Chapter 18 Chemokinesis

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1 Introduction

Paramecia are sensitive to many kinds of changes in their environment. The stimuli can take the forms of heat, mechanical jostling, or chemicals. The response to each is generally a change in swimming that causes cells to approach or move away from the stimulus source. This chapter will trace the progress of the study of organic chemical stimuli on *Paramecium* behavior. The tracing will lead from some fairly remarkable observations using simple equipment to modern membrane biochemistry. From this will emerge a picture, albeit incomplete, of the chemosensory transduction pathway in *Paramecium* from receptor to motile apparatus, perception to response. At the end we will offer some models to fill the gaps in our picture of how detection of a chemical cue ultimately results in altered swimming behavior.

This chapter will deal primarily with organic stimuli as opposed to inorganic cations, which have well-documented effects on *Paramecium* behavior (Kung and Saimi 1982). The reason for the dichotomy is that the cells are likely to have evolved receptors on the cell surface to specifically detect the presence of these organic stimuli. Inorganic ions have been studied extensively and the effects of inorganic ions on cell physiology and behavior will be examined in other chapters of this volume.

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Paramecium. Ed. by H.-D. Görtz

2 Assays of Chemoresponse

For a unicellular organism such as *Paramecium*, life in a freshwater environment presents a number of problems. Aside from the unremitting need to regulate and expel water from the cell interior, the organism is subject to rapid changes in external temperature and ion composition that are characteristic of the small bodies of stagnant water in which *Paramecium* abounds. In addition, most species of *Paramecium* are wholly dependent on external sources of nutrition, the availability of which may show considerable seasonal variation.

Clearly, it would be of advantage for the survival of the organism if it were to possess the capacity to both sense these environmental changes and then respond by moving toward or away from the stimulus. As previously discussed, the surface of *Paramecium* is covered with several thousand motile cilia. The metachronal beating of these organelles propels the cell at up to 2 mm s^{-1} through its surrounds; the cell is thus readily capable of a locomotory response to environmental change. That *Paramecium* is able to sense such change has been recognized since the turn of the century, when H.S. Jennings concluded a series of observations on the behavior of a variety of protists. These studies culminated in the publication of his treatise *Behavior of the Lower Organisms* (1906), a work still relevant to modern research on protist behavior. Many of Jennings' studies focused on the behavior of *Paramecium*, providing precise details of its responses to touch, gravity, electric and water currents, acidity, and chemicals. His experimental approach to the study of chemosensation was simple but effective. A suspension of paramecia was layered on a glass slide beneath a cover glass, supported by thin, glass rods. The cells could be seen randomly swimming in all directions until a drop of a test solution was pipetted into the center of the suspension. The drop was initially free of cells, and if the test solution were repellent to the cells, would remain this way. However, if the introduced solution were a positive stimulus, the drop would become filled with cells and the density of cells would be higher in the test solution than in the surrounds. Jennings noted that a strongly attractant solution would "trap" the cells within the confines of the drop, the cells turning upon leaving the area of stimulation and hence behaviorally defining its perimeter (Fig. 1). Jennings called this stimulus-induced turning an "avoiding reaction", a term that persists to this day in describing either stimulus-induced or spontaneous changes in swimming direction.

Jennings' studies were important in many respects. Firstly, he recognized the existence of a concentration threshold that had to be crossed for a cell to

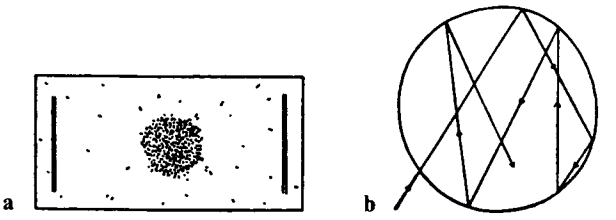

Fig. 1 a, b. Jennings' studies of chemoresponse. **a** Collection of paramecia in a drop of 0.02% acetic acid; **b** path followed by a single *Paramecium* in a drop of acid (Jennings 1906)

show a response to a stimulus. Secondly, he noted that responses to environmental stimuli are mediated through generation of an avoidance reaction; the avoidance response brings about a change in the direction of a cell's swimming path that causes eventual accumulation in attractants and prevents a cell from entering a repellent area. Thirdly, he emphasized that cells tend to collect in areas of optimal stimulation, and that it is movement away from this optimum that triggers a behavioral response. The importance of the latter is that it recognizes the fact that the terms attractant and repellent are relative. Thus, a weak repellent is attractive relative to a stronger repellent. Conversely, when presented with two attractant stimuli, cells will tend to collect in the solution that elicits a stronger positive response. The second solution is thus perceived by an observer as being repellent, despite the fact that the same solution may elicit a positive response when tested against a neutral or noxious solution. In concluding his studies, Jennings noted that paramecia are generally attracted to areas of weak acidity and aeration, and are repelled by a variety of monovalent and divalent cation salts.

Despite such eloquent beginnings, the study of *Paramecium's* responses to chemical stimuli languished until 1957, when Ferguson described a dark field photographic technique that could be used to record the swimming paths of single cells over the course of several seconds exposure. This technique was subsequently refined by Dryl (1958) in first attempts to quantify the behavioral responses to chemicals. Paramecia trace a helix as they swim, so that a two-dimensional photographic image of their swimming path appears as a now-familiar undulating white line against a black background. Since the time of exposure was known, the length of this line was a direct measure of the cells' swimming speed, and by widening the lens aperture during the final second of exposure, the last cell image (and hence direction) of the path is recorded as a brighter spot (Fig. 2). The simplicity of the recording system belies its ability to faithfully and clearly reproduce the individual swimming paths of many cells and was later used in a complete description of the characteristic behaviors exhibited by paramecia in response to stimuli (Dryl and Grebecki 1966).

