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# Biotin chemoresponse in *Paramecium*

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**Abstract** *Paramecium tetraurelia* locate their foodsource by detecting bacterial metabolites and altering swimming behavior to congregate near bacterial populations on which they feed. Several attractants, such as folate, glutamate, cAMP and acetate have been identified and various aspects of chemoreception, signal transduction and effector mechanisms have been described. Here we characterize the *Paramecium* chemoresponse to biotin. An essential enzymatic cofactor in all cells, biotin is secreted by a large number of bacterial species during growth phase. *P. tetraurelia* are strongly attracted to biotin with a half-maximal behavioral response at 0.3 mmol · $l^{-1}$ in T-maze assays. Physiological recordings from whole cells show that cells hyperpolarize in a concentration-dependent manner in biotin. Whole-cell binding assays utilizing $^{3}$H-biotin identify a saturable and specific binding site with an apparent dissociation constant of 0.4 mmol · $l^{-1}$. The biotin analogs desthiobiotin and biotin methyl ester are also strong attractants. Diaminobiotin fails to attract *P. tetraurelia* at 1 mmol · $l^{-1}$, but does interfere with the biotin chemoresponse and displaces $^{3}$H-biotin from whole cells. We hypothesize that the keto group and/or fidelity of the ureido ring of biotin are necessary for biotin chemoresponse.

**Key words** *Paramecium* · Chemoattractant · Biotin · Chemoreceptor · Chemokinesis

**Abbreviations** $B_m$ maximum binding capacity · *cpm* counts per minute · $I_{che}$ index of chemokinesis · $K_d$ equilibrium dissociation constant · $V_m$ membrane potential

## Introduction

*Paramecium tetraurelia* utilize a variety of bacterial metabolites in order to locate their bacterial food source (Van Houten 1994). At least three different chemosensory pathways for attractants result in a hyperpolarization, which changes ciliary beating and thus swimming behavior (Van Houten 1994). Hyperpolarization can increase ciliary beating and reduce the frequency of calcium action potentials that cause "avoiding reactions," i.e., turns (Machemer 1989). We have previously shown that relatively fast and smooth swimming is characteristic of cells in attractants and that speed of swimming and frequency of turning, with adaptation, are sufficient to account for the accumulation of a population of cells up a gradient of an attractant (Van Houten and Van Houten 1982; Van Houten 1990).

Two attraction pathways, those initiated by acetate and glutamate, probably activate a plasma membrane calcium pump (Wright et al. 1993; Van Houten 1994), which then is responsible for the hyperpolarization (Preston and Van Houten 1987). The distinguishing feature between the two pathways is that glutamate acts through the second messenger cAMP (Yang et al. 1997). The means by which acetate and other attractants, such as folate and extracellular cAMP, activate the calcium pump is unknown. The stimulus for the third pathway is ammonium, which appears to diffuse into the cytoplasm as ammonia, causing an alkalinization of the cell and resulting in hyperpolarization (Davis 1994). The study of other chemoattractant molecules may allow for a more complete understanding of the mechanisms of *Paramecium* chemokinesis.

Biotin, a water-soluble vitamin, is required as an enzymatic cofactor in all cells, both prokaryotic and eukaryotic (Eisner et al. 1996). Biotin is synthesized by bacteria and is released by the cells into the surrounding medium (Noda et al. 1994), and has already been described as a chemoattractant in another organism, the nematode *Caenorhabditis elegans*, which also feeds on bacteria (Bargmann and Horvitz 1991). Due to the rapid advances in biotin/avidin technology, a wide variety of tools is available that may facilitate description of a biotin-mediated chemosensory pathway. Among these are a number of biotin analogs which allow for detailed

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structure/function analysis. In this paper we describe the nature of a biotin-mediated chemoresponse in *P. tetraurelia* in behavioral assays, electrophysiology and binding studies.

## Materials and methods

### Cell culture

*P. tetraurelia* strains *51S* (sensitive to killer) and trichocyst non-discharge mutant *nd6* (Sonneborn 1975) were grown at 28 °C in a wheat-grass medium inoculated with *Klebsiella pneumoniae* (Sasner and Van Houten 1989). *Nd6* was used in electrophysiological studies, and has been shown by Preston et al. (1990) to have normal membrane currents.

