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Countercurrent Separation: A New Method for Studying Behavior of Small Aquatic Organisms

(nematode/*Caenorhabditis elegans*/chemotaxis)

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ABSTRACT **A new method for the analysis of behavior in small free-swimming aquatic organisms is described. In this procedure, called countercurrent separation, a dense solution flows down along the bottom of an inclined chamber while a light solution flows in the opposite direction, upward along the top of the chamber. The attraction of animals (injected into the center of the chamber) to one solution or the other is then determined by observing the proportion of animals that emerges from the chamber in that solution. When used with the nematode *Caenorhabditis elegans*, it is estimated that the apparatus is equivalent to at least nine theoretical plates.**

There is growing interest in studying behavior and its biological basis in many types of small organisms, especially by the use of genetic techniques (1). This interest stems from two basic advantages of small organisms. First, their size generally coincides with relative simplicity, and second, they reproduce relatively rapidly, a property that greatly facilitates genetic investigations. In order to take full advantage of these characteristics it is desirable to have methods for analyzing behavior in large populations. Such methods allow behavioral parameters to be determined with a minimum of error due to individual variation and, in addition, should provide mechanisms for isolating behavioral mutants. The technique of countercurrent distribution as applied to *Drosophila* (2) is a good example of this kind of method. However, it is not easily applied to aquatic organisms, and many of the most interesting organisms are aquatic, e.g., bacteria, protozoa, and nematodes. In this report, I describe a new technique called countercurrent separation that is similar to countercurrent distribution but is somewhat simpler in its operation and is appropriate for aquatic organisms.

The new method is based on analogous techniques for the extraction of molecules, organelles, or cells from one phase to another immiscible phase (3–5). The simplest countercurrent technique, used in the petroleum industry (6), uses a column in which a dense phase continuously flows down through a rising light phase. However, because of the limited rate of diffusion, for high efficiency it is usually necessary to intersperse phase-mixing operations with phase-separating operations (7). This process is commonly accomplished in one of two ways: (*i*) a continuous-flow column comprising alternate mixing and separating stages (7), or (*ii*) a series of chambers in which the phases are mixed, separated, and transferred in temporally discreet operations (3). In applying these concepts to studies of behavior in aquatic organisms it seemed that the relatively high rate of movement of a swimming animal, compared to the rate of diffusion of a molecule, would make a simple continuous method feasible.

In the method of countercurrent separation developed here for nematodes, two solutions are made to flow through a chamber in opposite directions toward separate collection vessels. Conditions are designed so that the solutions in the chamber have a large area of contact with one another, yet flow stably past each other. One solution is then made attractive relative to the other (e.g., by addition of an attractive chemical), and animals are inserted into the center of the chamber at the interface between the two solutions. The greater the fraction of time they spend in the more attractive solution, the more rapidly they will be moved in its direction of flow, and the more likely they will be to leave the chamber with it, as opposed to the other solution. By measuring the proportion of animals exiting in the two solutions and the rate of the exiting process, the direction and intensity of the animals' response may be quantitated.

This method should be generally applicable to the study of responses to heat, light, chemical stimuli, and perhaps other stimuli by various aquatic organisms. Initially, it has been used to study chemotaxis in the nematode, *Caenorhabditis elegans*, as described below.

MATERIALS AND METHODS

The nematode *C. elegans*, strain N2 was used. It was grown at 20° in petri dishes with a lawn of *Escherichia coli* OP50 (a uracil-requiring strain) on a medium consisting of 1.7% agar, 0.25% peptone, 0.3% NaCl, 25 mM K-PO_4 (pH 6.0), 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 5 μg/ml of cholesterol. These organisms and their method of culture were obtained from Dr. Richard L. Russell; the culture methods are derived from those worked out in the laboratory of Dr. Sydney Brenner. Nematode salts medium consisted of 50 mM NaCl, 25 mM $KHPO_4$, 1 mM $CaCl_2$, and 1 mM $MgSO_4$. Low-salt medium was identical, except that NaCl was 10 mM. K-PO_4 was a mixture of K_2HPO_4 and KH_2PO_4 at pH 6.0. The methyl cellulose used was Fisher 1500 centipoise.

