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Rates of Digestion of Bacteria by Marine Phagotrophic Protozoa: Temperature Dependence†

BARRY F. SHERR,[1]* EVELYN B. SHERR,[1] AND FEREIDOUN RASSOULZADEGAN[2]

University of Georgia Marine Institute, Sapelo Island, Georgia 31327,[1] and Station Zoologique, 06230 Villefranche-sur-mer, France[2]

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The effect of temperature on length of time for digestion of bacteria was evaluated, by using fluorescently labeled bacteria (FLB), for phagotrophic flagellates and ciliates isolated from coastal northwest Mediterranean waters. Accumulation of FLB in protozoan food vacuoles was followed until a plateau of FLB per cell occurred; then after a 1:10 dilution of FLB with unlabeled bacteria, disappearance of FLB in food vacuoles was monitored. For both 3- to 5-μm flagellates and 10- to 40-μm ciliates, the absolute linear slopes of FLB uptake and disappearance were nearly identical in individual experiments over a temperature range of 12 to 22°C. We inferred from these results that the leveling off of the uptake curves resulted when equilibrium between ingestion and digestion of bacteria was attained. The time to leveling off then represented the average time needed for complete digestion of the bacteria ingested at the start of the experiment, and the inverse of this time represented a bacterial digestion rate. The digestion rate increased exponentially from 12 to 22°C for both a mixed flagellate assemblage and the oligotrichous ciliate *Strombidium sulcatum*, with a Q_{10} of 2.8 for the flagellates and 2.0 for the ciliate. Although bacterial ingestion rates varied greatly, depending on protozoan cell size, total bacterial abundance, and temperature, digestion times appeared to be significantly influenced only by protozoan cell size (or type of protozoan) and by temperature.

Studies to date of digestive processes in bacterivorous protozoa have for the most part concerned rates of formation and disappearance of food vacuoles in species of the hymenostome ciliates *Tetrahymena* and *Paramecium* (6, 8). In addition, previous studies of the processes of phagotrophy and digestion in bacterivorous protozoa have typically involved quantification of uptake and/or egestion of dye particles and glass or plastic microspheres (5, 6–8, 16). Use of inert dye particles and microspheres likely does not provide an optimal model for phagotrophy in all protozoa. At least some marine bacterivorous flagellates and spirotrichous ciliates take up plastic microspheres at much lower rates than they do bacteria (10, 15). Also, retention of inert materials can provide information only on the processing of food vacuoles, not on the actual rate of digestion of food.

A few workers have utilized natural foods to investigate protozoan digestion processes. Fenchel (3) directly measured disappearance of bacteria and algae in food vacuoles of several species of freshwater ciliates to estimate digestion rates of food. Taneda and Ohno (19) used heat-killed bacteria stained with Congo red to investigate food vacuole formation in *Paramecium caudatum*. Nilsson (9) studied the time course of events in digestion of *Escherichia coli* cells in food vacuoles of *Tetrahymena* sp. via electron microscopy.

We have suggested that uptake of fluorescently labeled bacteria (FLB) may be used as a sensitive assay of protozoan bacterivory (15). Since FLB can be metabolized to support growth of flagellates and ciliates at rates similar to those on unstained bacteria, we also proposed that FLB would be a useful tool for the analysis of rates of bacterial digestion in protozoa (15). Our preliminary experiments showed that linear uptake of FLB ceased after 10 to 30 min at room temperature (23 to 25°C) (15). We inferred that this occurred when uptake of FLB equilibrated with digestion of FLB in the protozoa. Other workers, monitoring the ingestion of cyanobacteria and microspheres by phagotrophic protozoa, have obtained similar patterns of uptake; however, they speculated that digestion-egestion of bacteria or spheres also occurred during linear uptake (7, 16), leading to underestimation of actual ingestion rates.

To address the potential problem of rapid digestion of bacteria in protozoan grazing assays based on uptake of FLB, we compared the rates of uptake and of disappearance of FLB in food vacuoles of both flagellates and ciliates to determine the actual time spans required for digestion of bacteria in marine phagotrophic protozoa. We analyzed bacterial digestion times over a 10°C range of temperature as well as over different concentrations of bacteria. Our study is the first of which we are aware in which digestion of bacteria has been directly analyzed in phagotrophic flagellates and spirotrichous ciliates.

