BEHAVIORAL MUTANTS OF DROSOPHILA ISOLATED BY COUNTERCURRENT DISTRIBUTION

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Complex as it is, much of the vast network of cellular functions has been successfully dissected, on a microscopic scale, by the use of mutants in which one element is altered at a time. A similar approach may be fruitful in tackling the complex structures and events underlying behavior, using behavioral mutations to indicate modifications of the nervous system. *Drosophila* offers the same advantages to such a study as it did to classical genetics, namely, large numbers and short generation time, to which may now be added an enormous store of accumulated knowledge concerning the organism. Containing about 10^5 neurons, the fly's nervous system is roughly halfway, on a logarithmic scale, between a single neuron and the human brain, and the fly is possessed of a rich repertoire of behavior. The considerable literature on *Drosophila* behavior since Carpenter's 1905 paper[1] has recently been reviewed by Manning.[2]

Multiple T-mazes and related devices have been used to fractionate populations according to their geotactic or phototactic responses, or spontaneous activity.[3-6] This was pioneered by Hirsch and his colleagues,[3] who started with a genetically heterogeneous pool obtained by mixing diverse wild strains and showed that progressive selection yields strains showing hereditary changes in behavior. Such selection depends upon the recombination and additive effects of multiple genes that can prove quite difficult to disentangle. Since it is now possible to produce very high mutation rates in *Drosophila*, one can, instead, use an inbred strain and isolate mutants in which a behavioral change occurs by a single step, so that direct relationships between individual genes and the nervous system may be investigated.

The method described here for fractionating populations is analogous to countercurrent distribution,[7] an eminently effective method of separating molecules from a mixture by their partition between two solvent phases. Using, instead, the relative preference of an organism for two behavioral alternatives, one obtains rapid, simultaneous measurements on many subjects. The present paper describes the procedure and the isolation of mutants showing changes in phototactic behavior.

Materials and Methods.—(a) *Subjects: Drosophila melanogaster* stocks were supplied by Dr. E. B. Lewis and grown on cornmeal medium at 25°C, in constant light. Testing was done in a dark room at 20°C. The ages at testing ranged from several hours to several days. Unless otherwise stated, mixed males and females were used.

(b) *Mutagenesis* followed a protocol of E. B. Lewis and F. Bacher, utilizing ethyl methane sulfonate (EMS), a potent mutagen for *Drosophila*.[8] Males of the Canton, Ohio-Standard (C-S) wild strain, aged 0–48 hr, were fed on 0.025 *M* EMS in 1% sucrose for 18 hr, then mated to virgin females of a strain having attached-X chromosomes (also homozygous for yellow body color and forked bristles). The males' sex chromosomes were XY, the females' were $\overset{\wedge}{XX}Y$, yielding progeny types XY, $\overset{\wedge}{XX}Y$, $\overset{\wedge}{XX}X$, and YY. The last two are nonviable. Each male received his X from his EMS-treated father, so that mutations induced in that X would be fully expressed. A female, on the other hand, received her $\overset{\wedge}{XX}$ from her untreated mother, so that the only mutations that would be evident would be relatively infrequent autosomal dominants from the father. An index

to the effectiveness of the mutagen is the reduced proportion of male progeny due to sex-linked recessive lethal mutations. Under the conditions used, the ratio of progeny males to females was, typically, 0.4, corresponding to an average of about one lethal mutation per male X.

(c) *Apparatus:* Two 18 × 150-mm Pyrex test tubes were joined by a celluloid sleeve and laid horizontally in slots of a black rack. A 15-watt fluorescent lamp (warm-white) was the light source, the lamp being horizontal at table level, and perpendicular to the tubes. The intensity of illumination at 3 cm from the surface of the lamp was 600 ft-c; at 30 cm it was 60 ft-c. A clean set of test tubes was used for each run.

(d) *Countercurrent distribution:* The flies are placed in a double-tube as in Figure 1. To start, the flies are brought to one end, B. They now either remain in the B part or move toward A. After a time, A is shifted to join a new mate (dotted in Fig. 1), while being replaced by a fresh tube (also shown dotted). Thus, all the flies that moved into A are transferred. To start the second cycle, all the flies are again brought to the B end, and the procedure is repeated. By staggering the starts, the partitioning times are made constant for all tubes.