The general idea behind the apparatus is to obtain an inclined chamber in which a dense solution flows downward on the "floor" of the chamber, while a light solution (floating on the dense solution) flows upward along the "ceiling" of the chamber. This can be accomplished, as shown in Fig. 1, by pumping the dense solution into the chamber near the floor at the high end and allowing it to exit near the floor at the

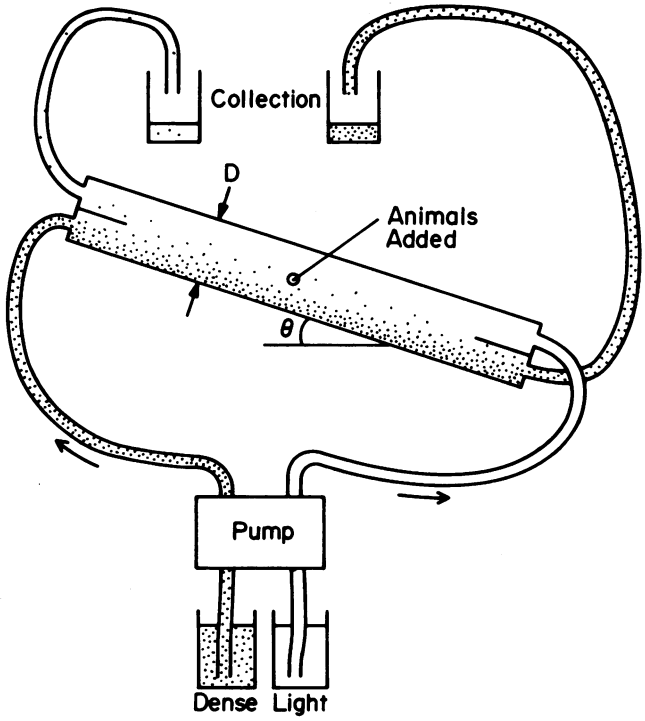

FIG. 1. Schematic diagram of the countercurrent separator. The *stipling* indicates the dense solution.

low end, while the light solution is pumped (at the same rate) into the chamber near the ceiling at the bottom end and allowed to exit near the ceiling at the top end. Tubing is used to release the two emerging solutions at the same height so that there is an equal flow of each. When the various parameters (rate of flow, angle of inclination, density difference, viscosity, and thickness of chamber) are properly adjusted, the pattern of countercurrent flow is stable and symmetrical. This may be seen in Fig. 2, a photograph of nine circular cross-section chambers in which the dense and light solutions have been differentially stained. Apart from diffusion, little—if any—mixing occurs throughout most of the length of the chamber. Some mixing is generally apparent at the ends, but does not hinder proper functioning of the apparatus.

The necessary relation among the above parameters for the establishment of stable countercurrent flow between parallel plates can be derived by the following argument. In the countercurrent flow pattern there is a surface in the center of the chamber, between the two solutions, at which the solutions are stationary. It is desired that both layers flow as if this surface were a solid boundary, and because this surface has the same effect on the flow pattern (producing a stationary layer) as a solid boundary, one may apply the well known equations for flow between parallel plates to each half of the chamber. The only complication is that the density difference must "drive" two solutions.

The equation for steady, laminar flow of a liquid driven only by gravity between two parallel plates is (8), with $\eta = \mu$, $g\rho = \gamma$, $d = 2b$, and $p = 0$:

$$V = \frac{d^2 g\rho}{12\eta} \cdot \frac{dh}{ds}$$

where V = mean velocity of the liquid (absolute value); d = separation of plates; η = dynamic viscosity of the liquid; s = distance in direction of flow; g = acceleration of gravity; ρ = density of the liquid, assumed to be constant; and h = elevation of plates with respect to gravity. In terms of the angle of inclination of the plates, θ, this becomes:

$$V = d^2 g\rho \sin\theta/12\eta.$$

From this the rate of flow, F, per unit width of the stream is calculated:

$$F = Vd = d^3 g\rho \sin\theta/12\eta.$$

In the case of countercurrent flow each solution must displace the other. As a result, only the difference in densities, $\Delta\rho$, between the solutions enters into the equation. Because this difference must drive both solutions, only half of it is effective in driving one solution. Thus, for each solution in countercurrent flow:

$$F = d^3 g\Delta\rho \sin\theta/24\eta$$
$$= D^3 g\Delta\rho \sin\theta/192\eta,$$

where $D = 2d$ = the separation of flooor and ceiling in a countercurrent chamber.

