GENETIC DISSECTION OF BEHAVIOR

By working with fruit flies that are mosaics of normal and mutant parts it is possible to identify the genetic components of behavior, retrace their development and locate the sites where they operate

by Seymour Benzer

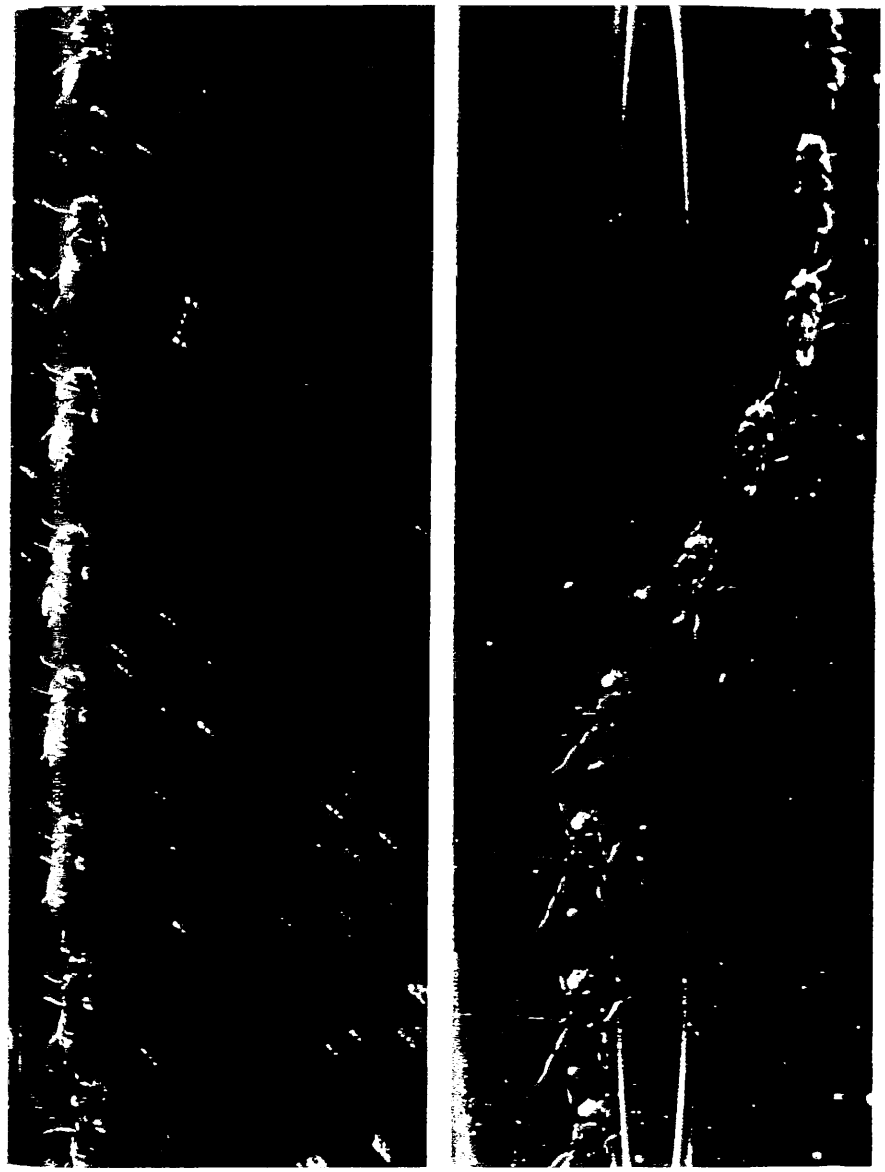
When the individual organism develops from a fertilized egg, the one-dimensional information arrayed in the linear sequence of the genes on the chromosomes controls the formation of a two-dimensional cell layer that folds to give rise to a precise three-dimensional arrangement of sense organs, central nervous system and muscles. Those elements interact to produce the organism's behavior, a phenomenon whose description requires four dimensions at least. Surely the genes, which so largely determine anatomical and biochemical characteristics, must also interact with the environment to determine behavior. But how? For two decades molecular biologists were engaged in tracking down the structure and coding of the gene, a task that was pursued to ever lower levels of organization [see "The Fine Structure of the Gene," by Seymour Benzer; SCIENTIFIC AMERICAN, January, 1962]. Some of us have since turned in the opposite direction, to higher integrative levels, to explore development, the nervous system and behavior. In our laboratory at the California Institute of Technology we have been applying tools of genetic analysis in an attempt to trace the emergence of multidimensional behavior from the one-dimensional gene.