### Solutions

All solutions contained 1 mmol · $l^{-1}$ $Ca(OH)_2$, 1 mmol · $l^{-1}$ citric acid and approximately 1.3 mmol · $l^{-1}$ TRIS base and additional compounds as noted, at pH 6.7 (all chemicals from Sigma Chemical, St. Louis unless otherwise noted). Solutions differed only in the type of biotin or analog (Fig. 1) used against $Cl^-$ control. For example, 1 mmol · $l^{-1}$ Na-biotin in buffer was tested in T-mazes compared to a 1 mmol · $l^{-1}$ NaCl buffer.

### Behavioral assays

T-maze assays were conducted as described by Van Houten et al. (1982). Briefly, *Paramecium* in control solution were placed in a stopcock and allowed to swim freely between the two glass arms of the T-maze apparatus which contained the test and control solutions. After 30 min the stopcock was turned, isolating the test and control arms and aliquots from each were counted. The number of animals in the test arm was divided by the total number of animals in both arms to yield an index of chemokinesis ($I_{che}$). Values greater than 0.5 indicate attraction. In interference assays, potential competitors or controls were added equally to both arms of the apparatus in addition to either Na-biotin or NaCl. Data were analyzed with the Mann–Whitney U-Test to assess statistical significance.

### Binding assays

Cells (0.5–1.5 mg · $ml^{-1}$ total protein) were incubated in 1 ml of chemokinesis buffer with 290 pmoles of $^3$H-biotin (Dupont-NEN) and various concentrations of unlabelled biotin or analog for 60 min in order for equilibrium to be reached. The cells were then pipetted onto a 5-μm SM filter (Millipore) and washed under vacuum with 20 ml of buffer containing the appropriate amount of biotin or biotin analog. Filters were dissolved overnight in 3 ml of Ready Protein (Beckman) scintillation cocktail before counting. Counts per minute (cpm) were converted into moles of $^3$H-biotin bound per milligram protein. Protein was quantified with the BCA protein assay (Pierce). Binding kinetic analyses were accomplished with the KELL radioligand binding analysis software package (Biosoft 1997).

### Electrophysiology

Membrane potential ($V_m$) measurements were made as previously described (Preston and Van Houten 1987). Briefly, cells were placed in a Plexiglass recording bath (capacity 1 ml) mounted on the stage of an Olympus CK inverted microscope. The bath was perfused continually with test or control solutions, driven by a Buchler 'polystaltic' pump at a flow rate of 1 ml · $min^{-1}$. The bath was held at ground by means of a 2% agar bridge, into which was inserted an Ag/AgCl wire. Intracellular recording glass capillary microelectrodes were filled with 0.5 mol · $l^{-1}$ KCl, tip resistance 60–80 MΩ. Potential signals from the recording microelectrode were amplified using a WPI M701 amplifier, and were displayed on a Tektronix 5112 dual-beam oscilloscope. Permanent records were made using a Gould 220 chart recorder. Membrane potential changes are the steady-state differences between the membrane potential in the test solution and in control solution.