It must be remembered that this discussion applies only to conditions of laminar flow. In general this restriction requires that the Reynolds number ($V\rho d/\eta$), must be less than 10^3.

This theory was tested using rectangular chambers 93 cm long, 2.5 cm wide, and 0.5 cm high. This width is sufficiently large that edge effects are negligible. The addition of 10 μg/ml of the dye Light Green SF Yellowish to the light layer permitted visualization of the flow pattern. The parameters for two quite different situations that were tested are presented in Table 1. In both cases the predicted flow rates did indeed produce stable countercurrent flow. This pattern of flow was sufficiently stable that changes in the rate of flow over a range of a factor of two produced minimal changes in the pattern. For this reason the equation was not tested in detail.

In addition to the rectangular cross-section chambers de-

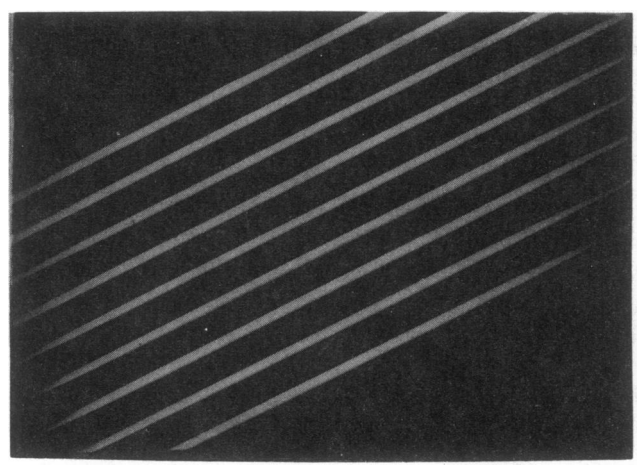

FIG. 2. Photograph of nine independent, circular cross-section chambers during countercurrent flow. Both solutions of each chamber contained 0.5% (w/v) methyl cellulose. The light solutions also contained 100 μg/ml of the green fluorescent dye Fluoresceine, while the dense solutions contained 100 μg/ml of the red fluorescent dye Rhodamine B, in addition to 2.0% (w/v) sucrose. The chambers were illuminated by ultraviolet light and photographed through a yellow cut-off filter (Corning 3-68).

scribed here, chambers with circular cross-section have been used in experiments with nematodes. These were glass tubes of 0.6-cm internal diameter and either 123 or 62 cm long. They performed well at an angular inclination of 25° and flow rates of 0.3 ml/min.

RESULTS

The method of countercurrent separation has been used to study some aspects of chemotaxis in the nematode *Caenorhabditis elegans*. Data from a typical set of experiments with the long, circular cross-section chambers are presented in Table 2, which shows the number of nematodes that have emerged with each of the two solutions and the number remaining inside the chamber 5 hr after they were injected into the center of the chamber. Note first that when the two solutions differ by a factor of only two in K-PO_4 concentration, the majority of the animals emerge in the solution of higher concentration, whether this is dense or light. Secondly, the nematodes have a bias in favor of the dense solution (probably because they tend to sink). Finally, it may be noted that more worms remain in the chamber when there is less asymmetry in the response. The latter observation can be explained if a typical nematode makes many choices between the solutions while still in the apparatus. When the two solutions are about equally attractive, some time is spent in each, and net movement away from the injection point is consequently slow; when the choice is clearer, most of the time is spent in the favored solution and the animal moves away from the injection point at nearly the rate of flow. A rough estimate of the number of choices made by a typical nematode can be obtained by analyzing the performance of the apparatus in terms of theoretical plates, as is common in chromatography (9). In this method the apparatus is considered to be a series of identical "plates" each of which, after a certain interval of time, transfers an animal to one or the other of the two adjacent plates with certain probabilities. (In the present case the probabilities are determined by the relative amount of time the animal spends in each of the two layers, and it is assumed that this is constant during the experiment.) The problem is then to determine what these probabilities are and how many plates are required to reproduce the degree of separation obtained and the length of time taken for the animals to emerge from the apparatus.