MATERIALS AND METHODS

Preparation of FLB. FLB were prepared as described by Sherr et al. (15) from a yeast extract enrichment culture inoculated with a mixed bacterial assemblage from protozoan wheat grain cultures. The cultured bacteria were grown up to about 10^8 cells ml^{-1} and concentrated by centrifugation. For one experiment with an in situ assemblage of pelagic ciliates, we prepared FLB from bacterioplankton (10^6 cells ml^{-1}) present in 10 liters of 0.8-μm-screened surface seawater. The bacterioplankton were concentrated with an Amicon hollow-fiber filter (15). By measuring dimensions of DAPI-stained (11) bacteria at ×2,000 magnification, we determined that cultured bacteria had an average bio-

* Corresponding author.
† Contribution no. 600 of the University of Georgia Marine Institute.

volume of 0.3 μm^3 and that in situ bacterioplankton had an average biovolume of 0.06 μm^3.

Protozoan cultures. All protozoa used in the ingestion-digestion experiments were isolated from surface seawater at point B, a routine sampling station at the mouth of Villefranche Bay on the coast of the northwestern Mediterranean Sea. The water at this site can be characterized as meso- to oligotrophic, according to classifications in the literature (see reference 18 for a review). A diverse population of phagotrophic flagellates and ciliates is typically observed in these waters (12, 13).

A mixed assemblage of 3- to 5-μm colorless flagellates was cultured from 5-μm-screened seawater amended with 11 mg of yeast extract $liter^{-1}$. The flagellate culture, which attained a population density of 7×10^4 cells ml^{-1}, was dominated by ovoid monads with one short and one long flagellum and a spherical flagellate with two short flagella of equal length.

Ciliates used in the experiments included two species originally isolated from point B water and maintained on wheat grain culture by F. Rassoulzadegan: *Strombidium sulcatum*, a 20- by 30-μm spirotrich, and *Uronema marina*, an 8- by 22-μm scuticociliate. These ciliate cultures attained densities of 100 to 1,000 cells ml^{-1}. We also cultured a mixed assemblage of phagotrophic ciliates from point B water to a density of 5 cells ml^{-1} by holding 20 liters of 50-μm-screened water in the laboratory for several days. The ciliate assemblage was dominated by 15- to 40-μm spirotrichs.

Experimental protocol. The rationale of the experimental design was to monitor uptake of FLB until FLB per protozoan cell stopped increasing with time and then to effectively inhibit further uptake of FLB by the protozoa via a 10-fold dilution with seawater containing the same concentration of unlabeled bacteria as found in the initial culture. After dilution, the decrease in FLB per cell with time should correspond to digestion or egestion of FLB by the protozoa. Since a low rate of uptake of FLB would still be expected in the diluted treatment, a dilution control was set up for each experiment. The control consisted of protozoan culture to which the same absolute amount of FLB initially used was added only after dilution with seawater plus bacteria; the FLB per cell was followed over time as in the diluted treatment.

Protozoan cultures in the stationary phase of growth were held overnight at the incubation temperature at which the ingestion-digestion experiment was to be run. Room temperatures of 19.5 to 20°C were used for some experiments. Higher and lower temperatures were attained by using a Huber (W. Germany) type 302 temperature-controlled water bath and a Bioblock Scientific Polysta 22 incubator.

Each experiment was conducted in two 400-ml Whirl-pak bags presoaked in 10% HCl and copiously rinsed. One bag received 100 ml of protozoan culture, and the second, used for the dilution control, received 50 ml of culture. FLB prepared from cultured bacteria were inoculated into the first bag at concentrations of from 5 to 50% of the total live bacteria, precounted in the culture by using DAPI staining and epifluorescence microscopy (11).

After the addition of FLB, 5-ml samples were taken over 1 to 4 h. Samples were fixed with a Lugol-Formalin decoloration technique, which prevented preservation-induced egestion of FLB by protozoa (16) and reduced the lysis of ciliates normally observed in samples preserved with Formalin alone (F. Rassoulzadegan, E. Sherr, and B. Sherr, manuscript in preparation). Preserved protozoan samples were stained with DAPI and filtered onto Nuclepore black membrane filters (0.8-μm pore size). Filters were mounted onto slides with immersion oil and inspected via epifluorescence microscopy with a Zeiss photomicroscope outfitted with a 50-W mercury lamp. DAPI-stained protozoa were located by using Zeiss filter set 47 77 02 at a magnification of ×1,250 for flagellates and of ×100 for ciliates, and then Zeiss filter set 47 77 09 was used to enumerate FLB within each protozoan at a magnification of ×1,250 (15).

