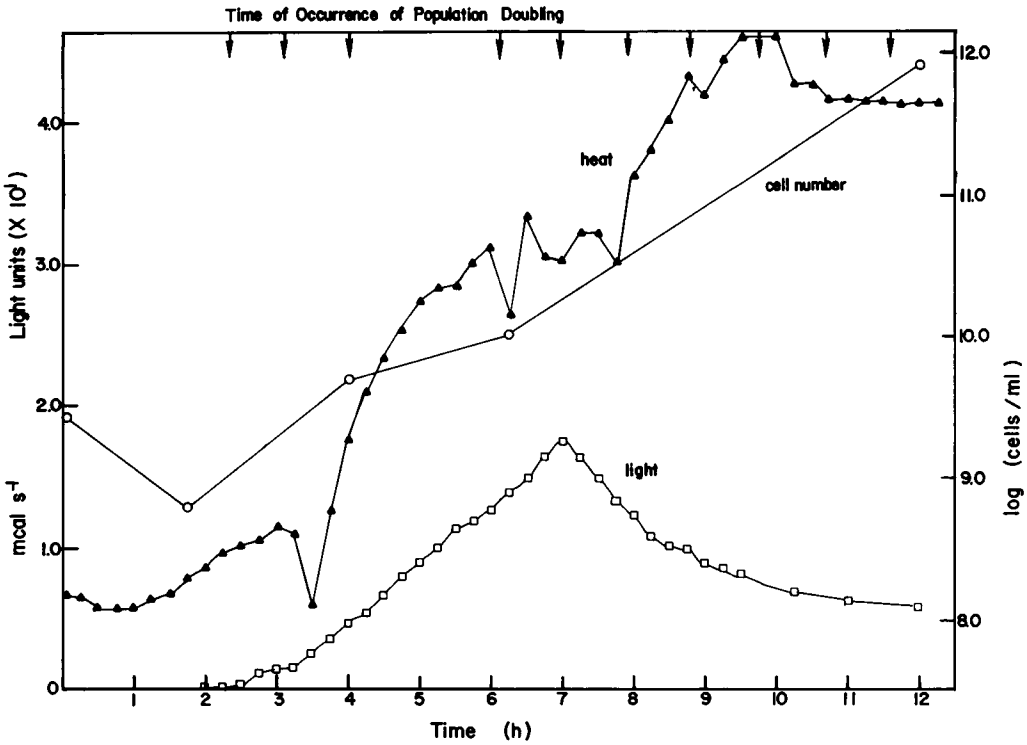

FIGURE 3 Combined thermogram for *B. harveyi* in conditioned medium. See legend for Fig. 2 for details.

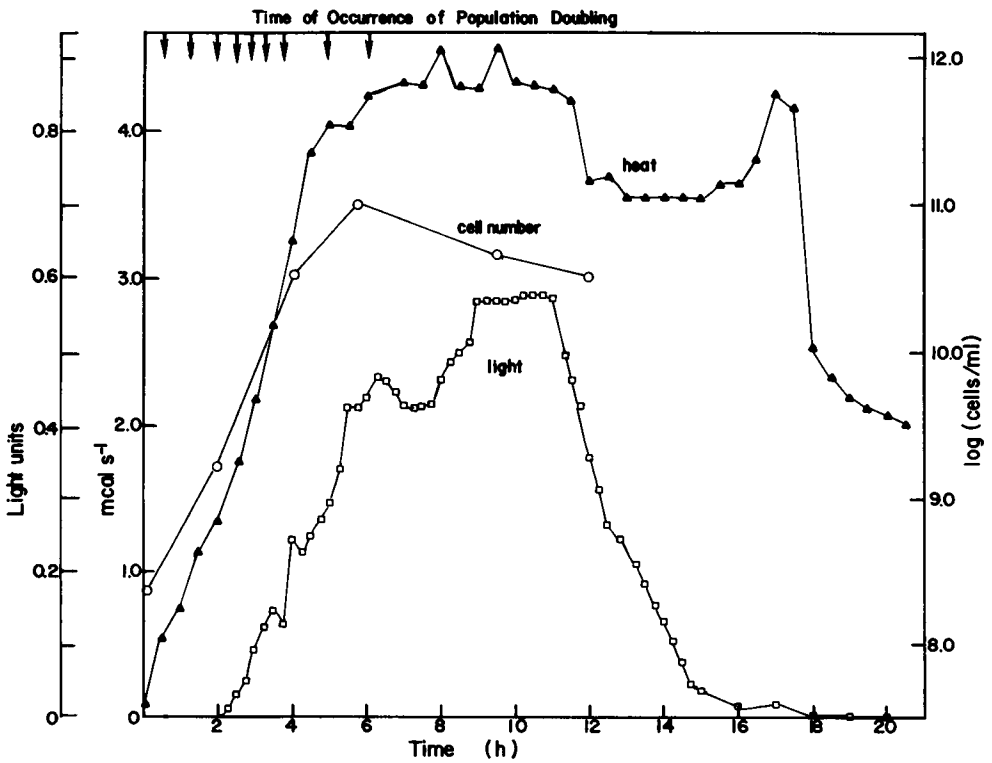

FIGURE 4 Combined thermogram for *B. harveyi* strain MB-20 in complete medium. See legend for Fig. 2 for details.

