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Chemotaxis by the Nematode *Caenorhabditis elegans*: Identification of Attractants and Analysis of the Response by Use of Mutants

(cAMP/anions/cations/hydroxyl ions/klinotaxis)

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ABSTRACT **The nematode *Caenorhabditis elegans* is attracted by at least four classes of attractants: by cyclic nucleotides, cAMP and cGMP; by anions, Cl^-, Br^-, I^-; by cations, Na^+, Li^+, K^+, Mg^+; and by alkaline pH values. The nematode's behavioral response to gradients of these attractants involves orientation and movement up the gradient, accumulation, and then habituation. Comparison of the tracks of wild-type and mutant animals responding to gradients of attractants indicates that sensory receptors in the head alone mediate the orientation response and that the direction of orientation is determined by the lateral motion of the head. Therefore, the orientation response is a klinotaxis.**

The study of behavioral mutants of an animal can lead to correlation of the behavioral alterations with underlying alterations in anatomy, physiology, or biochemistry (1). These correlations will help identify the neural circuity mediating the behavior and may reveal molecular mechanisms by which these nerves operate. If the behavioral mutants are due to single gene defects, their interpretation may eventually indicate how individual genes can act to specify patterns of behavior.

The nematode, *Caenorhabditis elegans*, is an excellent organism for such studies. It is easily maintained in the laboratory, and many mutants have been isolated and genetically characterized (S. Brenner, unpublished). There are less than 300 neurons in the nervous system of the nematode, and extensive work has already been done to determine the detailed anatomy and connections of these cells by reconstruction from serial section electron micrographs (White, Ward, Thomson, and Brenner, unpublished).

Before selection of mutants with defined behavioral alterations it is necessary to characterize behavioral responses of the wild type. This paper reports experiments that define the chemotaxic behavior of *C. elegans*. Several attractants have been identified, and the behavioral response to gradients of these attractants has been specified. Comparison of the wild-type behavior with that of some mutants suggests the location of the sensory receptors mediating the responses and defines the mechanism of the orientation response.

METHODS AND MATERIALS

Organism. The nematode used for these studies was *C. elegans* (var. Bristol). All mutant strains were derived from this parent by ethylmethane sulfonate mutagenesis, and were obtained from the collection of S. Brenner. Methods of mutant isolation and general methods of culture and handling of the nematode will be described elsewhere.

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Establishment of Gradients. The two methods used for studying chemosensory behavior depend upon the establishment of defined and reproducible gradients of attractants. Radial gradients were established in a thin layer of agarose or Sephadex gel beads spread on petri plates. The agarose or gel beads stabilize the liquid against convection and also provide a medium on or through which the worms move rapidly. When the diffusion of $^{32}PO_4$ and [^{14}C]phenylalanine were measured, the gradients established at different times were found to decrease exponentially as predicted by theory (ref. 2; Eq. 3, 10), and the diffusion coefficients of the phosphate and phenylalanine agreed with the published diffusion coefficients for these molecules in water. Gradients of identical shape were established for molecules with different diffusion coefficients by adjusting the time allowed for diffusion inversely to the diffusion coefficient; diffusion coefficients were either estimated from molecular weight (3) or calculated from ionic free mobilities (4). Unknown attractants were assumed to have a diffusion coefficient of 10^{-5} cm^2/sec.

Chemosensory Assays. The first assay for chemosensory behavior measures the fraction of worms that accumulate in the center of a gradient of attractant. Gradients were established by applying 5 μl of attractant to the center of a 4-cm petri plate spread with 1.25 ml of a slurry of Sephadex (Pharmacia) G-200 superfine gel beads swollen in buffer [0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid (Hepes) pH 7.2–0.25% Tween 20]. Worms from an agar plate that had been spread with bacteria, innoculated with 5–10 worms, and grown for 6 days at 20° were washed off with 5 ml of the above buffer. They were rinsed three times on an 8-μm pore size SCWP Millipore filter to remove bacteria, then suspended for 15 min in buffer and counted. About 2000 worms were filtered again, washed into a centrifuge tube containing 1 ml of Sephadex gel beads, centrifuged at low speed for 10 sec to sediment the worms with the Sephadex, then immediately applied to the edge of the assay plates with a 50-μl Eppendorf pipette. The fraction of the population of worms accumulating in the center was then determined by counting under a dissecting microscope.