The mathematical analysis of this problem is identical to the problems of one-dimensional random walk with absorbing barriers and "gambler's ruin." The general solution is given by Feller (10). Using his notation, let p be the probability of moving in the direction of flow of the light solution, q the remaining probability of movement in the opposite direction, a the total number of plates in the apparatus, z the number of plates between the point of injection and the end from

TABLE 1. *Conditions used for testing countercurrent flow*

η (poise)	$\Delta\rho$ (g/cm³)*	θ (deg)	F (ml/min per cm)	Reynolds number
0.16†	0.0078	37	2.8	140
0.01	0.0039	10	5.3	530

* The density difference was produced by adding 2.0 or 1.0% (w/v) sucrose to the dense solution.

† The viscosity was increased by adding 0.5% methylcellulose.

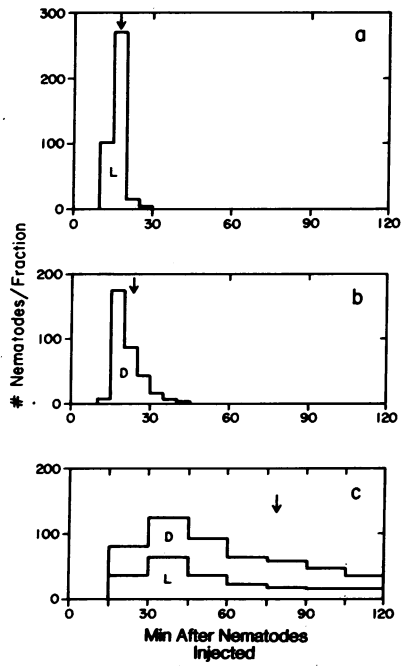

FIG. 3. The rate at which nematodes emerged from short, circular cross-section chambers in three different situations: (*a*) Nematode salts medium in light solution and low-salts medium in dense solution. After 2 hr, 392 nematodes in light solution and 4 in dense. Mean time for emergence is 17.5 min. (*b*) Low-salts medium in light solution and nematode salts medium in dense layer. After 2 hr, no nematodes in light solution and 349 in dense solution. Mean time for emergence, 24 min. (*c*) 1 mM NaCl in light solution and no salt in dense. After 5 hr, 240 nematodes in light solution and 362 in dense. Mean time for emergence, 78 min. In all cases both solutions contained 0.5% (w/v) methylcellulose with 10 μg/ml Light Green SF Yellowish in the light solution and 2.0% (w/v) sucrose in the dense solution.

which the dense solution emerges, q_z the probability of emerging with the dense solution, and D_z the mean number of steps (duration) before an animal emerges from the chamber. The relevant equations are as follows:

for $q \neq p$

$$q_z = \frac{(q/p)^a - (q/p)^z}{(q/p)^a - 1} \qquad [1]$$

TABLE 2. *Chemotaxis of C. elegans to potassium phosphate*

K-PO_4 (pH 6.0)		No. of animals		
Light	Dense	Light	Dense	Remaining
40 mM	20 mM	260	43	140
20	40	0	352	23
20	10	272	28	40
10	20	0	385	2
10	5	249	22	27
5	10	0	227	0

Both solutions contained 0.5% (w/v) methylcellulose. In addition to this and potassium phosphate, the light solution contained 10 μg/ml of the dye Light Green SF Yellowish and the dense solution contained 2.0% (w/v) sucrose.

$$D_z = \frac{z}{q-p} - \frac{a}{q-p} \cdot \frac{1-(q/p)^z}{1-(q/p)^a} \quad [2]$$

and for $q = p = 1/2$

$$q_z = 1 - \frac{z}{a} \quad [3]$$

$$D_z = z(a-z). \quad [4]$$

For the extreme values of p and q the following approximations may be made:

$$D_z \simeq z \text{ for } q \gg p \quad [5]$$

$$\simeq a-z \text{ for } q \ll p \quad [6]$$

The mean durations under conditions approximating the special conditions of Eq. **4**, **5**, and **6** can be determined by assuming that each transfer between plates takes the same amount of time. These equations therefore provide a basis for estimating the number of plates, a.