After n cycles, the flies are distributed in $n + 1$ fractions according to tendency to move into A. If the tube positions are numbered, starting with zero at the left, the fraction in which a subject appears corresponds to the number of positive responses. Information as to the sequence of positive and negative responses is lost. If the flies are identical, act independently, and have constant probability p of moving into A in each trial, they should be distributed, after n transfers, according to the binomial distribution: $\frac{N_r}{N} = \frac{n!}{(n-r)!r!} \cdot p^r \cdot (1-p)^{n-r}$, where N is the total and N_r is the number in fraction r. Such a "behaviorally pure" population would give a single peak, as illustrated in Figure 2, for $n = 15$ transfers and various values of p. If the population is heterogeneous or the probability of response not constant, departures from the ideal may occur.

Results.—(1) *Preliminary experiments:* Six wild strains of *Drosophila*, of various origin, maintained in the collection at the California Institute of Technology, were examined. They showed very great differences, some being poorly phototactic,

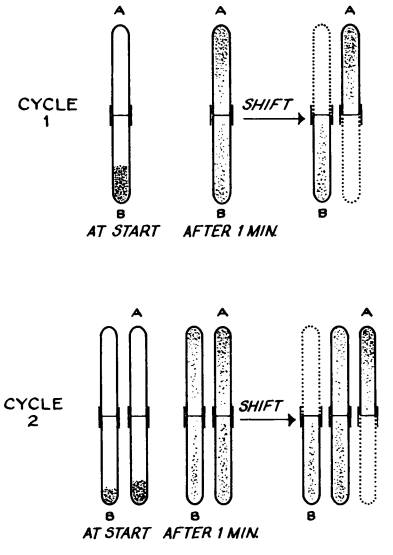


FIG. 1.—Countercurrent distribution procedure for fractionating a *Drosophila* population. In each cycle, the flies are partitioned between two alternatives. Dotted lines indicate new tubes introduced at the end of each cycle. Only the first two transfer cycles are shown.

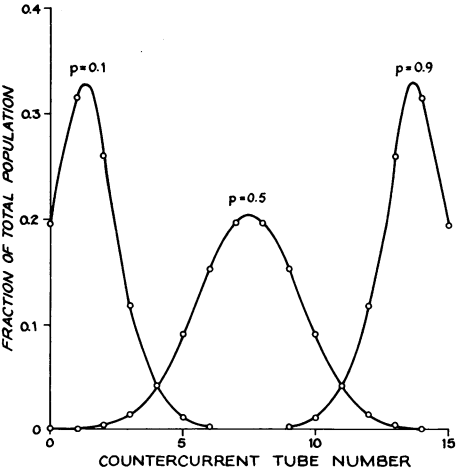


FIG. 2.—Theoretical distribution curves, after 15 transfers; p = probability of response per trial.

some being highly heterogeneous in response. The Canton, Ohio-Standard (C-S) strain produced homogeneous populations with strong positive behavior and was chosen for further work.

To determine appropriate conditions for phototaxis tests by the countercurrent procedure, preliminary experiments were done on C-S flies, using one double-tube. Flies tend to accommodate to a constant environment, but are much more responsive to light when startled.[9] The procedure was to admit about 100 flies, lay the tube in a horizontal position, and allow several minutes for accommodation under the conditions to be used. The tube was then held in a vertical position and tapped on sponge rubber, bringing the flies to one end and arousing them. The tube was then returned to horizontal (time zero). In complete darkness, the flies move at a certain rate toward the opposite end. With light, two phototactic responses can be distinguished. If the lamp is distal to the starting position, there is an increase in movement toward the light. Conversely, if the lamp is placed at the starting end, the movement away from that end decreases. The different signs show that the behavior observed is phototactic and not merely an increase in random movement with light intensity.

Figure 3 shows these effects for C-S flies, using a distance of 3 cm between the lamp surface and the end of the tube. The half time for reaching a plateau in the *to-light* response is of the order of 15 seconds. The results are almost the same at a lamp distance of 30 cm. Therefore, a 3-cm lamp distance and cycling time of one minute were chosen as standard in further experiments, as adequate (for normal flies) to saturate the response. The responses are fairly reproducible for 15 or more repetitive trials. While there is some tendency for the initial rate to slow down, the level reached by 1 minute remains reasonably constant. For countercurrent experiments, the conditions chosen were therefore 3-cm lamp distance, 1-minute cycle time, and 15 transfers.

(2) *Countercurrent distribution experiments:* (a) *To-light versus from-light:* Figure 4*A* shows results for C-S flies, performed as in Figure 1, the lamp being placed at the tube end distal to the start. Flies showing many responses *to-light* appear at the right of the distribution. In Figure 4*B*, the *from-light* response is shown. The great difference between these curves shows good phototaxis.