Dryl (1959 b) extended Jennings' observations of *Paramecium's* responses to chemicals by pipetting drops of test solution to each of the four corners of a glass plate supporting a thin suspension of cells. The number of cells collecting within these areas was counted from a time exposure photograph, and these data expressed as a percentage of the number of cells collecting within a central, control drop of distilled water. Inevitably a degree of mixing occurred when the test and control solutions were introduced to the cell suspension, but Dryl (1959 b) was able to demonstrate reproducible repulsion of *P. caudatum* from ten lower alcohols. The concentration of alcohols required to both repel and kill paramecia was proportional to their molecular weight, perhaps reflecting a direct interaction of the chemicals with the lipid portion of the plasma membrane. In later studies, Dryl (1961 a) determined the optimum pH for the motility of *P. caudatum*, showed quinine to be a strong repellent (1961 b), and described an "adaptation" phenomenon, whereby a prolonged (24 h) incubation in high concentrations of $MgCl_2$, $CaCl_2$, NaCl, or KCl caused a slowly reversible insensitivity of the cells to subsequent cation stimulation (Dryl 1959 a).

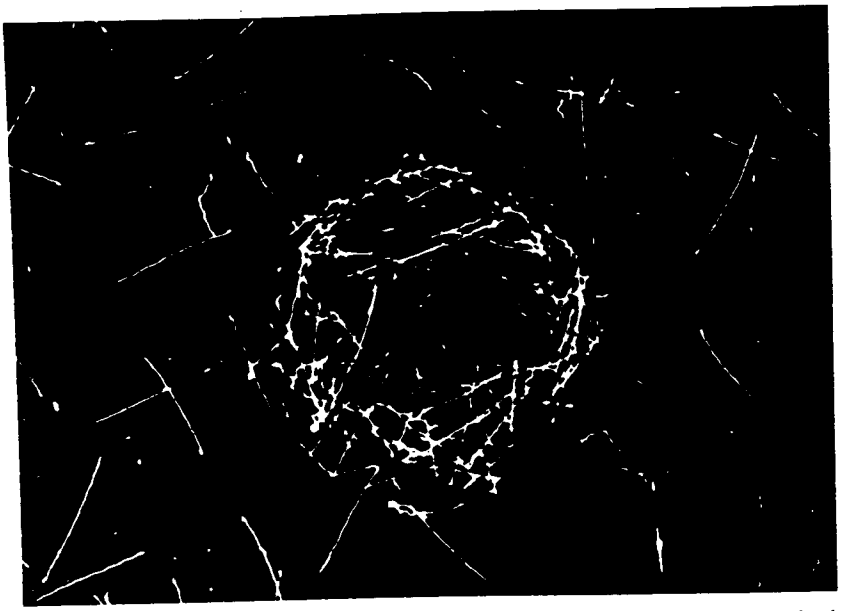

Fig. 2. Photomacrographic assay of attraction of paramecia to slightly acidic medium (Dryl and Grebecki 1966)

Recognizing the inherent diffusion and dilution problems associated with adding drops of a test solution to a layer of cells, Nakatani (1968) developed a capillary tube assay for testing the relative attractiveness of starch, milk, latex particles, acids, ethanol, and ions to *P. caudatum*. Capillary tubes are routinely used to assay the responses of bacteria to chemicals (Adler 1966), but have since been deemed less reliable for use with the larger, faster-swimming ciliates (Levandowsky and Hauser 1978). Nakatani's (1968) assay involved filling 0.6 mm diameter capillary tubes with test solutions, and then placing one end in a trough containing paramecia. Cells attracted to the test solution swam into the capillary and then became trapped as they migrated upwards under a negative geotactic influence. At 5-min intervals, the upper ends of the capillaries were photographed and the number of accumulated cells estimated by densitometric analysis of the resultant negative. Of all the various compounds tested, only $BaCl_2$ elicited a strong negative response, while the effects of acetic acid and ammonium chloride were concentration-dependent, being attractant at concentrations below the millimolar range, but repellent above these concentrations. Such diphasic behavior is also observed for bacteria capillary tests and may be due to the geometry of the assay rather than due to the cells making a discrimination between high and low concentrations (Adler 1969; Adler et al. 1973). Salts of acetic acid at neutral pH have subsequently been shown to be attractant to *P. tetraurelia* (Van Houten 1978), so it is likely that Nakatani's observations were of responses to changing pH rather than to acetic acid as a specific ligand. These studies emphasize the need to transfer paramecia to a buffered salts solution for experimentation, while previous authors had used culture fluid or tap water.

The chemoresponse assays of Jennings, Dryl, and Nakatani each provided some improvements over previous assays and significantly contributed to the progress of understanding *Paramecium* chemoreception. The macrophotographic method of Dryl made it clear that the avoiding reaction must figure into the behavioral mechanism of repulsion and attraction, but the draw back of this assay is that it is semiquantitative. Nakatani's capillary method later proved useful for the rapid screening of mutants and progeny of crosses (Van Houten 1977; DiNallo et al. 1982). However, in order to infer more information about the chemoresponse pathway, it was necessary to have a more quantitative assay. In response to this need, Hansma designed an assay (Van Houten et al. 1975) based on the countercurrent flow method used by Dusenbury (1973) for isolating chemoresponse mutants of *Cenorhabditis elegans.* Another method, described by Van Houten in the same article (Van Houten et al. 1975), used a T-maze made from a modified three-way stop cock. Of the two methods, the T-maze has been routinely used and adapted to other organisms because it was simple, fast, and uncomplicated conceptually and physically. The T-maze assay satisfied the need to carry out many replicate studies for concentration and structure-activity studies, for example.