The second assay determines the orientation of a worm in a gradient of attractant by recording its tracks. 8-cm petri plates were spread with 3-4 ml of melted 1.5% agarose (BDH, electrophoresis grade) in the above buffer. After the agarose cooled, 5 μl of attractant was twice applied to the center, at time intervals adjusted to give a gradient that does not flatten at the center. Worms were prepared as for the previous assay, omitting the transfer to Sephadex, and individual worms were applied to the plates with a 5-μl

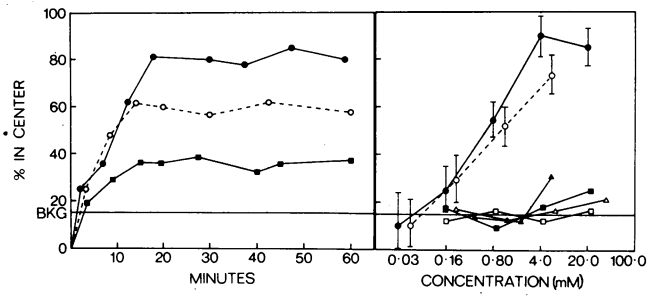


FIG. 1 (*left*). Rate of accumulation in response to cAMP. 5 μl of cAMP at different concentrations was applied to the centers of plates spread with Sephadex beads and allowed to diffuse for 7 hr. Then, about 100 worms were applied to the edges of the plates and the number of worms collecting within 0.8 cm of the center was determined. The background (*BKG*) is the percentage expected in the center for a uniform distribution of worms. The gradient of cAMP changes only slightly during the time of the worms' response. The concentration in the center for each curve was calculated from the diffusion equation. ●——●, 3.2 mM; ○——○, 0.8 mM; ■——■, 0.2 mM.

FIG. 2 (*right*). End-point of accumulation in response to cAMP and other nucleotides. The gel contained 0.01 M $NaCH_3COO$ to eliminate response to the sodium in the salt of dibutyryl-cAMP. The percentage of the population in the center 30 min after the addition of worms is shown plotted against the concentration of attractants. *Error bars* show the counting uncertainty. The *lower curves* have similar uncertainties. ●——●, cAMP; ○- - -○, cGMP; ▲——▲, 3′-CH_2-cAMP; ■——■, $N,^6O^{2\prime}$-dibutyryl-cAMP; △——△, 3′-AMP; □——□, 5′-AMP.

Eppendorf pipette. Their tracking was initiated by withdrawing the excess liquid surrounding them with a fine capillary pipette. When it was found that the preparation of the worms did not affect their subsequent response, worms were transferred directly to the assay plates from the growth plates with sharpened applicator sticks. After the desired tracking time, the worms were killed in their tracks by inversion of the plates over a few drops of chloroform. The tracks are visible grooves in the agar and were recorded permanently by placing the plate on a sheet of Kodak Kodalith Ortho Type 3 film in a dark room and exposure to parallel light from an enlarger, thus making a contact negative of the plate (K. Harvey and R. Freedman, personal communication). Tracks were subsequently analyzed from prints or by direct projection of negatives.

Chemicals. All chemicals used as attractants were reagent grade when available. Stock solutions of salts were carefully adjusted to neutral pH with their conjugate acid or base. Other reagents were neutralized with ammonium hydroxide, triethylamine, or acetic acid, because the ions of these do not attract the nematode. Nucleotides were obtained from Sigma, London, except for the cAMP methyl phosphonate derivatives, which were generously provided by J. G. Moffat of the Syntex Institute of Molecular Biology. All were obtained as free acids and neutralized with triethylamine, except $N^6,O^{2\prime}$-dibutryl-cAMP, which was obtained and used as the sodium salt.