(b) *Reproducibility:* This can be tested by reassembling the distributed population and running it again. C-S flies showed almost the same curve for a second and even a third run. However, this was not always observed with other strains, some of which showed apparent fatigue effects.

(c) *Effect of population size:* Flies in a tube are hardly independent. When packed together, they disperse; there are also attractive influences, as between males and females. Such interactions could affect the results if the density of population were too large. To test for the effects of crowding, various-sized groups of C-S flies were run. The average response diminished somewhat as the size of the group increased, implying that the flies behaved quasi-independently. However, in the 18 × 150-mm tubes employed, several hundred flies could be run at a time without excessive distortion of the distribution.

(d) *Separation of a mixed population:* A mixture was made of C-S and the wing-deficient mutant *vestigial* (*vg*), which shows poor response to light.[10] Figure 5 shows the results for *vg* run alone (curve *A*), for C-S alone (curve *B*), and for a mix-

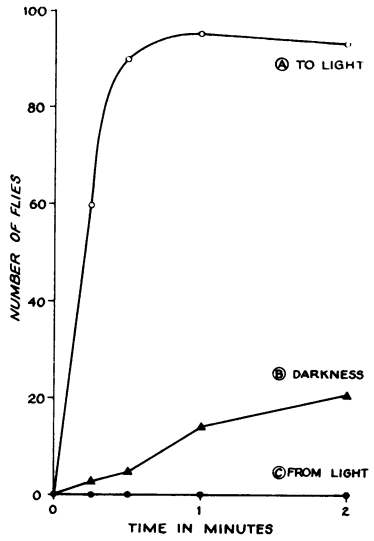


FIG. 3.—Kinetics of phototaxis of normal flies (C-S strain). A horizontal double-tube is used. At time zero, the flies are started at one end and the number of flies in the tube *opposite* the start is plotted vs. time. Curve (*A*), flies started at the end distal to the white lamp and must move *to-light* to score. Curve (*B*), no white light. Curve (*C*), flies started at end proximal to the white lamp and must move *from-light* to score. Dim yellow overhead illumination was used in these three experiments so that the flies could be seen in absence of the white light. (N = 115; age, 1–2 days.)

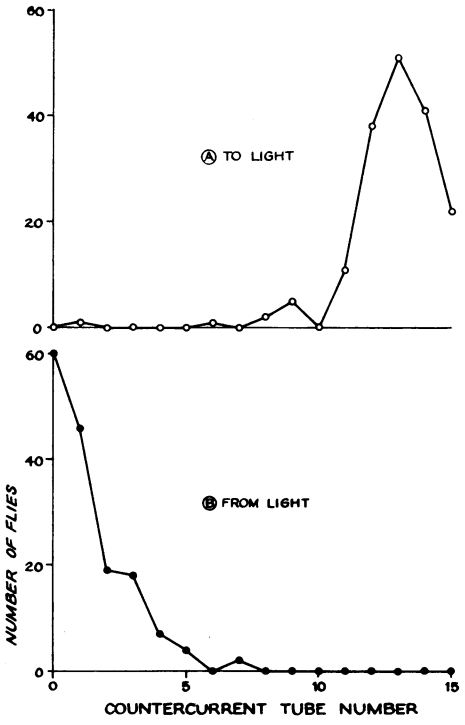


FIG. 4.—Countercurrent distribution of C-S flies showing the number of flies in each tube after 15 transfers. Curve (*A*) shows high frequency of movement toward light. Curve (*B*) shows low tendency to move away from light. (N = 172 in curve (*A*), 156 in curve (*B*); age, 5–6 days.)

ture of the two (curve *C*). Again, the flies behaved quasi-independently. The existence of some interaction is indicated by a slight "retardation" of the C-S flies and some "advancement" of the *vg* flies. The countercurrent technique can thus be used to obtain "behaviorally pure" fractions of a population in precisely the manner used for biochemical purifications.