The T-maze originally had cells loaded into the shortened perpendicular arm of a three-way stop cock. When the stop cock was opened, cells entered the plug and distributed between the two longer side arms. An index of chemokinesis was calculated based on the fraction of cells that distributed into the arm with the test compound. B. Diehn suggested a modification of using a two-way plug in the three-way stop cock. This change made the T-maze even more convenient (Fig. 3). Kinetic studies showed that cells distribute rapidly between the two arms of the T-maze, and that the distribution of cells in the arm with attractant, acetate, for example, was not due to a subpopulation of cells that could detect acetate with the cells in the control arm representing another subset of cells that were unable to detect the attractant (Van Houten et al. 1982). Instead, all the cells in the population seemed able to detect the attractants tested and distribution depended upon the average amounts of time spent by the cells in test and control solutions.

Van Houten used the T-maze to systematically search for organic attractants of *P. tetraurelia.* In this search, the test and control solutions of the T-maze were identical in pH and ionic strength and only one compound being tested was

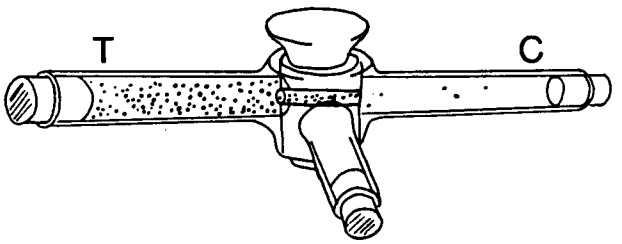

Fig. 3. T-maze apparatus for assaying chemoresponse. This T-maze is modified from the original (Van Houten et al. 1975) by the use of a two-way plug in the three-way stop cock

varied, e.g., the acetate anion was tested against Cl^- (Van Houten et al. 1975). This approach was essential because inorganic cations varying in ionic strength or pH would affect the *P. tetraurelia* swimming behavior and hence would complicate the search for a response to an organic compound for which *P. tetraurelia* could have evolved a receptor-response pathway. The T-maze assays demonstrated that attraction behavior was saturable and specific (Van Houten 1976, 1978). For example, acetate was attractive relative to Cl^- even when saturating amounts of another attractant, lactate, were included in all solutions as a competitor (Van Houten 1976, 1978). Saturability and specificity could be accounted for by specific surface receptors for stimuli. Doughty later used a modification of the T-maze in similar "jamming" experiments to argue for specific receptor sites for nicotinic cholinergic ligands (Doughty 1979).

Once a few organic attractants, such as acetate, lactate, and folate, were identified, the stage was set to select mutants defective in chemokinesis and to use these in comparison with normal cells to determine the behavioral mechanism of attraction and repulsion. Clones of mutants were screened for among mutagenized populations using capillaries filled with K-acetate and KCl (Van Houten 1977). The mutant that proved to be most useful in behavioral analysis was d4-530, a line of cells that were repelled from and not attracted to acetate relative to Cl^-.

3 Swimming Behavior

The previous work of Jennings and Dryl firmly established the avoiding reaction or turn as an important behavioral component in keeping *P. caudatum* cells accumulated or dispersed. Nakatani (1970) recognized that chemoeffectors also change the swimming speed of *P. caudatum.* In this study, cells were transferred to a test solution where they remained for 10–20 min, a time required for the "chemotactic response of *Paramecium* to reach a maximum" (Nakatani 1970). A few specimens in test solution were then placed under the objective of a microscope and their motion photographed under stroboscopic illumination. Detailed analyses of these records revealed that compounds that had previously been noted to repel paramecia (Nakatani 1968) also decreased their swimming speed, while chemicals that were attractant to *P. caudatum* increased swimming speed. Nakatani (1968) had shown that acetic acid and NH_4Cl become repellent at high concentrations; paradoxically these same concentrations caused even greater increases in cell swimming speed. Thus, there is a point at which an increase in swimming speed acts to disperse paramecia rather than attract them to the area of stimulation. This relationship is shown in Fig. 4.

Nakatani's observation of increased speed of swimming did not intuitively fit into the long accepted framework of the mechanism of attraction and repulsion by turning frequency. Dryl even considered speed changes a "contamination" of a pure "chemotactic" response (Dryl 1973). However, speed had to be incorporated somehow into a comprehensive mechanism of chemoresponse. Using both long exposure macrophotographs to measure speed and ob-