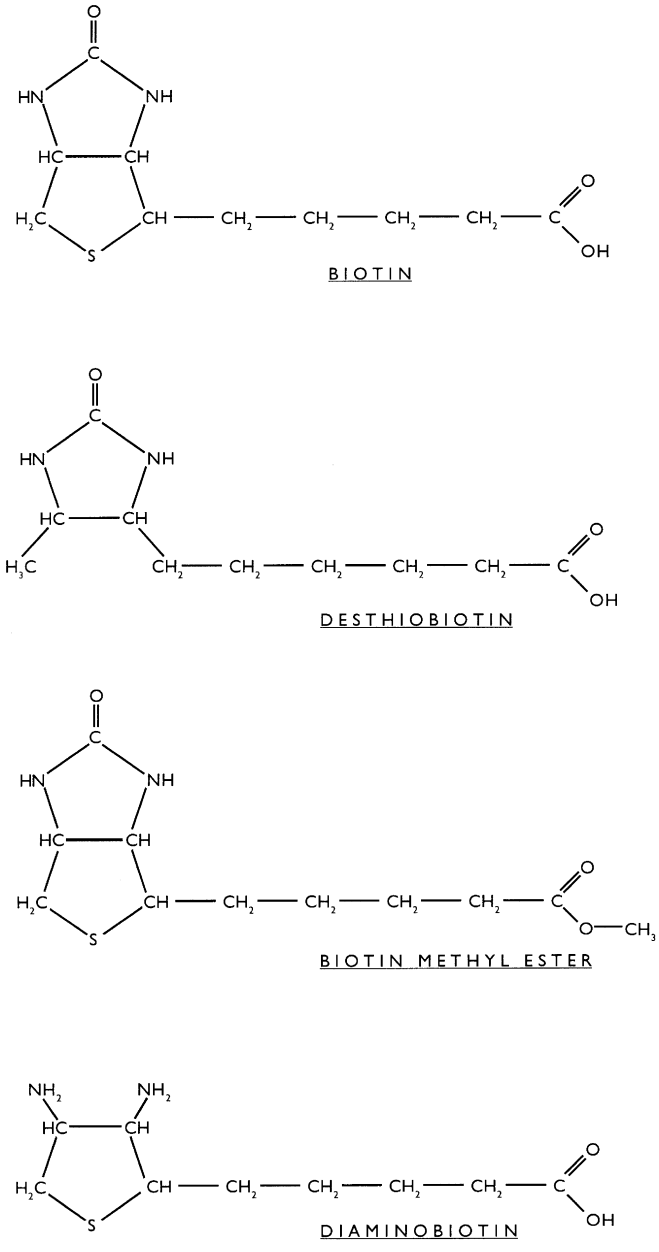


**Fig. 1** Biotin and structural analogs

## Results

### Biotin is a chemoattractant of *Paramecium*

In T-maze assays, *P. tetraurelia* show a strong attraction to Na-biotin versus the NaCl control (Fig. 2), with an apparent half-maximal response at approximately

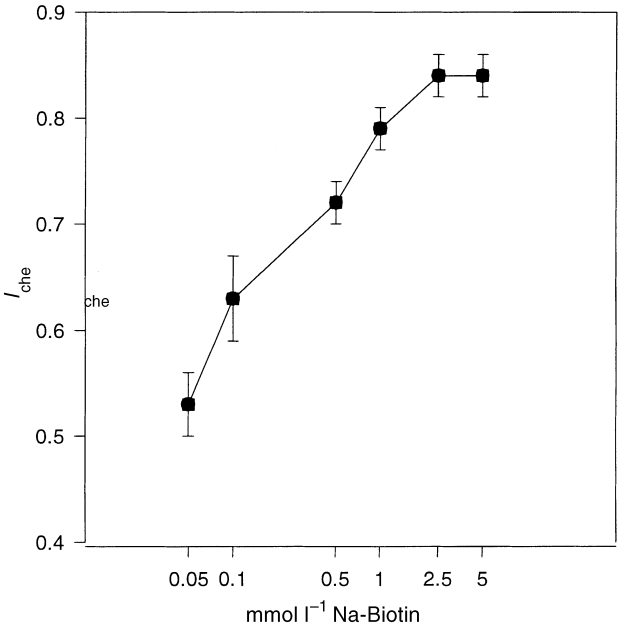


**Fig. 2** Na-biotin is an attractant of *P. tetraurelia* in T-maze assays. Each point represents the mean of nine T-mazes ± one standard error

**Table 1** Behaviour of *Paramecium* to biotin analogs in T-mazes. Data are the means of nine T-mazes ± one standard deviation. Concentration of biotin or analog tested was 1 mmol · $l^{-1}$ versus 1 mmol · $l^{-1}$ NaCl. Na-biotin versus NaCl in T-mazes was run in parallel for each analog as a control. Statistical significance tested with the Mann-Whitney U test

| Analog | $I_{che}$ Analog | $I_{che}$ Biotin control | Significance from control |
|---|---|---|---|
| Methyl ester | 0.87 ± 0.05 | 0.90 ± 0.05 | No |
| Desthiobiotin | 0.79 ± 0.07 | 0.78 ± 0.10 | No |
| Diaminobiotin | 0.40 ± 0.09 | 0.77 ± 0.11 | Yes $P < 0.01$ |

0.3 mmol · $l^{-1}$. Cells are also attracted to K-biotin at pH 6.7 although the response at pH 7.0 in $K^+$ was reduced compared with the response to Na-biotin (data not shown).

Biotin analogs allow us to carry out coarse structure/activity studies. Desthiobiotin, which lacks the sulfur molecule, and biotin methyl ester, in which the carboxyl group is replaced by a methyl ester, are attractants of *P. tetraurelia* at 1.0 mmol · $l^{-1}$ (Table 1). The diaminobiotin analog, which has a disrupted ureido ring due to removal of the keto group, is not an attractant at 1.0 mmol · $l^{-1}$ in $Na^+$ solutions (Table 1). The T-maze result for diaminobiotin is 0.40 ± 0.09 which indicates not only lack of attraction, but slight repulsion. In our experience, this effect is slight and probably not significantly different from NaCl versus NaCl neutral controls.