(3) *Genetic differences in behavior:* Strikingly different countercurrent profiles are obtained for various genotypes. As mentioned earlier, even wild strains from diverse sources vary. The Lausanne-S strain gave only slightly more response *to-light* than *from-light*. In the Swedish-C strain all the flies moved very little *from-light*, but the *to-light* test produced a distribution with three peaks, corresponding to high, medium, and zero phototaxis. It is possible that the original strains in nature were more alike and that many generations of inbreeding in the laboratory have led to accumulation of genetic changes. Since the various available mutants of *Drosophila* have a range of genetic backgrounds, it is hazardous to ascribe their behavioral characteristics solely to the genes for which the mutants are noted. In a proper study of behavioral genetics it is important to backcross each mutant repeatedly with a standard normal strain, or to isolate the mutants from a single

strain. Notwithstanding uncertainties in their genetic backgrounds, many of the stock *Drosophila* mutants have been studied by countercurrent and give a wide variety of results. Some are of particular interest here.

In *sine oculis*, the eyes are entirely missing. Figure 6 shows curves for this mutant. The blindness of the flies is expressed in the similarity of the *to-light* and *from-light* curves, although the flies are quite active, showing much movement in both cases. The peak is much *narrower* than a binomial distribution would predict, showing that the assumptions (constant probability of response per trial, independence of the flies) cannot be valid for this strain.[11] Another mutant, *tan*, has eyes that appear entirely normal, yet gives the same results as *sine oculis*. This mutant has long been known to be nonphototactic,[10] and histological examination[12] has revealed no obvious abnormality in the structure of the eye or its associated neural apparatus. Thus, a subtle genetic alteration may produce as profound an effect as complete elimination of the optic system.

Phototaxis is profoundly affected by the condition of the fly's wings, even though the response, in a narrow tube, involves mostly walking rather than flying. McEwen[10] showed that removal of the wings, either by surgery or by mutation, greatly reduced phototaxis. These observations were confirmed by countercurrent measurements on C-S flies with clipped wings and on the wingless mutants *vg* (Fig. 5) and *nubbin*. Some sort of sensory feedback from the wings appears to play an important role. As McEwen also showed, the negative *geotactic* response of flies deprived of

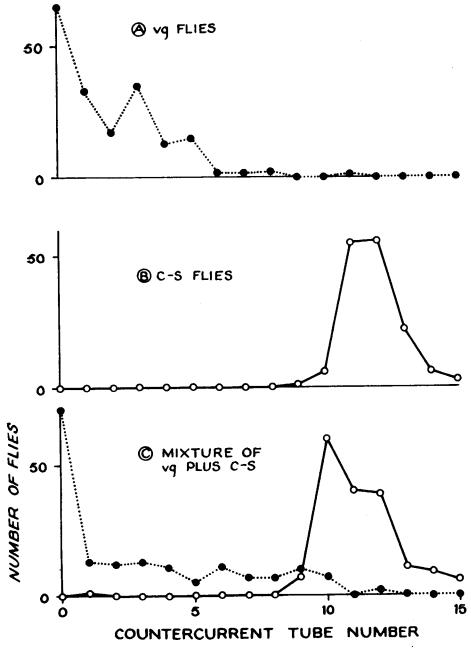


FIG. 5.—Separation of different behavioral types in countercurrent distribution *to-light*. (*A*) Vestigial-wing flies (N = 185); (*B*) C-S flies (N = 149); (*C*) mixture of 169 vestigial flies plus 173 C-S flies. Age of flies, 2–5 days.

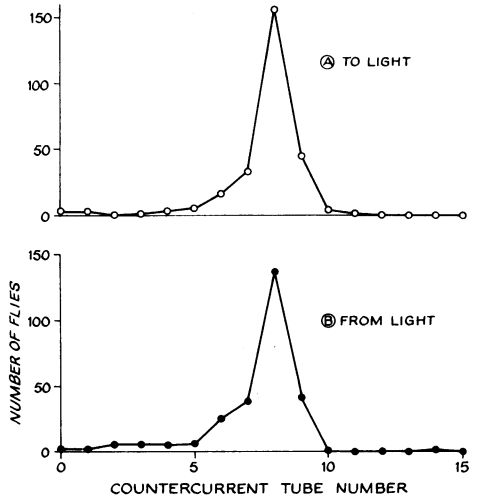


FIG. 6.—Distribution curves for the eyeless mutant strain, *sine oculis*. Lack of phototaxis is evident in the identity of the two curves. N = 270 in (*A*), 268 in (*B*).

their wings remains unimpaired. This also has been confirmed by countercurrent experiments on the climbing response in darkness.