RESULTS

The Nematode Is Attracted to cAMP. Fig. 1 shows the rate of accumulation of worms in the center of plates with gradients of cAMP. The worms accumulate in the center until a steady-state end point is reached. This end point is stable for several hours. The rate of accumulation is not dependent on the concentration of attractant in the center, and varies considerably from plate to plate, so it cannot be used as a quantitative measure of attraction. In contrast, Fig. 1 shows that the fraction of worms in the center at the end point is dependent on concentration. It is also reproducible and was, therefore, used to quantitate the strength of an attractant.

Fig. 2 shows the end-point response plotted as a function of concentration for cAMP and related nucleotides. The slope of the cAMP response shows that the accumulation assay is sensitive over a 10-fold range of concentration, with a threshold of 0.2 mM. For ten independent assays done over the past 12 months, the mean and standard deviation of the concentration of cAMP that caused 50% of the worms to accumulate was 0.65 ± 0.1 mM, indicating that the assay is reproducible to better than 20%.

Fig. 2 shows that cGMP is as strong an attractant as cAMP, and that 3′-CH_2-cAMP may be 10% as attractive. N_6,O_2'-dibutyryl-cAMP, 3′-AMP, 5′-AMP, and 5′-CH_2-cAMP are less than 1% as strong attractants. In addition, ADP, ATP, or deoxy 5′-AMP are not attractive. When cAMP was distributed uniformly at 2.5 mM in the Sephadex before establishment of a gradient of cGMP, the subsequent response to cGMP was eliminated (the opposite experiment gave the same result), suggesting that both these molecules are interacting with the same receptor site. In contrast, N_6,O_2'-dibutyryl-cAMP and 3′-AMP, 5′-AMP, 5′-CH_2-cAMP, and 3′-CH_2-cAMP do not interfere with the response to cAMP, suggesting that these nucleotides do not bind to the receptor site.

Identification and Classification of Additional Attractants. Several hundred chemicals have been screened with the accumulation assay for attraction, at concentrations up to 10 mM. Sugars, including pentoses, hexoses, and disaccharides, gave uniformly negative results. Products of

TABLE 1. *Summary of attractants*

Class	Attractant	Accumulation threshold (mM)
1. Cyclic Nucleotides	cAMP	0.2
	cGMP	0.2
	Not AMP, Bu_2-cAMP	
2. Anions	Cl^-	2
	Br^-	20
	I^-	20
	Not CH_3COO^-, F^-	
3. Cations	Na^+	2
	Li^+	4
	K^+	15
	Mg^{2+}	20
	Not NH_4^+, $CH_3NH_3^+$	
4. Basic pH	OH^-	∿ 0.001
Unclassified	lysine	∿10
	histidine	∿10
	cysteine	∿10

The attractants are shown grouped into classes, according to competition experiments. The accumulation threshold is the concentration of attractant at the center of the plate that causes 25% of the population (twice the background) to accumulate. Because of their high threshold, the amino acids have not been extensively studied except to show that the amino acids, and not their counter-ions, are the attractants.

bacterial catabolism, organic acids, alcohols, ketones, etc., did not attract. Many other chemicals were also not attractants. Strong attraction was found to several inorganic salts and to basic pH. Weaker attraction was found to several amino acids.