The relevant experiments are presented in Fig. 3, which shows the time dependence with which nematodes emerged from the short, circular cross-section chambers under three different conditions. In part "*a*" the worms were strongly attracted to the light solution ($q_z = 0.01$), in part "*b*" the worms were strongly attacted to the dense solution ($q_z = 1.00$), and in part "*c*" they were nearly evenly distributed between the two solutions ($q_z = 0.60$). The arrows indicate the mean times at which the worms appeared in each of the three experiments. Taking into account an observed 5-min delay between an animal's leaving the countercurrent chamber and its reaching the fraction collector, one finds that the mean duration in part "*a*" was 13 min, in part "*b*" 19 min, and in part "*c*" 73 min. Use of these values in Eq. **4**, **5**, and **6** yields:

$$z = 73/13 = 5.6$$

$$a-z = 73/19 = 3.8$$

$$a = 9.4$$

By substituting these values in the exact Eq. **1** and **2** it is possible to test whether the conditions of the experiments meet the requirements of the approximations. It is found that in part "*c*," the distribution is well within the range that gives the maximum duration. On the other hand, the asymmetric cases could be a factor of two or more from the shortest duration. It is not feasible to measure q_z with sufficient precision to determine this point with much more accuracy. Thus, it can only be concluded that the apparatus is equivalent to at least nine theoretical plates when used with these animals under these conditions. This calculation, with its assumptions, indicates that the response of most individual nematodes is tested several times during the separation process.

DISCUSSION

The foregoing data demonstrate that it is possible, indeed easy, to generate the desired countercurrent flow pattern, and that this pattern does provide a fairly simple and sensitive means of determining an animal's response to certain stimuli. It is anticipated that this method will prove useful in various studies on many different types of animals. In addition to the simple identification of attractants and repellants, the fact that a response can be obtained for two closely spaced and well defined concentrations indicates that various quantitative experiments can be performed. Furthermore, the effects of drugs and other agents on a particular response will be an important application. Finally, the most distinctive potential of this method is for the isolation of genetic mutants which are abnormal in some aspect of behavior. In this regard it is important to note that at least 1×10^4 *C. elegans* can be injected into the small tubes without any decrement in performance.

NOTE ADDED IN PROOF

Recently this technique has been successfully used to isolate behavioral mutants of *C. elegans*.

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1. Benzer, S. (1971) "From the gene to behavior," *J. Amer. Med. Ass.* **218**, 1015–1022.
2. Benzer, S. (1967) "Behavioral mutants of *Drosophila* isolated by countercurrent distribution," *Proc. Nat. Acad. Sci. USA* **58**, 1112–1119.
3. Craig, L. C. (1944) "Identification of small amounts of organic compounds by distribution studies. II. Separation by countercurrent distribution," *J. Biol. Chem.* **155**, 519–534.
4. Karlstam, B. & Albertsson, P.-A. (1972) "Isolation of different classes of spinach chloroplasts by countercurrent distribution. A methodological study," *Biochim. Biophys. Acta* **255**, 539–552.
5. Baird, G. D., Albertsson, P.-A. & v. Hofsten, B. (1961) "Separation of bacteria by countercurrent distribution," *Nature* **192**, 236–239.
6. Brandt, R. L. (1930) "The Edelanu Process for refining petroleum," *Ind. Eng. Chem.* **22**, 218–223.
7. Cornish, R. E., Archibald, R. C., Murphy, E. A. & Evans, H. M. (1934) "Purification of vitamins. Fractional distribution between immiscible solvents," *Ind. Eng. Chem.* **26**, 397–406.
8. Rouse, H. (1961) in *Fluid Mechanics for Hydraulic Engineers* (Dover Publications, New York), p. 152, Eq. 75.
9. Morris, C. J. O. R. & Morris, P. (1963) in *Separation Methods in Biochemistry* (Interscience Publishers, New York), p. 47.
10. Feller, W. (1957) in *An Introduction to Probability Theory and Its Applications* (John Wiley & Sons, New York), Vol. I, 2nd ed., chap. XIV.