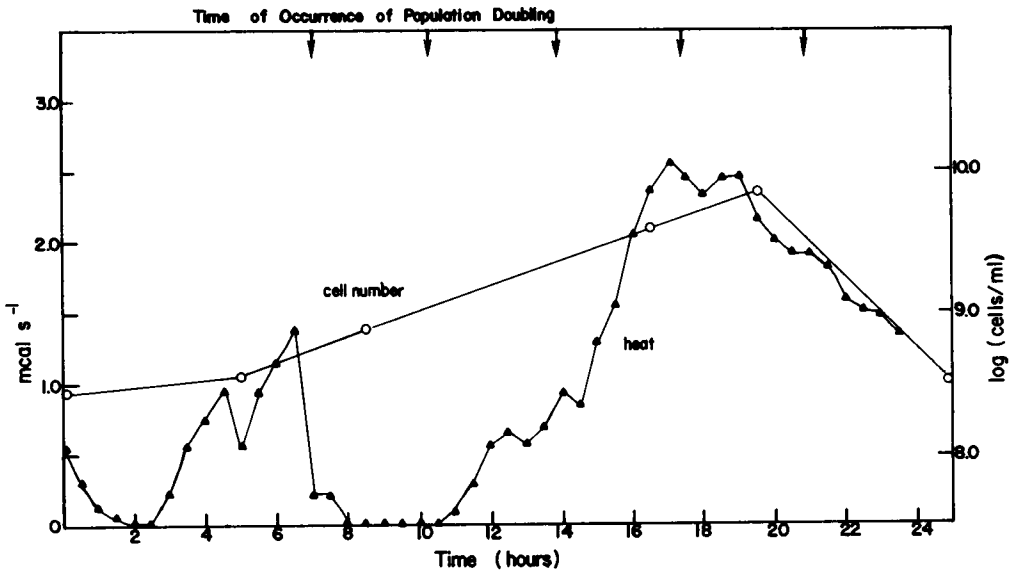

FIGURE 5 Combined thermogram for *E. coli* in nutrient broth. See legend for Fig. 2 for details.

this species was obtained from Dr. S. Ulitzur, Visiting Professor, Harvard University) was found to produce the growth curve shown in Fig. 6. Interestingly, this species does not exhibit the repression-derepression shown by *B. harveyi* and does not show any dramatic decrease in heat flux during the early stages of growth. For comparison, a dark variant of this species was isolated and grown in the calorimeter. These data are shown in Fig. 7.

The heat flux values observed in these experiments ranged from 0.5 to 6 mcal $\cdot s^{-1}$. It is reasonable to determine what portion of this heat is due to emitted light being reabsorbed by the opaque walls of the calorimeter. At a total light flux of 200 light units or 7×10^{12} photons s^{-1}, the maximum heat flux is 0.7 μcal s^{-1}, a very small portion of the total signal. This is not surprising since the total number of moles of substrate being used by the bacteria must be greatly in excess of the 1×10^{-11} mol of photons being released each second. Previous work on *B. harveyi* (Mangold and Langerman, 1975) has demonstrated that at least 50% of the available free energy in the in vitro luminescent system, i.e., reduced flavin, aldehyde, and O_2, is used to produce a photon, the remainder being released largely as heat. Even under these conditions, reabsorbed photons make an insignificant contribution to the total heat flux.

It is useful to examine a major contribution to the observed heat flux. If we assume that the net oxygen flux gives a good indication of the overall substrate utilization, then we can calculate the heat flux due only to oxygen utilization. With a value of 1 nmol O_2/ml-min-10^6 cells (obtained at Po_2 = 1 atm, S. Ulitzur, personal communication), an exothermic heat flux of 17 mcal $\cdot s^{-1}$ is calculated. This value appears quite large when compared to the numbers we actually observed, but it must be