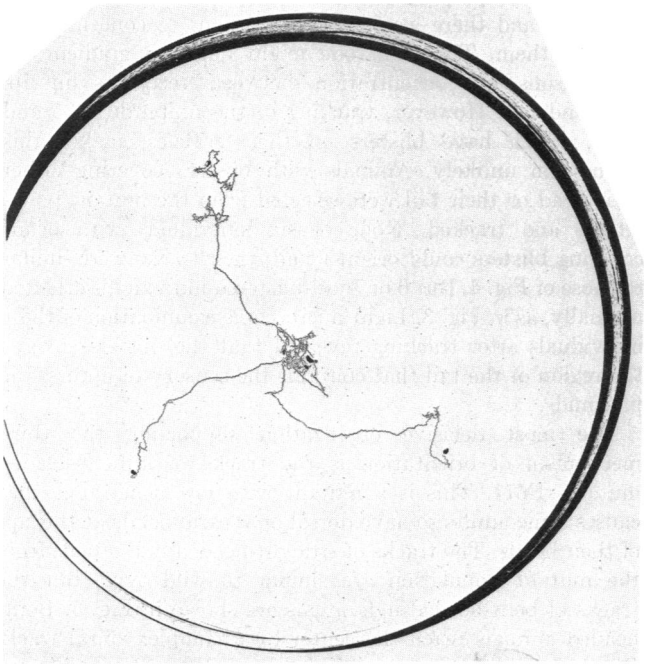
In order to determine whether the response to salts was due to cations, anions, or both, gradients of one ion alone were established. This was done, for example, by making the gel slurry to 0.1 M $Na^+CH_3COO^-$ before spreading, so that the gel would have a uniform concentration of Na^+ and CH_3COO^- ions. Then, when a solution of 0.1 M Na^+Cl^- was applied to the center of the plate, no gradient of Na^+ could form because Na^+ was already uniformly distributed throughout the plate. Therefore, only a gradient of Cl^- would be established. (The deficiency of CH_3COO^- ions initially at the center would be quickly eliminated by diffusion of the surrounding CH_3COO^-.) When worms are added to such a plate they accumulate in the center, showing that the worm can detect and respond to the Cl^- ion alone. Similar experiments showed that NH_4^+ and CH_3COO^- do not attract (nor do they interfere with the response), so the response to individual cations could be further studied by use of acetate salts, and the response to anions could be studied with ammonium salts.

Most of the attractants identified are listed in Table 1. They all give end-point response curves similar to those for cAMP, but differing in their concentration range. The worms are repelled by salt concentrations above 0.3 M.

Table 1 groups the various attractants into classes based on competition experiments between the attractants, as has been done for bacterial chemoattractants (5). For example, as described above, the worm responds to a gradient of Cl^- ions in the presence of $Na^+CH_3COO^-$. The worm also responds to a gradient of Na^+ ions in the presence of NH_4Cl. This result shows that Cl^- does not compete with the response to Na^+, and also vice versa. Therefore, these attractants must be detected by different receptor sites. Similar competition experiments were performed among most of the attractants listed in Table 1, and the results allow classification of the attractants as shown. All the pairwise competitions gave reciprocal results, except for cAMP and Cl^-. The presence of cAMP does not affect the response to Cl^-, but the presence of Cl^- at concentrations above 25 mM abolishes the response to cAMP.

Juvenile and adult worms respond to each of the classes of attractants similarly. In particular, the *dauer* larvae, specialized third-stage juveniles that accumulate when cultures are starved, have responses similar to adults.

The Nematode Initially Orients Up Gradients of Attractants. The accumulation assay used to identify the attractants does not determine how the nematode finds its way to the center of the gradient. In order to determine whether *C. elegans* can orient up a gradient of attractant, the tracks of individual worms responding to gradients of attractant in a thin layer of agarose were followed. Tracks of three adult worms responding to gradients of NH_4Cl are shown in Fig. 3. A control plate is shown in Fig. 4. It is obvious from Fig. 3 that the tracks are directed up the gradient, so the worm must orient its body up the gradient. Therefore, the behavioral response is a chemotaxis. This orientation response is very reproducible: 73 of 82 (89%) adult animals tracked showed unambiguous orientation to NH_4Cl. In addition, first-stage juveniles, *dauer* larvae, and males all show similar orientation. The adults orient to all four classes of attractants listed in Table 1 and to the amino acids. In addition, they orient and move up gradients of Ca^{++} ions, although Ca^{++} does not cause accumulation.