At the end of the initial time course of sampling, 50 ml of the remaining culture was diluted with 450 ml of 0.2-μm-filtered seawater containing appropriate numbers of bacteria grown in wheat grain culture to approximate the total concentration of bacteria initially in the protozoan culture. This treatment served to decrease further uptake of FLB to a 10-fold-lower rate while maintaining the same rate of bacterivory by keeping the total bacterial concentration constant. The second bag containing 50 ml of protozoan culture without FLB was also diluted with 450 ml of seawater plus bacteria, and at the same time FLB were added at half the amount that was originally inoculated into the 100 ml in the first bag. Thus the second, dilution control bag would have the same concentration of FLB as in the diluted treatment experimental bag, and actual uptake of FLB at the diluted concentration could be monitored. Samples (20 ml) were taken over a second time course of 1 to 4 h to follow the decrease of FLB per protozoan cell with time in the experimental bag and increase of FLB per cell with time in the dilution control bag.

The experimental design was modified in the case of the mixed ciliate assemblage to allow for the lower numbers of ciliates per milliliter. A 100-ml sample of ciliate culture was added to each of eight Whirl-pak bags, and FLB prepared from natural bacterioplankton were added to a final concentration of 10^5 ml^{-1} to seven of the bags at the start of the experiment. Samples (50 ml) were taken from separate bags at 20-min intervals for 2 h. Then 50 ml was removed from the eighth bag, and all bags were diluted with 450 ml of 5-μm-screened seawater taken from the 20-liter carboy in which the ciliates had been cultured. At the same time, FLB were added to the eighth (control) bag to yield 10^4 FLB ml^{-1}. The total 500-ml samples in each bag were sequentially preserved over a second 2-h time course. The sample in the dilution control bag was preserved after 60 min. Samples were examined for FLB per protozoan cell as described above, except that the samples were filtered onto 3-μm-pore-size Nuclepore filters because of the greater sample volume. Although the majority of ciliates in the mixed spirotrich population took up FLB, some species were never observed with ingested FLB; these species were not included in calculating FLB per cell for the mixed-ciliate experiment.

In all cases, from 30 to 80 protozoa were inspected for each time period in each experiment to determine average FLB per cell. The FLB disappearance curves were corrected for FLB uptake at the 1:10 dilution by using values of FLB per cell obtained with the dilution control samples. Slopes of increase and decrease of FLB per cell were determined via regression analysis for the linear portions of the curves for each ingestion-digestion experiment.

RESULTS

In all experiments, there was a linear rate of increase of FLB per protozoan cell to a maximum value, after which FLB per cell remained constant or showed a slight increase with time (Fig. 1 and 2). After dilution with unstained bacteria, FLB per cell declined at a linear rate for a period of time corresponding approximately to the time of duration of

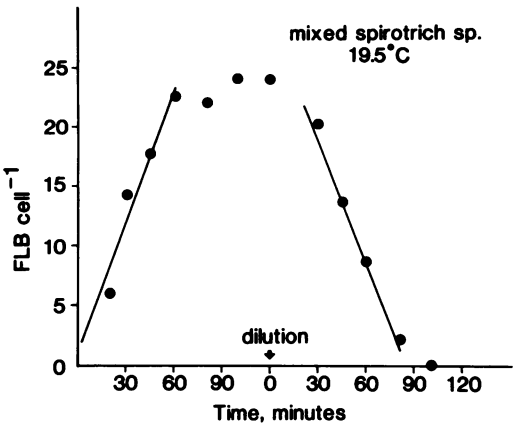

FIG. 1. Representative time course of ingestion-digestion for marine ciliates feeding on bacteria.

the linear portion of the uptake curve. However, FLB per cell typically showed a leveling off toward the end of the disappearance curve (Fig. 1 and 2). Visually, FLB within protozoan food vacuoles became noticably thinner and fluoresced less intensely before disappearing. We do not know whether disappearance resulted from loss of the stain or from egestion of the undigested portion of the FLB. Tiny fluorescent particles were often observed within food vacuoles; we interpreted these as fragments of FLB.

Calculated rates of FLB uptake and FLB disappearance for the flagellate and ciliate experiments are presented in Table 1. The correlation coefficients for the linear regression fits to the data (Statpak ONEVREG program; Northwest Analytical, Inc., Portland, Oreg.) were >0.97 in all cases. There was no significant difference in absolute rate between each pair of uptake-disappearance slopes, determined by the F test for the difference between the absolute regression coefficients (17). The elapsed time between the addition of FLB and leveling off of the uptake curve ranged from 25 min for *U. marina* at 22°C to 95 min for *S. sulcatum* at 12°C (Table 1).

Since the rates of FLB uptake and disappearance were essentially the same, the leveling off of the FLB uptake curve can be interpreted as the attainment of equilibrium between ingestion and digestion-egestion of FLB. The time from initiation of the experiment to leveling off of the uptake curve (Table 1) is thus a measure of the time required to completely process, or digest, bacterial cells within protozoan food vacuoles.