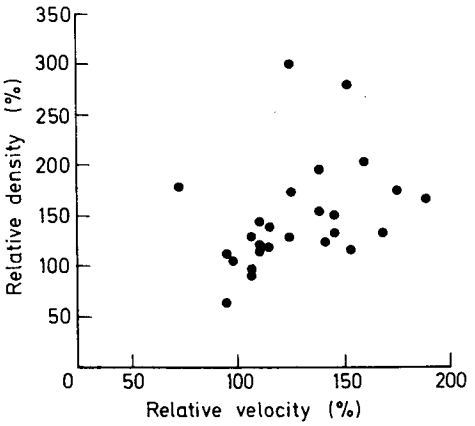

Fig. 4. Nakatani's measurements of speed correlated with density of cells accumulating in capillaries of chemical stimuli (Nakatani 1970)

servations of turns over time, Van Houten was able to build a coherent picture of both speed and turning in chemoresponse behavior for *P. tetraurelia* that had not been done for *P. caudatum*. *P. tetraurelia* both decreased frequency of turning and increased speed in attractants like acetate and increased frequency of turning and decreased speed in repellents like quinidine. However, these two behavioral changes were at odds when fit into the two mechanisms of accumulation and dispersal described for small organisms (Fraenkel and Gunn 1961). There was no evidence of oriented movement to chemical stimuli in *P. caudatum* or any ciliate and, therefore, the cells must have accumulated or dispersed by a kinesis mechanism: klinokinesis or orthokinesis. Klinokinesis requires the modulation of turning frequency, the equivalent of the biased random walk so nicely described in bacteria (Berg 1986). Orthokinesis requires the modulation of speed with accumulation occurring where cells move slowly. Somehow in *Paramecium* these two kineses must combine because both frequency of turning and speed were changed with chemical stimuli, but a complication arose for *Paramecium* in that the change in turning frequency and speed seemed to be at cross purposes: In attractants such as acetate, cells slightly decreased frequency of turning, which would cause attraction by klinokinesis, but also increased speed, which by orthokinesis would work against attraction. Conversely, repellents increased frequency of turning, consistent with repulsion by klinokinesis, but also decreased speed inconsistent with repulsion by orthokinesis. There were at least two possible explanations: (1) There were conditions under which the speed change was nonconsequential and the klinokinesis mechanism was all important and other conditions under which the speed change was all important; or (2) the combining of turning frequency and speed changes produced yet a different mechanism of attraction and repulsion not described by Fraenkel and Gunn.

In order to explore these possibilities, pawn, fast-2 (Kung et al. 1975) and mutant d4-530 (Van Houten 1976, 1977) were tested for attraction and repulsion (Van Houten 1978). Pawn mutants could not turn for lack of ciliary Ca influx, which would transiently reverse ciliary beating and make normal cells turn. However, pawns could modulate speed with external stimuli. Pawns were not

attracted to acetate or similar attractants, implicating turning as essential for these attractants. However, pawns could be repelled from high pH in which the cells swim extremely fast. Fast-2 mutant showed smooth, fast swimming in Na solutions and relatively normal behavior in K solutions. Fast-2 mutants could not be attracted to Na salts of organic anion attractants such as acetate, but were normally attracted to the K salts, in which the cells showed normal turning control and speed. Mutant d4-530 was repelled from Na-acetate and, therefore, it was expected that this mutant would show the converse of the wild-type behavioral responses in acetate, that is, it would turn more frequently and swim more slowly in acetate than in chloride. Instead, mutant d4-530 moved faster and more smoothly in Na-acetate than the normal cells. This appeared to be a repulsion by orthokinesis and was reminiscent of the unusual observation by Nakatani of repulsion from high concentrations of attractants that induced fast swimming in *P. caudatum.*

From these mutant studies, it was clear that turning was essential in some responses but that speed control was sufficient for others. Therefore, there must be the potential for two mechanisms of response, and both must be compatible with *Paramecium* physiology. When the behavior of cells in attractants and repellents was plotted as frequency of turns vs speed, normal *Paramecium* behavior fell into two quadrants: a small increase in speed and decrease in frequency of turning was associated with attractants such as acetate; a large increase in speed and a large decrease in frequency of turning was associated with repellent action of high pH; a small decrease in speed and increase in frequency of turning was associated with repellents such as low pH or quinidine (Fig. 5).

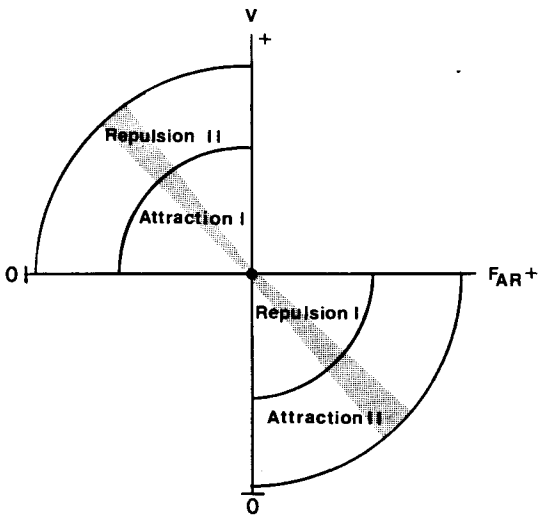

Fig. 5. Graphical description of *Paramecium* behavior. Behavior of cells in control solution is at the origin. An increase or decrease in velocity (V) from control is plotted on the y-axis. An increase or decrease in the frequency of avoiding reactions (F_{AR}) that cause turns is plotted on the x-axis. Behavior of normal animals falls in the *upper left* or *lower right* quadrants. Behavior is restricted to an area represented by a *shaded sector* that is determined by the membrane potential (ΔVm) (Van Houten 1978)

It was clear from this plot that the key to the behavior in attractants and repellents was the membrane potential of the cell. A change in membrane potential of *Paramecium* controls both the frequency of Ca action potentials, hence turns, by setting the potential closer to or farther from the threshold for action potential; and a change in membrane potential from rest changes swimming speed by altering the frequency and angle of ciliary beating (Machemer 1976; Machemer and de Peyer 1982).