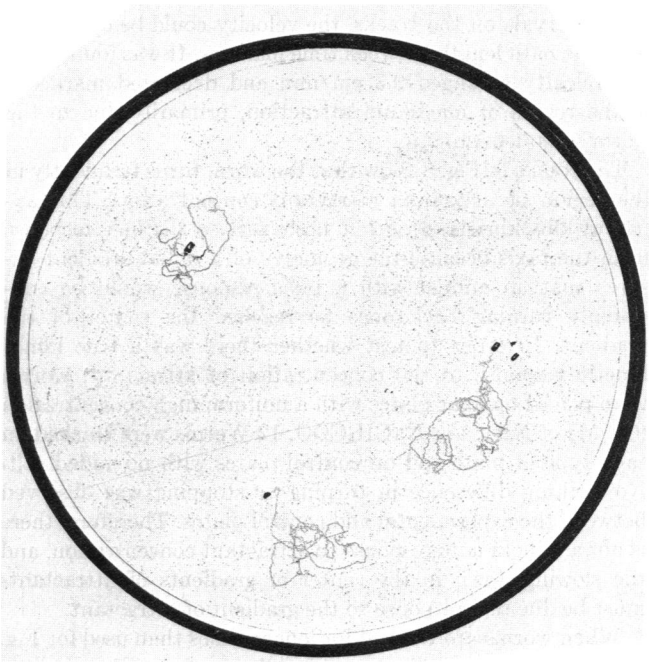


FIG. 3 (*left*). Tracks of wild-type adults responding to gradients of NH_4Cl. 5 μl of 0.5 M NH_4Cl was applied to the center of a 8.5-cm petri plate coated with 4 ml of 1.5% agarose. 12 hr later, another 5 μl was applied. 3 hr later three worms were placed on the plate at the points marked by *dots* (at the outer periphery of the tracks). They were allowed to make tracks for 15 min. The actual gradient on the plates extends from 50 mM in the center to 0.05 mM at the edge, and it changes little during the time of tracking.

FIG. 4 (*right*). Tracks of wild-type adults in the absence of an attractant. Plates were prepared as for Fig. 4, but water was applied to the center rather than NH_4Cl.

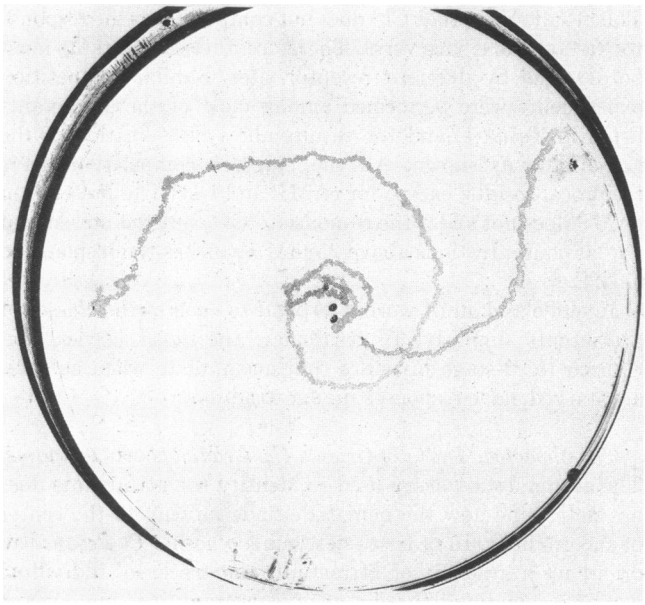


FIG. 5. Tracks of mutant E611. Plates were prepared as for Fig. 3, but two bent-headed animals from mutant E611 were used. The animals required about 1 hr to reach the center. The more-direct track was given by the animal with a lesser bent head.

The threshold concentration for orientation can be estimated roughly by establishing gradients on 12-cm petri plates and placing worms at increasing distances from the center until their initial tracks are randomly oriented. The approximate threshold of orientation for cAMP is 5 μM and for Na^+ or Cl^- is 10 μM.

By tracking worms with a camera lucida and marking equal time intervals on the tracks, the velocity could be calculated from the path length between time markers. It was found that the velocity averaged 0.3 cm/min and decreased markedly in the region of maximum attraction, primarily due to the worm's frequent stopping.