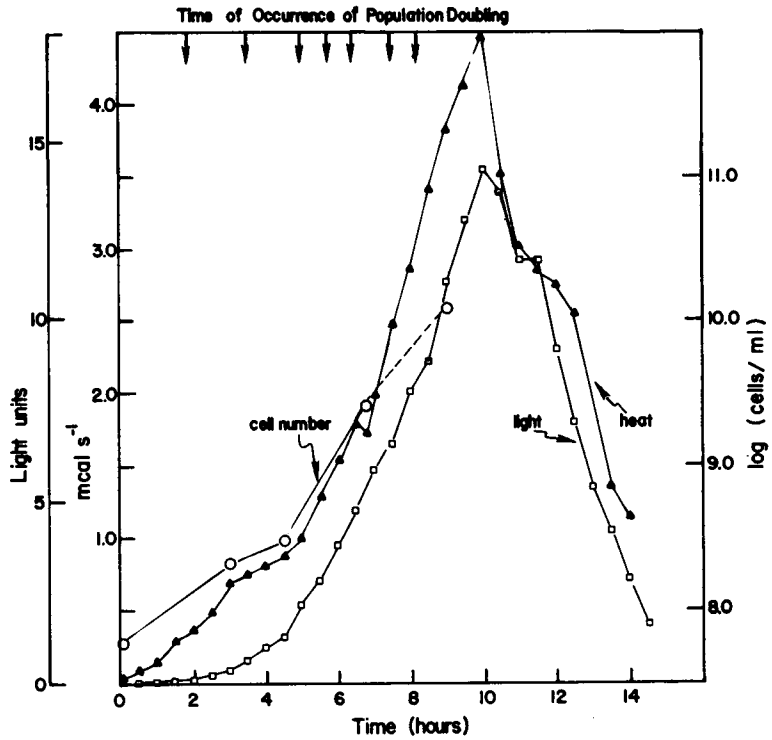

FIGURE 6 Combined thermogram for *P. leiognathi* in complete medium. See legend for Fig. 2 for details.

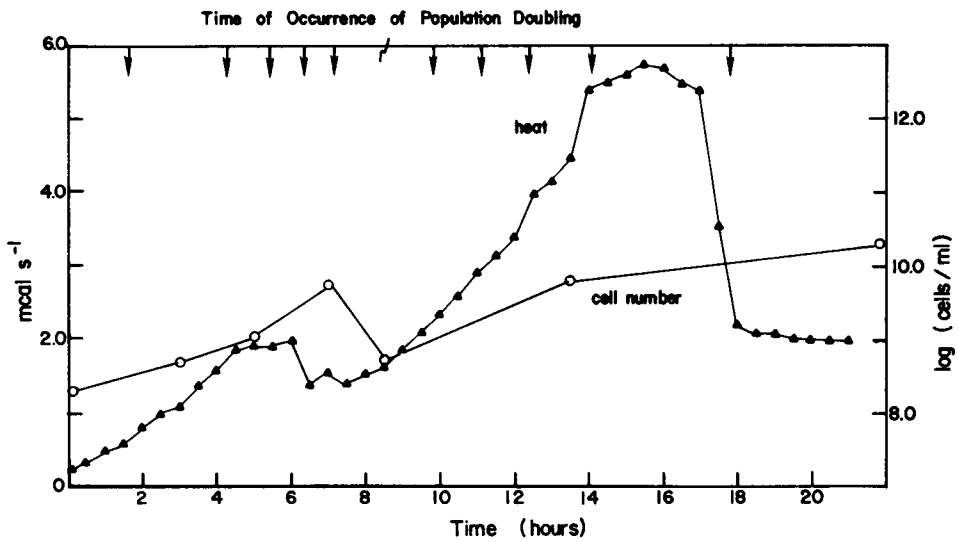

FIGURE 7 Combined thermogram for the dark variant of *P. leiognathi* ("USU-1") in complete medium. See legend for Fig. 2 for details. The break in the upper scale is a result of the marked decrease in population at 7 h.

realized that anabolic processes require energy, thus further *lowering* the metabolic heat flux observed.

Data similar to ours have been extensively examined for *E. coli, Staphylococcus aureus* (Bayne-Jones and Rhees, 1929), and *Aerobacter aerogenes.* (Stoward, 1962*a*,*b*) The original interpretation of these data was that "young cells produce more heat than old ones" (Bayne-Jones and Rhees, 1929). However, Forrest et al. (1961) disagreed with both the data and the conclusion. Stoward (1962*a*) later supported the original observation and extended the interpretation to test the theorem of minimum entropy production. Our data fully support Bayne-Jones and Rhees and substantiate the experimental work of Stoward, which was seriously challenged by Forrest and Walker (1962). The most straightforward interpretation of the heat production per cell, based on the work of Prigogine, (1956) and the theorem of minimum entropy production, is that living systems tend toward a state of minimum metabolism per unit mass.

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