From Fig. 5 it could be predicted that cells in attractant acetate would experience a small hyperpolarization relative to control and this ΔVm would cause the behavioral changes that lead to attraction; a larger hyperpolarization as in KOH (high pH) would cause repulsion. Conversely, a small depolarization of cells in repellent quinidine relative to control would lead to repulsion; a larger depolarization as in $BaCl_2$ relative to KCl would cause attraction. These predictions of change in membrane potential (ΔVm) were replotted as in Fig. 6a. Here, it is more evident that there should be a continuum of change in membrane potential relative to control and that depending on the magnitude of the ΔVm, a cell would be attracted or repelled. Direct testing using standard electrophysiological techniques gave credence to this hypothesis of ΔVm control of chemoresponse (Fig. 6b) (Van Houten 1979, 1980; Van Houten et al. 1984a). Therefore, even a relatively complex population behavior like chemoresponse could be explained on the basis of the known physiology of individual cells.

Frequency of turning and speed were the primary parameters that were necessary to describe *P. tetraurelia* behavior using computer simulations (J. L.

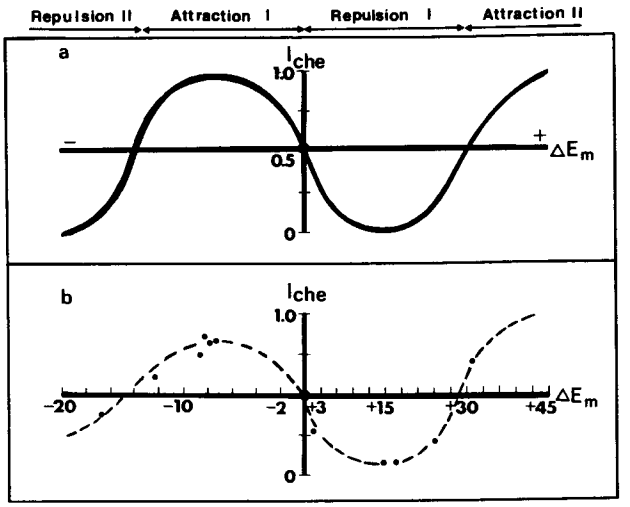

Fig. 6a, b. Membrane potential control of chemokinesis. **a** Graphical description of membrane potential control of chemokinesis. Change of membrane potential (ΔE_m or ΔVm) from control at origin is plotted against the index of chemokinesis: $I_{che} > 0.5$ indicates attraction; < 0.5 indicates repulsion. As chemical stimuli change E_m relative to control, animals will be attracted or repelled, depending on the magnitude and direction of the E_m change. **b** Data from direct recordings of ΔE_m from individual cells (Van Houten 1979) are plotted as ΔE_m produced by the attractant or repellent versus I_{che}. Scale of ΔE_m is different for depolarizing and hyperpolarizing stimuli (Van Houten 1979)

Van Houten and J. Van Houten 1982). Empirically determined speeds and turning frequencies were used to simulate accumulation in T-mazes. To properly mimic *Paramecium* behavior, however, adaptation had to be built into the simulation because the cells' behavior adapted and changed toward a basal level despite the continued presence of a stimulus. Adaptation seemed to prepare the cells for an immediate response at the boundary between solutions (Van Houten 1976, 1978; Machemer and de Peyer 1979; Van Houten et al. 1981; J. L. Van Houten and J. Van Houten 1982). The exact kinetics of this adaptation were not crucial to the outcome of computer simulation of attraction and repulsion for mutants and normal cells. However, the immediate response of the cells upon encountering the change in solution was very influential in the behavioral result.

4 Stimuli

For the most part, attractants of *P. tetraurelia* were found among fermentation or other products of bacteria, *Paramecium*'s food. At neutral pH, acetate and lactate anions, folic acid (a vitamin for the ciliate), cAMP, NH_4^+, and amino acids were among the compounds found to be attractive (Van Houten 1976, 1978; Schulz et al. 1982; Preston 1983). Repellents of *P. tetraurelia* include extremes of pH and salt and organic compounds that were "membrane active" and elicit action potentials (Van Houten 1976, 1978). Toxicity per se did not disperse the cells (Van Houten 1976). It appeared that *P. tetraurelia* accumulate in areas of food and optimal conditions for pH and osmotic balance and did not use organic stimuli as repellent cues. Compounds like the repellents quinine and quinidine (Dryl 1961 b; Van Houten 1978) or attractant cholinergic ligands (Doughty 1979) may not normally occur in *P. tetraurelia*'s environment, but were useful in perturbing the sensory transduction pathway for study. *P. caudatum* had a slightly different array of attractants and repellents. Alcohols were repellent to *P. caudatum* as were areas of high salt and extremes of pH (Dryl 1959 b). However, *P. caudatum*'s pH optimum was more acidic than that for *P. tetraurelia.*

Generally, the stimuli need to be present in high, near millimolar concentrations to elicit responses from *Paramecium*, which may be advantageous to the ciliates as they swim through a noisy aqueous environment. Exquisite sensitivity would lead the cells to many nonproductive areas for food gathering. Fermenting *E. coli* were found to be capable of producing lactate in millimolar concentrations around the soft agar into which they were inoculated. It was also nearest to this plug of agar that *P. tetraurelia* accumulated (Van Houten, unpubl. results).

Mating-reactive paramecia do not appear to release any diffusible mating substances for the complementary mating type (Van Houten et al. 1981) nor do paramecia release diffusible products detected by its predator *Didinium.* In T-mazes, *Didinium* was attracted to bacterial products of low molecular weight and, therefore, sought out its prey's food and not the prey itself (Antipa et al. 1983).