The tracks in Fig. 3 show that the worm turns frequently in the region of maximum attractant concentration. This apparent klinokinesis (6) is not necessarily a distinct response from the taxis because the geometry of a radial gradient ensures that an animal with a taxis response would be continually turning back once he reached the center of the gradient. In order to test whether there was a true klinokinetic response to the concentration of attractant, worms were placed on agar plates with a uniform high concentration (0.1 M) of NH_4Cl or $NaCH_3COO$. 12 Worms were tracked on each type of plate and on control plates with no added salt. No striking difference in turning or stopping was observed between the experimental and control plates. Therefore, there is not a klinokinetic response to attractant concentration, and the slowing down in the center of gradients of attractants must be due to a response to the gradient of attractant.

When worms are tracked for longer times than used for Fig. 3, it was found that after reaching the center of the gradient the worm remains there briefly, then its behavior alters and it swims away from the center. After remaining briefly on the periphery of the plate it returns to the center and repeats the cycle. If the concentration of attractant in the center is varied, then the lower the attractant concentration the less time the worm remains in the center. This behavior is observed for every class of attractant.

The Mechanism of Orientation. Orientation in a chemical gradient requires that the nematode compare the concentration of attractant at different points to determine the direction of the gradient. This determination could be done in any of at least three ways: (*i*) single or multiple receptors could compare concentrations successively in time at points separated by the forward movement of the nematode; or (*ii*) concentrations could be compared simultaneously by use of two receptors separated on the body. These could be located anteriorly and posteriorly or symmetrically about the long axis (tropotaxis, 6); or (*iii*) successive comparisons in time could be made by side-to-side (actually dorso–ventral) displacement of receptors (klinotaxis, 6). The orientation behavior of several mutants shows that the third mechanism is used by *C. elegans* to detect the direction of a chemical gradient.

If the first mechanism were involved in the orientation response, then the accuracy of orientation should depend on the worm's forward velocity. But a mutant (E444) that moves slowly because its body muscles degenerate gives tracks identical to the wild type, although these worms take 2 hr to reach the center instead of 15 min for the wild type. In shallower gradients, which test more sensitively for orientation, the slow-moving animals again oriented as well as the wild type. Therefore, the orientation is not affected by the forward velocity of the worm, so the first mechanism of orientation is unlikely.

The nematode could not detect the direction of the gradient by the second mechanism, which uses two receptors placed symmetrically about the long axis, because the animal swims on its side and its only likely anterior chemoreceptors, the amphids, are located laterally (7). Therefore, these receptors are positioned perpendicular to the plane of motion of the nematode and there would be no gradient of concentration between them. The nematode might detect a gradient by comparisons of concentration between receptors in its head and tail. However, tracking of the mutants E935 and E937, which have blisters on their cuticles, makes this mechanism unlikely. Animals with blisters covering either their head or their tail were selected from the mutant populations and tracked. None of six individuals with head-covering blisters could orient at all; their tracks were similar to those of Fig. 4. But 6 of 7 tail-blistered individuals oriented normally, as in Fig. 3. Light microscopic examination of these individuals after tracking revealed that the blisters covered the region of the tail that contains the sensory receptors, the phasmids.

The most decisive observation supporting the third mechanism of orientation is the tracks of a bent-headed mutant, E611. This is a mutant with low penetrance that causes some adults to have dorsal or ventral bends at the tips of their heads. The tracks of straight-headed individuals from the mutant population are similar to wild type, but the tracks of bent-headed individuals are shown in Fig. 5. Bent-headed animals reach the center by a complex spiral track. Observation of 10 of these animals while tracking revealed that the hand of the spiral is such that direction of bend of the head is towards the center. On two occasions, animals flipped over while tracking and the hand of the spiral reversed. This observation shows that the worm orients so that its head points directly up the gradient. When it does so, the body is at

an angle to the gradient so that the direction of motion is at an angle to the gradient generating the spiral track. The track is a complex series of loops and turns because the bent head serves as a rudder at the front of the animal, causing it to turn as it moves forward. The worm continually turns back again to maintain its orientation up the gradient. Note that the angle of deviation of the track from the line of the gradient corresponds roughly to the angle of bend of the head, and is much greater than would be expected if the gradient were measured between the head and the tail.