We tested the ability of these compounds to interfere with biotin chemoresponse by adding them to both arms of a T-maze assay. The T-maze normally has Na-biotin in buffer in one arm and NaCl in buffer in the other. Controls for interference assays include addition of extra NaCl in both arms to control for ionic strength effects or the addition of extra Na-biotin in both arms as a control assay for the ability of biotin to interfere with biotin response (Table 2). In interference assays NaCl does not affect the cells' ability to respond to biotin, but Na-biotin does, as expected for a positive control. Diaminobiotin eliminated the ability of *P. tetraurelia* to respond to biotin in the T-maze, where an $I_{che}$ of 0.5 indicates a neutral response. A structurally unrelated attractant, Na-glutamate, did not interfere in the cells' ability to respond to biotin (Table 2).

## Stimulation with biotin results in hyperpolarization of the cell membrane

Whole-cell recordings of *P. tetraurelia* in K-biotin show a significant hyperpolarization relative to resting $V_m$ in KCl in a dose-dependent manner (Fig. 3), with a maximum of 11.5 mV at 1.0 mmol · $l^{-1}$ biotin and half-maximal response of 5.0 mV at 0.3 mmol · $l^{-1}$. Recordings in $Na^+$ solutions also show hyperpolarization of the cell, however, with a hyperpolarization of 6.1 mV at 2.5 mmol · $l^{-1}$ Na–biotin relative to NaCl (data not shown). $K^+$ solutions yielded more reliable data than $Na^+$ because the $V_m$ traces are much less noisy in $K^+$ than in $Na^+$ solutions.

**Table 2** Behavioral interference in T-maze assays. Assessment of ability of diaminobiotin (DAB) to interfere with *P. tetraurelia* chemokinesis to biotin in T-maze assays. DAB or NaCl controls were added equally to each side of the T-maze. Experiments 1 and 2 are controls for ionic strength and interference by biotin itself. Experiment 4 with glutamate is a negative control. Statistical significance tested with the Mann-Whitney U test. Data are means ± one standard deviation

| Exp. | Control solution | Test solution | $I_{che}$ | $n$ |
|---|---|---|---|---|
| 1 | 0.5 mmol · $l^{-1}$ NaCl | 0.5 mmol · $l^{-1}$ Na-biotin | | |
| | 0.5 mmol · $l^{-1}$ NaCl | 0.5 mmol · $l^{-1}$ NaCl | 0.72 ± 0.09 | 9 |
| 2 | 0.5 mmol · $l^{-1}$ NaCl | 0.5 mmol · $l^{-1}$ Na-biotin | | |
| | 0.5 mmol · $l^{-1}$ Na-biotin | 0.5 mmol · $l^{-1}$ Na-biotin | 0.47 ± 0.12* | 9 |
| 3 | 0.5 mmol · $l^{-1}$ NaCl | 0.5 mmol · $l^{-1}$ Na-biotin | | |
| | 0.5 mmol · $l^{-1}$ Na-DAB | 0.5 mmol · $l^{-1}$ Na-DAB | 0.54 ± 0.14* | 9 |
| 4 | 0.5 mmol · $l^{-1}$ NaCl | 0.5 mmol · $l^{-1}$ Na-biotin | | |
| | 0.5 mmol · $l^{-1}$ Na-glutamate | 0.5 mmol · $l^{-1}$ Na-glutamate | 0.75 ± 0.06 | 10 |

* Statistically significant from control ($P < 0.01$)