5 Chemoreceptors

A general model for a sensory transduction pathway begins with binding of ligand to receptor and this binding is subsequently transduced into a second messenger and response. Receptors can account for at least some of the specificity and saturability of the chemosensory systems and would be expected to be affected by surface-acting agents such as glutaraldehyde (Doughty and Dodd 1978). However, the task of identifying receptors is difficult and to date there has been only one external chemoreceptor protein identified, the receptor for cAMP in *Dictyostelium* (C. Klein et al. 1986; P. Klein et al. 1985). In *Paramecium*, the chemosensory behavior and hyperpolarization are saturable and specific, which argues for a receptor-mediated system. The attractant stimuli were effective in a charged form (Schulz et al. 1984; Preston et al. 1987) and, therefore, they cannot act internally without first binding to a site on the surface for transport into the cell. Therefore, regardless of whether the stimuli act exclusively externally or must enter the cell to be active, there must be a specific binding site, which we will refer to here as a receptor.

The initial description of receptors generally comes from structure-activity studies of the behavior that determine the important site on the stimulus that is detected by the receptor and from binding studies that provide information on the affinity, number, and location of receptor sites. The attractant molecules studied for most of the preliminary work on *P. tetraurelia* and *P. caudatum* were too small for or not amenable to manipulation for the structure-activity studies that would be necessary to make inferences about the nature of the receptor from behavioral studies. Therefore, the discovery of folate as an attractant molecule for *P. tetraurelia* provided a useful stimulus for receptor studies (Van Houten 1978). The folate molecule has three distinct moieties, making possible competition experiments (Van Houten 1976, 1978) to determine that the pterin group was necessary and sufficient for attraction (Schulz et al. 1984). Folate was found to bind specifically to whole cells and this binding was diminished to nonspecific, low levels in mutants (Schulz et al. 1984) and in the presence of a weak competitor for the behavioral response, cAMP (Schulz et al. 1984; Preston et al. 1987). The half-maximal values for behavior and ΔVm were approximately 100 μM, while the binding Kd was measured at approximately 30 μM. Discrepancies between binding and response data are not uncommon, but part of this discrepancy could be due to the need to measure whole cell binding as instantaneous binding (Schulz et al. 1984). Folate was found to be transported into cells and cold temperatures would not eliminate this transport to make equilibrium binding measurements possible. Therefore, instantaneous binding was used and though it should not have underestimated the Kd, it might have underestimated the binding capacity of the cell. Nonetheless, the instantaneous binding measured by centrifugation methods using [^{3}H]-folate had strong correlations with behavior and hyperpolarization in stimulus and probably represented binding involved in chemoreception: mutants did not show this binding; cAMP affected attraction, and binding and characteristic hyperpolarization (Schulz et al. 1984; Preston et al. 1987); binding was primarily to the cell body and not cilia (Schulz et al. 1984). This latter point was signifi-

cant because electrical recording from deciliated cells indicated that the cell body membrane had all the components necessary for the characteristic hyperpolarization in folate and other attractants (Preston and Van Houten 1987b).

The folate receptor should reside among the folate binding proteins of the cell body membrane. Affinity chromatography has been used to identify approximately 20 proteins of the cell body membrane that bind folate specifically. Only five were accessible to [^{125}I] labeling and, therefore, externally facing and among these only two were much more abundant in the cell body membrane compared to the ciliary membrane (Schulz et al. 1985a, b; J.L. Van Houten et al. 1986; Van Houten and Preston 1987c). However, low affinity posed a problem for the use of traditional affinity chromatography in identifying chemoreceptors. It was likely that the folate binding protein of interest would bind too weakly to be specifically eluted and thereby display differences in affinity chromatography between normal and mutant. Therefore, antiligand antibody was developed to circumvent the problems of low affinity. The rationale for its use was as follows: when folate was cross-linked onto whole cells, they specifically lost their attraction response to folate, but not to acetate (Schulz et al. 1985a, b; Sasner 1986; Van Houten and Preston 1987a, c). Therefore, the receptors should have been among the surface proteins that were cross-linked with folate. To detect the proteins that were cross-linked, a polyclonal antifolate antibody was used to develop electroblots (Sasner et al. 1986) and to precipitate folate cross-linked proteins from [^{35}S]-labeled cells. This alternative approach will be potentially useful for other low affinity receptor systems.

A parallel picture has emerged for cAMP, which was found to be an attractant to *P. tetraurelia* (Schulz et al. 1982; Smith et al. 1987). Cyclic AMP and not a hydrolysis product acted externally as an attractant (Smith et al. 1987). Cyclic AMP attraction and characteristic hyperpolarization could be blocked by 5′-AMP but not by 5′-GMP or cGMP (Smith et al. 1987). Likewise, binding of cAMP to whole cells was saturable, specific, and blocked only by 5′-AMP. Binding once again was primarily to the cell body membrane with a Kd of approximately 250 μM, which was approximately one-fourth of the concentrations for hyperpolarization and behavioral half-maximal responses.

There was primarily one protein of approximately 48000 daltons that specifically eluted from a cAMP affinity column with cAMP or 5′-AMP, but not with cGMP or 5′-GMP (Van Houten and Preston 1987a, c). This protein appeared to be missing from affinity column fractions of a mutant, cyc^{-1}, that was not attracted to cAMP (Smith et al. 1987). The 48000 dalton protein was close in molecular weight to the immobilization antigen-related proteins in the 40000 molecular weight range (Eisenbach et al. 1983). However, immunoblots of whole cell body membranes and cAMP affinity column fractions have demonstrated that they are not the same proteins (Sasner and Van Houten, unpubl. results).