The times of duration of digestion were converted to bacterial digestion rates. These rates showed a highly significant (correlation coefficients, >0.98), exponential increase with temperature (best described by the equation $y = a \times e^{(b \times x)}$; Statpak ONEVREG program) (Fig. 3). Two separate relationships were obtained for the mixed flagellate species and for *S. sulcatum* and mixed spirotrich species (Fig. 3). The flagellate plot of digestion rate versus temperature was characterized by higher digestion rates as well as by a steeper slope (regression coefficient [*b*], 0.106) compared with the ciliate plot (*b*, 0.069). Calculated Q_{10} values between 12 and 22°C were 2.8 for the flagellates and 2.05 for the spirotrichs. The digestion rate obtained for *U. marina* at 22°C was considerably higher than that found for *S. sulcatum* at this temperature (Fig. 3).

The per cell clearance and total bacterial ingestion rates (FLB plus unstained bacteria) calculated from the uptake slopes in each experiment are presented in Table 2. For flagellates, clearance varied from 1.4 to 4.3 nl cell^{-1} h^{-1}, and bacterial ingestion varied from 5.2 to 27.4 bacteria cell^{-1} h^{-1}. For ciliates, clearance ranged from 57 to 413 nl cell^{-1} h^{-1}, and bacterial ingestion varied from 380 to 1,095 bacteria cell^{-1} h^{-1}. The cell clearance and ingestion rates appeared to be influenced by total bacterial abundance as well as by temperature (Table 2), although we did not carry out sufficient separate experiments at each temperature to define these relationships.

DISCUSSION

The similarity of absolute rates of FLB uptake and disappearance found in individual experiments strongly supports the interpretation of the plateau in FLB per cell as an equilibrium between ingestion and digestion or egestion of FLB. The leveling off of the FLB uptake curve should occur at the moment at which the first FLB ingested have been completely digested, or egested, so that they are no longer visible in the protozoan food vacuoles. After this time, for every FLB taken in, another FLB which had been in the protozoan cell for the required digestion-processing time would no longer be visible, and the FLB per cell would be relatively constant so long as the protozoa maintained the same rate of feeding. The time from addition of FLB to the leveling off of the FLB uptake curve then represents the average duration of digestion of bacteria in the protozoa.

Fok and Valin (5) identified a two-phase digestive cycle in *P. caudatum*: a processing period, during which food contents of recently formed vacuoles are digested, and a vacuole defecation period, during which the digestive vacuoles are egested. If this model is applicable to other protozoa, then the processing period may be identified with the duration of linear FLB uptake in our experiments. For *P. caudatum*, few if any digestive vacuoles are egested during the processing period (4, 6). We suggest, then, that newly ingested FLB remain in the protozoan cell for at least as long as the processing period and are egested thereafter only if they have not been completely digested by that time.

The duration of bacterial digestion, or the processing period, was on the order of tens of minutes for both flagellates and ciliates within the 10°C span of temperature investigated. In our earlier study, we found a plateau in FLB per cell after 15 min for mixed 10- to 15-μm spirotrichous ciliates at 25°C (15). This is the shortest inferred time of

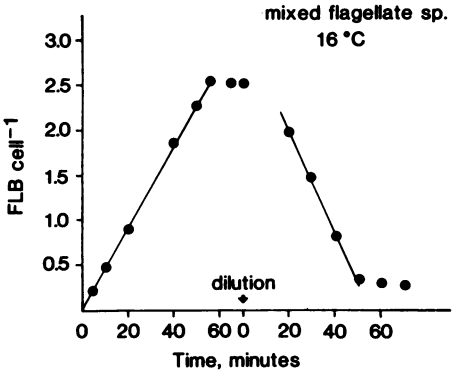

FIG. 2. Representative time course of ingestion-digestion for marine flagellates feeding on bacteria.

TABLE 1. Summary of uptake of bacteria by bacterivorous flagellates and ciliates incubated at 12 to 22°C

Protozoan prepn	Temp (°C)	Bacteria (10^6 ml^{-1})		Rate (FLB cell^{-1} min^{-1}) of:		Time to leveling off of uptake curve (min)
		Live[a]	FLB	Uptake	Disappearance	
Mixed 3- to 5-μm flagellates	12	2.8	1.0	0.023	−0.020	80
	16	5.3	0.9	0.050	−0.060	55
	19.5	1.8	0.7	0.025	−0.030	38
	20	4.0	1.1	0.078		34
	20	4.5	4.7	0.234		34
S. sulcatum	12	6.6	0.1	0.11		95
	16	10.4	0.1	0.13	−0.13	70
	19.5	2.6	0.1	0.62	−0.60	60
	22	8.6	0.2	0.28		45
Mixed spirotrichs	19.5	1.4	0.1	0.37	−0.35	60
U. marina	22	14.0	0.4	0.34	−0.30	25