(4). *Isolation of nonphototactic mutants by countercurrent:* Male C-S flies were exposed to EMS (see *Methods*), then mated to virgin attached-X females. The progeny were run in countercurrent *to-light*, selecting male flies that showed little or no response. This class should include mutants in which the X chromosome of the treated fathers has been affected. However, since EMS produces many mosaic offspring, a particular fly might be phenotypically nonphototactic but genetically normal. Also, damaged flies (in the wings, for example) could show poor response for nongenetic reasons. Each candidate was therefore mated to virgin attached-X females and its progeny examined by countercurrent.

Out of 26 suspects examined in this way, two turned out to be true mutants showing striking heritable alterations in behavior. Figure 7 shows the countercurrent curves for the progeny of one of these "strange behavior" mutants, SB8, crossed to attached-X females. The change shows up only in the males, since the females receive attached-X chromosomes from their normal mothers. Thus, the total progeny population produces a bimodal distribution, but when each tube is scored for males and females, two distinct curves are obtained. (The normal females are a convenient internal control.) The countercurrent curve, *from-light*, of the mutant

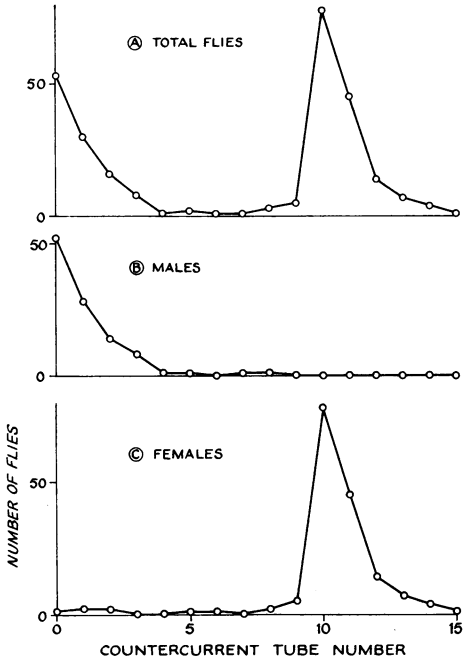


FIG. 7.—Distribution curve *to-light* for progeny of sex-linked nonphototactic mutant SB8 males mated to attached-X females. The progeny are mutant males and normal females. Curve (*A*), total population of 106 males and 163 females; age, 0–5 days. In curves (*B*) and (*C*) the data for males and females are plotted separately.

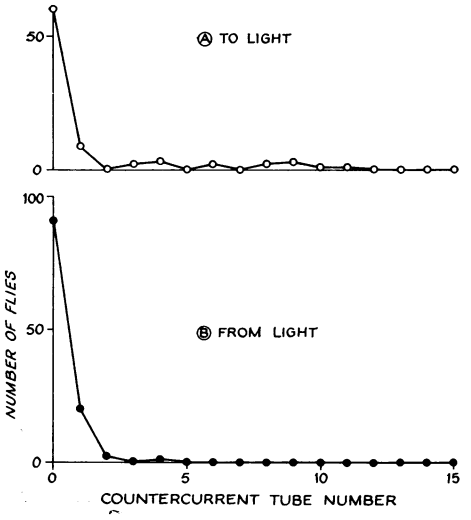


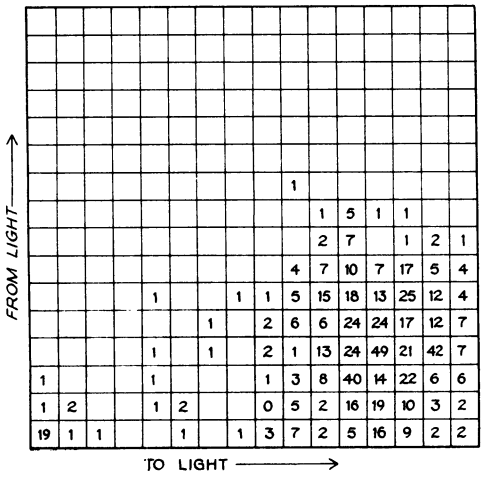
FIG. 8.—Comparison of *to-light* and *from-light* responses for flies of the nonphototactic mutant SB6. (N = 83 mutant males plus 84 normal females. Data for the males only are plotted.)

males shows little difference from the *to-light* curve. The mutation has therefore produced a fly that is nonphototactic. No anomaly in external morphology has been detected in this mutant and histological examination has so far revealed no obvious abnormality in the eye or its associated neural apparatus. When observed in a small chamber, the mutant flies show reduced general locomotor activity and, unlike C-S flies, tend to bump into each other.