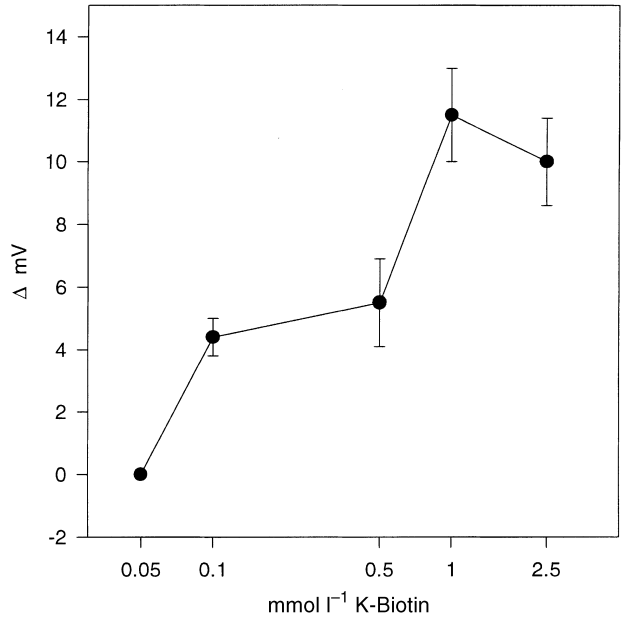


**Fig. 3** Degree of hyperpolarization. Whole-cell membrane potentials ($V_m$) were measured in cells in KCl and biotin by standard techniques. Data are the means of differences in $V_m$ in KCl versus K-biotin from three to five cells ± one standard error. No difference in $V_m$ between KCl and K-biotin was detectable at 0.05 mmol · l$^{-1}$

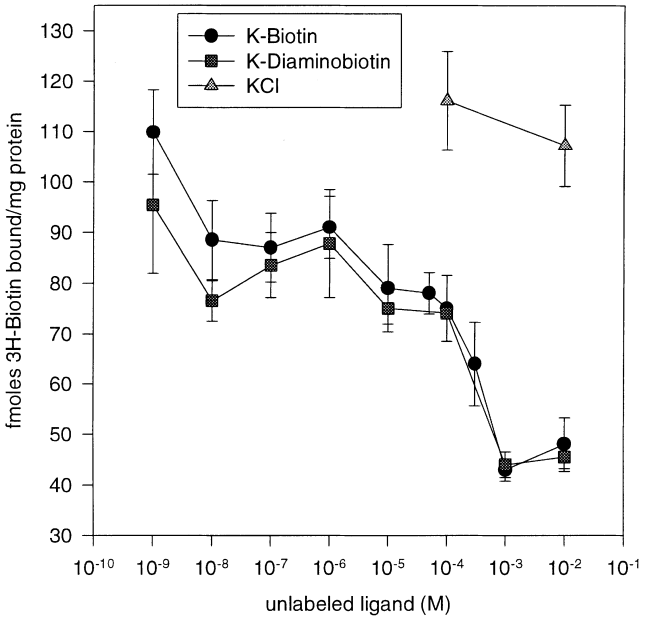


**Fig. 4** $^3$H–biotin binding was measured by displacement from *P. tetraurelia* whole-cell preparations using a vacuum filtration assay. Data are the means of three to six separate experiments ± one standard error and represent total binding

### Binding analysis of $^3$H-biotin with whole cells

Kinetics of binding of K-biotin to whole cells were determined by displacement of $^3$H-biotin with increasing amounts of unlabelled biotin or an analog. Time to equilibrium was estimated from experiments of binding of $^3$H-biotin to whole cells, in which cpm associated with the cells reached a plateau by 1 h with no apparent increase in association due to uptake after that time (data not shown). Nonetheless, we omit $Na^+$ from the binding buffer to eliminate contributions that $Na^+$ may make toward uptake.

Displacement of $^3$H-biotin from whole-cell preparations of *P. tetraurelia* with unlabelled biotin shows a saturable and specific component with an apparent equilibrium dissociation constant ($K_d$) of 0.4 mmol · l$^{-1}$ and $B_{max}$ of 0.6 pmoles/mg protein. Non-specific binding was calculated to be 38% of the total binding. As biotin concentration was increased in the displacement studies, the counter ion $K^+$ also was increased to control for the effects of ionic strength on binding. Therefore, as a control, binding in the presence of 0.1 and 10 mmol · l$^{-1}$ KCl was measured and the data show no significant displacement of $^3$H–biotin (Fig. 4). Our data suggest the existence of a second, high-affinity binding site, perhaps for transport. Because of the high variability in the data at low competitor concentrations and the need for ligand concentrations below those physiologically relevant for *Paramecium* chemoresponse, we have chosen to leave the study of possible high-affinity sites for future study.

The behavioral antagonist diaminobiotin, when substituted for biotin in displacement assays, shows an ability to displace $^3$H–biotin similar to biotin (Fig. 4).

## Discussion

We have demonstrated that biotin is a strong attractant of *P. tetraurelia* in T-maze assays. It also hyperpolarizes the cell membrane and binds to whole cells in a saturable and specific manner. Behavioral and binding analyses, including those utilizing biotin analogs, suggest that biotin chemoattraction is a receptor-mediated process and that the receptor requires integrity of the ureido ring structure to initiate a response.