The tracks of two other head-defective mutants, one with defective head muscles, E25, and one with a shortened head, E30, show that these animals do not orient as well as the wild type, although they accumulate normally. These results are consistent with a mechanism of orientation dependent solely on the motion of the head.

DISCUSSION

The reproducible gradients that can be established in slurries of Sephadex gel beads or in agarose make it possible to assay chemotaxic behavior reliably. The small deviation of the accumulation assay and the near 90% reliability of the orientation assay show that the behavior of genetically identical worms is highly invariant.

The two assays for chemosensory behavior measure different aspects of the behavioral response. The tracking assay determines the tendency of the worm to orient in a concentration gradient. That this response is distinct from accumulation is indicated by its 50- to 100-fold lower threshold for salts and cAMP, by the response to Ca^{++}—which induces orientation but not accumulation—and by the mutants that orient poorly but accumulate normally. The distinction is further confirmed by the recent isolation of two mutants that orient and move up gradients normally, but do not accumulate.

This distinction between orientation and accumulation may be important to understanding the host-finding behavior of parasitic nematodes. Chemical attractants that draw the parasites near the host may be different from those that keep the nematode there and allow host penetration. Some evidence for this distinction is discussed in ref. 8.

The alteration of the nematode's behavior after remaining in a region of maximum attractant resembles a classical habituation to repeated stimuli (9). The dependence of habituation on concentration provides an explanation of the concentration dependence of the accumulation assay. The fraction of worms accumulating in the center of a plate reflects the fraction of time that each individual worm spends in the center rather than the fraction of the population that responds to a given concentration. This conclusion has been confirmed by tracking individual worms during the accumulation assay with a camera lucida, and by collecting the worms that are not responding and observing that in a fresh assay they give the same fraction responding as the original population.

The attraction of *C. elegans* to cAMP is not surprising since bacteria are its normal food and all bacteria that have been examined thus far synthesize cAMP and release it into the medium (10). Slime molds are also attracted to bacteria by cAMP (11). However, preliminary experiments with *Escherichia coli* mutants deficient in adenylate cyclase (kindly provided by Drs. Ira Pastan and Larry Soll) show that the cAMP plays only a slight role in attracting the nematodes to *E. coli* under laboratory conditions. The bacteria must, therefore, release other—so far unidentified—chemicals that are stronger attractants. Whether this is so for the soil bacteria that are the natural food of *C. elegans* remains to be determined.

The chemosensitivity of other nematodes has been recently reviewed by Croll (7), Green (8), and Klingler (12). The attractant that has been most studied is CO_2, but oxidizing agents, reducing agents, some ions, and some amino acids have also been reported as attractants for various species. The response of *C. elegans* to specific anions and cations is more sensitive than that described for other nematodes, and a nematode response to basic pH or to cAMP has not been reported before. It is not clear what role the responses to ions play in the nematode's natural environment. The specificity of attraction of *C. elegans* to cAMP suggests that the conclusion that the host-finding mechanisms of plant parasitic nematodes are entirely nonspecific should be re-examined (7).

The orientation behavior of the mutants tracked shows that sensory receptors on the head alone mediate the orientation response, and that the length of the head and its motion are critical for orientation. It cannot yet be specified whether the orientation requires comparison of the concentration only at the extremes of head movement, or throughout the swing of the head. In either case the orientation requires successive side-to-side (actually dorso–ventral) comparisons of stimulus intensity and is, therefore, a *klinotaxis* (6).

Knowing the chemotaxic behavior of the wild type, it should be possible to select more mutants with defined alterations in this behavior. The fine structure of the receptors in the head that must mediate the chemotaxis is known and the axonal connections of the sensory neurons are presently being worked out (Ward, White, Thomson, and Brenner, unpublished). When these are known, mutants can be compared to the wild type to correlate behavioral and anatomical defects. In this way it may be possible to specify further how the nematode's nervous system transforms the sensory input from the head into its chemotaxic behavior.

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