A second mutant, SB6, gives countercurrent curves (Fig. 8) much like SB8. It has apparently normal eyes, but does show other morphological traits, resembling the known mutant *fused* in having two wing veins partially fused, wings held out at an abnormal angle, and ocelli missing. However, the Caltech *fused* mutant shows normal phototaxis, as does an *ocelliless* strain that was tested. Also unlike the Caltech *fused* mutant, the development of SB6 shows a marked temperature dependence. SB6 flies raised at 18°C, instead of the usual 25°C, are morphologically normal and also show normal phototaxis (tested, as usual, at room temperature).

(5) *Two-dimensional countercurrent distributions:* The above two mutants were really selected for low locomotor activity combined with lack of phototaxis, since lack of phototaxis alone would not necessarily place them in the zero tube of the countercurrent distribution. (This is evident from the behavior of *sine oculis* and *tan*, which show much spontaneous activity.) The two factors may be scored independently by use of a two-stage procedure. First, a distribution *to-light* is run. Then, the flies in each fraction are run a second time, *from-light*. The results may be plotted in a square array, as in Figure 9. Nonphototactic flies should give the same response to or from light and fall near the diagonal, the distance from the origin being a measure of general locomotor activity. Positively phototactic flies should appear on one side of the diagonal, negatively phototactic ones on the opposite side. This procedure, which can be used with any two variables, is being utilized for isolation for further behavioral mutants.

Discussion.—Phototaxis is a complex behavioral response. Light is absorbed by a pigment in the receptor cell, producing neural excitation, transmission at synaptic junctions, integration in the central nervous system involving comparison with other inputs, and generation of appropriate motor signals such that the fly walks in a particular direction. A defect in any one of these structures or processes



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1	2			1	2			0	5	2	16	19	10	3	2
19	1	1			1		1	3	7	2	5	16	9	2	2

FIG. 9.—Two-dimensional fractionation. Progeny of EMS-treated males mated to attached-X females (N = 665; age, 2–4 days). The flies were first distributed in a *to-light* countercurrent of 15 transfers, the results determining their horizontal coordinates. The flies in each position were then distributed in a *from-light* countercurrent, the results determining their vertical coordinates. The figure shows the number of flies in each of the final fractions. Note that the bulk of the population showed good phototaxis but that 19 flies showed no response to either test.

could lead to modification or elimination of the phototactic response. Thus, if one were to isolate many nonphototactic mutants, the collection should include ones having defects affecting the various possible elements of the system. For instance, mutation might eliminate an enzyme needed for the synthesis of one of the neural transmitter substances. In such a case, probing with electrodes could establish that the nervous impulse reaches the terminal in question, but that transmission fails. By biochemical fractionation and comparison with the normal strain, the missing substance might be identified. Thus, use of mutation as a microsurgical tool could conceivably lead to the identification of the various transmitters, about which little is presently known.

The countercurrent procedure is obviously adaptable to a wide range of stimuli, such as gravity, odor, sound, and special visual patterns, thus lending itself to the isolation of many kinds of behavioral mutants, including ones in which the wiring pattern of the nervous system is affected. Furthermore, as preliminary experiments have shown, the speed of the procedure permits its use in the study of short-term modifications of behavior.

Summary.—A countercurrent distribution method is described for fractionating *Drosophila* populations according to their behavioral responses on repeated trials and is applied to the analysis of phototaxis. Various genotypes show great differences. By application of the method to the progeny of mutagenized flies, sex-linked behavioral mutants, showing loss of phototaxis, have been isolated in one generation. A two-dimensional fractionation procedure is also described.

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[9] It is important to note a basic difference between the discontinuous countercurrent procedure, in which attention is revived at the start of each cycle, and a stationary maze through which the animals wander slowly. Even the sign of phototaxis can be changed by such considerations (Lewontin, R. C., *Am. Naturalist*, **93**, 321–328 (1959)).

[10] McEwen, R. S., *J. Exptl. Zool.*, **25**, 49–106 (1918).

[11] In the absence of a strong phototactic drive, other factors could become quite important. One consideration is the demonstrable fact that flies leave a repellent deposit in tubes which they have occupied. This can have the effect of retarding the "front" of the distribution, since flies at that end, to respond, must enter tubes that have been previously occupied. Flies at the trailing end of the distribution, on the other hand, are presented with fresh tubes. The over-all effect would be to narrow the distribution. Slackening of successive responses or tendencies to "gregariousness" would also tend to narrow the distribution.

[12] Both by McEwen and by the present author.