*P. tetraurelia* respond to biotin by hyperpolarization of the cell membrane which reduces the rate of turning and increases swimming speed (Van Houten 1978). This behavior should result in an accumulation of cells in the area of biotin concentration. Since biotin can be secreted by a growing population of bacteria, this chemokinesis will result in attraction of *P. tetraurelia* to it's food source. In that a large number of biotin secreting species of bacteria have been identified (Gyorgy and Pearson 1967) and chemoresponse to biotin has been described in *C. elegans* that also feeds on bacteria (Bargmann and Horvitz 1991), it is not unreasonable to hypothesize that bacterial feeders such as *Paramecium* might utilize this metabolite to locate food. It is possible that paramecia benefit from the uptake of biotin as does the protozoan *Ochromonas danica*, which requires biotin for growth and is so sensitive to its presence that it has been used in a bioassay to quantify biotin concentration (Baker 1985). Therefore, chemoresponse to biotin may provide the cells with a valuable vitamin as well as food.

Previous studies on yeast and mammalian cells indicated that a critical group for transport of biotin was the

carboxyl group (Wolf 1995). However, *O. danica* is able to utilize biocytin, in which the carboxyl group is bound to a lysine, an analog normally unavailable to cells for uptake (Baker et al. 1962), which suggests that this protozoan may bind a region other than the carboxyl group of biotin in its transport mechanism. We have no direct evidence for biotin uptake in *P. tetraurelia*; however, biotin is not required to sustain growth of *Paramecium* in defined media (Van Wagtendonk 1974).

The saturability, specificity, and kinetics of the binding data coupled with the behavioral and electrophysiological observations indicate that biotin chemokinesis in *Paramecium* is a receptor-mediated process. The ability to detect the desthiobiotin and biotin methyl ester analogs but not diaminobiotin suggests that the keto group and/or the integrity of the ureido ring are necessary for activation of the biotin signaling pathway. A similar receptor/ligand interaction may explain the ability of *O. danica* to utilize biocytin for growth. In that diaminobiotin interferes with the biotin chemoresponse in behavioral assays and displaces $^{3}$H-biotin from whole cells with similar kinetics to the parent compound, it is likely that diaminobiotin is directly competing with biotin at the level of receptor binding. These results also further support the hypothesis that biotin interacts with its receptor through the ureido ring and not the carboxyl side chain.

The binding of biotin to its putative receptor is a relatively low affinity interaction with a $K_d$ of 0.4 mmol $\cdot$ l$^{-1}$. The half-maximal concentrations for hyperpolarization (0.3 mmol $\cdot$ l$^{-1}$) and behavioral responses (0.3 mmol $\cdot$ l$^{-1}$) are close to the binding $K_d$, considering the variability of the assays. This is reasonable if one considers the amount of intrinsic attractant molecules in solution in the natural *Paramecium* environment, the pond. Although no quantification of biotin in pond water has been accomplished, it is present and probably abundant, since the breakdown of plant materials allows for a release of water soluble biotin (Lampen et al. 1942). Our results indicate that only exposure to a high concentration of biotin would result in chemokinesis, perhaps eliminating the useless pursuit by cells of sources of biotin other than bacteria. It is also likely that the physiological response is the result of a multi-ligand, or combinatorial effect, due to the presence of other bacterial attractant molecules such as glutamate, folate or cAMP. These ligands could ultimately all work on the same effector mechanism, the plasma membrane $Ca^{2+}$ pump.

Signaling molecules, known as quorum sensing factors, have been identified from numerous bacterial species and can effect a variety of responses including, but not limited to, population growth, gene regulation and virulence (Kaiser 1996). Amino acids act as quorum sensing factors in *Myxococcus xanthus* (Kuspa et al. 1992) and homoserine lactones function in quorum sensing in numerous species including *Vibrio fischeri* (Fuqua et al. 1994). In each case a population of bacteria must reach a minimal size before a function is triggered, and the quorum sensing factor concentration is an indicator to the bacteria of the state of the population. Since *P. tetraurelia* seems to respond primarily to high concentrations of stimuli that are bacterial metabolites, the paramecia will be attracted to dense and actively growing populations of bacteria rather than to declining populations. Therefore, it is possible that *P. tetraurelia* is carrying out quorum sensing, not of its own population densities, but those of its prey, bacteria. We do not have any evidence that biotin is a quorum sensing factor for bacteria, only that it is part of the negative regulator of its own operon (Cronan 1989). Nonetheless, paramecia may be using it to respond with hyperpolarization and chemokinesis to locate a bacterial source only when the population is dense and actively growing.

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