Like folate, cAMP can be covalently cross-linked to its receptor. 8-azido-cAMP will covalently bond to nearby proteins in the presence of *UV* light (Haley 1975). Treatment of cells with *UV* light in the presence of 8-azido-cAMP eliminated attraction to cAMP in the T-maze assay, while irradiation in the presence of cAMP did not. This inhibition was specific for cAMP; acetate

Table 1. Effects of 8-N_3-cAMP on chemoresponse

Pretreatment with UV irradiation in:	Test for chemoresponse		I_{che}[a]	n
	Test solution	Control solution		
2.5 mM NacAMP	2.5 mM NacAMP	2.5 mM NaCl	0.66 ± 0.05	25
2.5 mM N_3cAMP	2.5 mM NacAMP	2.5 mM NaCl	0.50 ± 0.06	14
	2.5 mM Na_2folate	5 mM NaCl	0.62 ± 0.07	11
	5 mM NaOAc	5 mM NaCl	0.66 ± 0.06	12

[a] Data are averages of n T-maze tests ± one standard deviation. $I_{che} > 0.5$ indicates attraction; = 0.5 indicates no response (Baez, unpubl. results).

and folate attraction were not affected (Table 1). This development makes possible the use of [^{32}P]-azido-cAMP to specifically label the chemoreceptors.

Chemoreceptors probably are not evenly distributed over the cell surface. There is a partitioning of components for the hyperpolarization between the ciliary membrane and the contiguous cell body membrane; generally, cells could be deciliated without loss of the attractant-induced hyperpolarization (see Preston and Van Houten 1987b for discussion and an exception). Additionally, there is partitioning between the anterior and posterior ends of the cell for folate reception. Pressure perfusion of folate onto deciliated cells elicited the largest hyperpolarization on the anterior end with decreased responses as the perfusion pipette was moved toward the posterior of the cell (Fig. 7) (Preston and Van Houten 1987b). Gradients of chemoresponsiveness anterior to posterior could not be distinguished as a gradient of fluorescence from normal cells stained with fluorescein-folate. Fluorescein-folate bound specifically to normal cells and normal cells could be distinguished in blind tests from folate chemore-

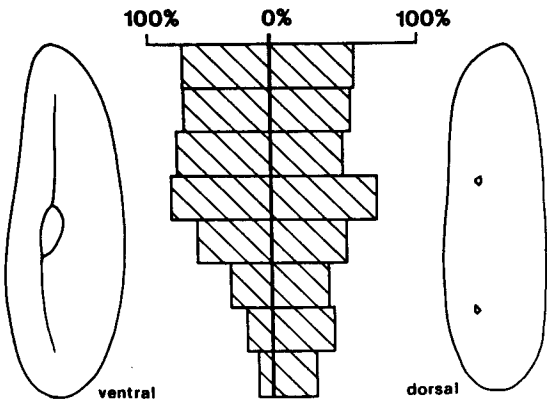

Fig. 7. Localization of chemosensitivity on the cell body membrane of deciliated *P. tetraurelia*. 2.5 mM K_2 folate was locally applied by pressure perfusion to 8 sites along the ventral and dorsal surfaces of cells. The resultant membrane hyperpolarizations are expressed as a percentage of the maximum response obtained from each cell. The data represent mean responses of 20 deciliated cells (Preston and Van Houten 1987b)

ception mutants and autofluorescence (Van Houten et al. 1975). However, this method of assaying chemoreceptor binding probably was not sensitive enough to detect gradients along the cell.

Once the identities of chemoreceptors for folate, cAMP, and others are verified, it should be possible to clone the genes for these receptors and thus obtain extremely useful reagents for their study. The isolation and use of mutants will assist in this effort. Mutants induced with nitrosoguanidine have been selected and characterized (DiNallo et al. 1982) and from these mutants there have been revertants that by genetic analysis appeared to be second-site suppressors (Van Houten, unpubl. results). However, the reversion of phenotype toward the wild type and hence instability has posed a problem with existing mutants. To address this, a different mutagenesis designed to produce null-phenotype mutants by a deletion or break in a structural gene, has produced mutants that are stable, perhaps due to lack of a functional gene product. Additionally, a newer screening procedure was developed. Previously, cells had been selected for their inability to remain in attractant (DiNallo et al. 1982). The alternative method selects for cells that do not bind folate, by scoring clones grown and stained with fluorescein-folate in microtiter (96-well) plates (Van Houten et al. 1985).

6 Characteristic Membrane Potential Changes in Chemoresponse: Models for Testing

The study of the ionic basis of the membrane potential change (ΔVm) has gone on concurrently with the receptor studies, with acetate and folate as the most intensely studied stimuli. The simplest working models for the hyperpolarization in attractants were a K or Na efflux triggered either directly by the ligand-receptor interaction or by ligand-dependent increase in Ca^{+}_{i}. These models were tested (Preston and Van Houten 1987a) by determining the effects of external ion substitution and resting ΔVm on the size of the attractant-induced hyperpolarizations. There was found to be a general dependence on ionic strength; increasing any divalent or monovalent cation to high levels decreased the size of the hyperpolarization. However, K_0 or Na_0 could be present, absent, and replaced by $Tris^+$, or present in amounts that should eliminate the driving forces for their net movement in or out of the cell with no effect on ΔVm. Therefore, ion substitution experiments indicated that simple efflux of K or Na could not account for the ΔVm, nor could influx of Cl^-. Ca_0 could not be reduced greatly without affecting cell viability and motility. However, the use of mutants with specific defects in Ca currents and W-7, a calmodulin inhibitor that has been shown to eliminate many Ca currents in *Paramecium* (Hennessey and Kung 1984), have ruled out most known Ca conductances in *Paramecium* that could trigger a hyperpolarizing ion efflux. Additionally, there appeared to be no voltage dependence and reversal potential for the hyperpolarizations in folate or acetate as measured either by current clamp or voltage clamp (Preston and Van Houten 1987a).