[a] Live bacteria were enumerated via DAPI staining. Heat-killed FLB do not stain with DAPI.

bacterial digestion we have observed. Our results are in general agreement with those of other workers. Fenchel (3) reported digestion times of 30 min to 5 h for two bacterivorous ciliates within the temperature range of 7 to 25°C. Fok and Shockley (4) found that the processing period in *Tetrahymena* sp. was 45 min at room temperature, and Nilsson (9) reported that mass digestion of bacteria occurred at around 30 min for *Tetrahymena* sp. at 28°C.

Plots of digestion rate versus temperature showed that the flagellates had faster rates of bacterial digestion than did the spirotrichous ciliates (Fig. 3), in keeping with the general axiom that for protozoa, as for other animals, metabolic activity increases with decreasing body size (1, 2). The 25-min digestion time found for *U. marina* at 22°C did, however, fall approximately on the flagellate plot, even though *U. marina* had a 20-fold-larger biovolume than the flagellates (700 μm^3 compared with 35 μm^3). It is possible that strictly bacterivorous protozoa are able to degrade bacteria more rapidly compared with protozoa such as *S. sulcatum*, which ingest both algae and bacteria (14).

Digestion rate must also be affected by the nature of the ingested food. Nilsson (9) reported that a few newly ingested bacteria were lysed in *Tetrahymena* sp. food vacuoles within 5 min and that some bacteria remained intact after 90 min. She related this in part to differential thicknesses of the cell walls of the bacteria. Our observations that FLB per cell often showed a gradual increase after attainment of the initial plateau (Fig. 1) and that FLB per cell did not always go to zero after dilution (Fig. 2) may be interpreted in a similar way.

The rates of per-protozoan cell clearance and bacterial uptake were affected by protozoan cell size and bacterial abundance as well as by temperature (Table 2). These parameters would undoubtedly be influenced by other factors such as the growth state of the protozoa. In this study, bacterial digestion rate appeared to be responsive only to cell type and to temperature. Further work will be needed to more precisely determine the effects of protozoan size, growth state, and feeding rate and the nature of the food on the digestion rate. It is clear, however, that FLB are not digested immediately or randomly and can be successfully used for analysis of rates of protozoan bacterivory and bacterial digestion.

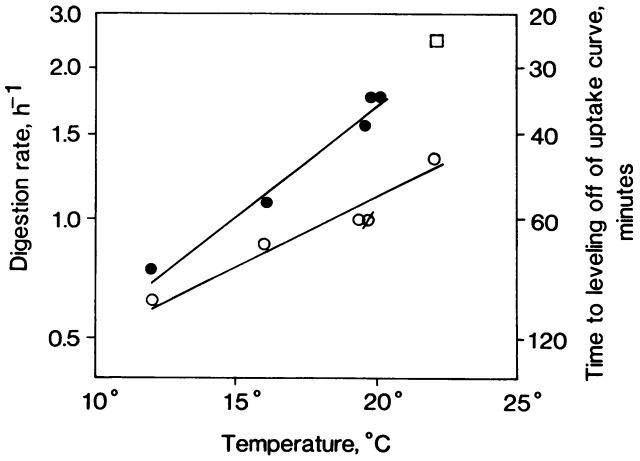

FIG. 3. Plot of bacterial digestion rate versus temperature for marine protozoa feeding on bacteria. Symbols: ●, flagellates; ○, *S. sulcatum*; ⌀, mixed spirotrich species; □, *U. marina*.

TABLE 2. Rates of clearance and bacterial ingestion calculated from rates of uptake of FLB by bacterivorous flagellates and ciliates incubated at 12 to 22°C

Protozoa	Temp (°C)	Total bacteria[a] (10^6 ml^{-1})	Clearance rate (nl cell^{-1} h^{-1})	Ingestion (bacteria cell^{-1} h^{-1})
Mixed flagellates	12	3.8	1.4	5.2
	16	6.2	3.3	20.6
	19.5	2.5	2.3	5.7
	20	5.1	4.3	21.5
	20	9.2	3.0	27.4
S. sulcatum	12	6.7	57.0	380.0
	16	10.5	86.0	900.0
	19.5	2.7	413.0	1,095.0
	22	8.8	94.0	825.0
Mixed spirotrichs	19.5	1.5	247.0	380.0
U. marina	22	14.4	49.0	710.0

[a] Live bacteria plus added FLB.

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