The lack of reversal potential and lack of effects of ion substitutions had at least two possible explanations. The ΔVm was due to surface charge changes or

to electrogenic pumps. Ruthenium red, a polycation that perturbs surface charge, did not affect the ΔVm; nor did a surface charge mutant (Satow and Kung 1981) display an altered ΔVm for folate or acetate (Preston and Van Houten 1987a). Therefore, efforts are being made to investigate Ca^{2+} and H^+ pumping and exchange during chemoresponse using voltage clamping, radiotracer, and Ca-sensitive fluorescent dyes. All of these methods can be applied to *Paramecium* and should clarify some aspects of the role of Ca in chemoreception.

In addition to attractants like folate, acetate, and cAMP, amino acids were found to hyperpolarize the cells (Preston 1983). This hyperpolarization of 5–6 mV is transient, lasting approximately 9 s. All amino acids tested, except ornithine and tyrosine at low concentrations elicited a variable attraction response from the cells, with thresholds in the nM range and each amino acid caused increased swimming speed (Preston 1983). The binding of [^{3}H]-amino acids to isolated cilia indicated the presence of binding sites with Kds in approximately the 10^{-7} M range (Preston 1983, 1984). The relation of the transient hyperpolarization in amino acids to chemoresponse remains to be established.

7 Second Messengers

Stimulation of a cell with attractant was transduced into a hyperpolarization. It was expected that a second messenger system was part of the transduction of an external chemical signal into an electrical one, to which the motor apparatus responded. Cyclic nucleotides were the most obvious second messengers, since internal levels of cAMP and cGMP have been linked with control of ciliary beating (Bonini et al. 1986; Majima et al. 1986; Schultz et al. 1986). Hyperpolarization from decreased ionic strength was associated with increased $cAMP_i$ and swimming speed (Bonini et al. 1986), and therefore, it was possible that the hyperpolarization associated with attractants could also be associated with $cAMP_i$. However, isobutyl methylxanthine (IBMX), a phosphodiesterase inhibitor, did not perturb chemoresponse when it was included in both test and control arms of the T-maze, even though it was present in concentrations high enough to elevate internal cAMP levels and to increase swimming speed (Van Houten et al. 1987). Also, measurements of $cAMP_i$ during chemostimulation indicated no significant change in internal levels other than an increase as would have been predicted from the size of the hyperpolarization alone (Van Houten et al. 1987). There remains the possibility that cyclic nucleotides are messengers in chemoreception because the final motor response of the pathway involves cilia. However, it does not seem likely that cAMP is a second messenger involved in the generation of the ΔVm. Other second messengers, such as $Ca^{2+}{}_i$, H^+, IP_3, and diacyl glycerol remain possibilities. There was no effect on chemoresponse by phorbol esters that should mimic diacyl glycerol as second messenger (Wright, unpubl. results), and no strong effect of external pH, which might perturb H^+ movement if it were involved in transduction. While none of these alternative second messengers has been eliminated, we favor an efflux pump or exchange for Ca^{2+} to account for the hyperpolarization and the 0.2 nA current that can be measured by voltage clamp.

Application of amiloride, a diuretic that is known to perturb Na^+/H^+ and Na^+/Ca^{2+} exchange (Zhuang et al. 1984; Kaczorowski et al. 1985) has been shown to inhibit chemoresponse to sodium salts of acetate and folate (Van Houten and Preston 1985). Likewise, lithium has been shown to inhibit chemoresponse to acetate and folate. The study of these two effects is ongoing and may hold key information about the mechanism of transducing stimulus binding into ΔVm or into the slower process of adaptation. Covalent modification may also be involved in adaptation, as is methylation in bacteria (Boyd and Simon 1982; Hazelbauer and Harayama 1983; Ordal 1985). There is a striking effect of S-adenosyl-methionine on chemoresponse that is inhibited by a battery of methyl transfer inhibitors in *Paramecium* chemoresponse (Van Houten et al. 1984b). Likewise, phosphorylation of membrane components could potentially be involved in the mechanism of adaptation, as in *Dictyostelium* chemoreceptors (C. Klein et al. 1985; P. Klein et al. 1985).

8 Summary

Paramecium is a bona fide receptor cell. It shows clear responses to organic stimuli, for which it seems to have evolved receptors and sensory transduction pathways. The mechanisms by which cells accumulate or disperse as a population depend upon the magnitude of the ΔVm the stimulus elicits. Cells can be attracted or repelled either by primarily modulating the frequency of turning or primarily modulating speed. Both are consistent with the known physiology of individual cells. The identity of receptors is being determined and will eventually lead to the cloning of the receptor genes. The ionic mechanism of the ΔVm appears to be due to a pump perhaps specific for Ca^{2+} or H^+. Other internal messengers such as cAMP are undoubtedly involved in the control of ciliary beating, but perhaps as only third or fourth messengers for chemoresponse. The roles for methylation, phosphorylation, and amiloride-sensitive ion fluxes are being explored.

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