Our objectives are to discern the genetic component of a behavior, to identify it with a particular gene and then to determine the actual site at which the gene influences behavior and learn how it does so. In brief, we keep the environment constant, change the genes and see what happens to behavior. Our choice of an experimental organism was constrained by the fact that the simpler an organism is, the less likely it is to exhibit interesting behavioral patterns that are relevant to man; the more complex it is, the more difficult it may be to analyze and the longer it takes. The fruit fly *Drosophila melanogaster* represents a compromise. In mass, in number of nerve cells, in amount of DNA and in generation time it stands roughly halfway on a logarithmic scale between the colon bacillus *Escherichia coli* (which can be regarded as having a one-neuron nervous system) and man. Although the fly's nervous system is very different from the human system, both consist of neurons and synapses and utilize transmitter molecules, and the development of both is dictated by genes. A fly has highly developed senses of sight, hearing, taste, smell, gravity and time. It cannot do everything we do, but it does some things we cannot do, such as fly and stand on the ceiling. Its visual system can detect the movement of the minute hand on a clock. One must not underestimate the little creature, which is not an evolutionary antecedent of man but is itself high up on the invertebrate branch of the phylogenetic tree. Its nervous system is a miracle of microminiaturization, and some of its independently evolved behavior patterns are not unlike our own.

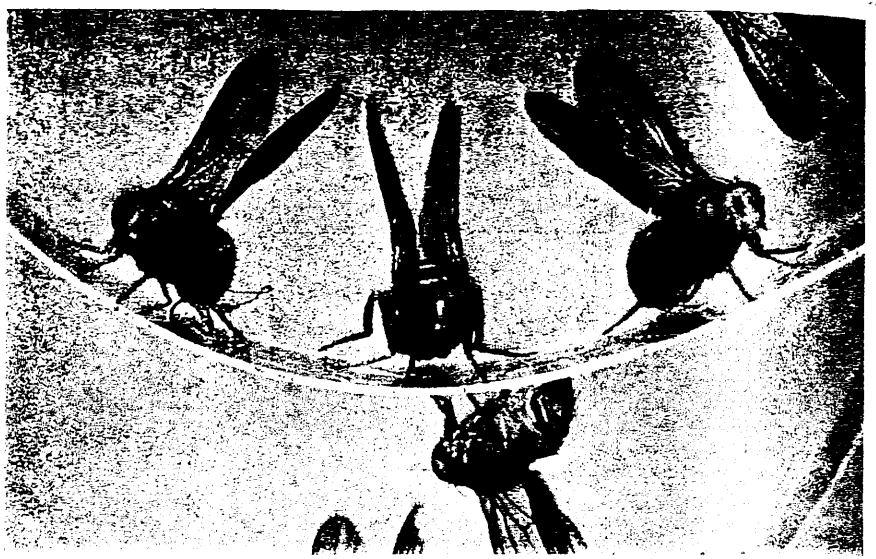
Jerry Hirsch, Theodosius Dobzhansky and many others have demonstrated that if one begins with a genetically heterogeneous population of fruit flies, various behavioral characters can be enhanced by selective breeding pursued over many generations. This kind of experiment demonstrates that behavior can be genetically modified, but it depends on the reassortment of many different genes, so that it is very difficult to distinguish the effect of each one. Also, unless the selective procedure is constantly maintained, the genes may reassort, causing loss of the special behavior. For analyzing the relation of specific genes to behavior, it is more effective to begin with a highly inbred, genetically uniform strain of flies and change the genes one at a time. This is done by inducing a mutation: an abrupt gene change that is transmitted to all subsequent generations.

A population of flies exposed to a mutagen (radiation or certain chemicals) yields some progeny with anatomical anomalies such as white eyes or forked bristles, and it also yields progeny with behavioral abnormalities. Workers in many laboratories (including ours) have compiled a long list of such mutants, each of which can be produced by the alteration of a single gene. Some mutants are perturbed in sexual behavior, which in normal *Drosophila* involves an elaborate sequence of fixed action patterns. Margaret Bastock showed years ago that some mutant males do not court with normal vigor. Kulbir Gill discovered a mutant in which the males pursue one another as persistently as they do females. The mutant *stuck*, found by Carolyn Beckman, suffers from inability to disengage after the normal 20-minute copulation period. A converse example is *coitus interruptus*, a mutant Jeffrey C. Hall has been studying in our laboratory; mutant males disengage in about half the normal time and no offspring are produced. Obviously most such mutants would not stand a chance in the competitive natural environment, but they can be maintained and studied in the laboratory.

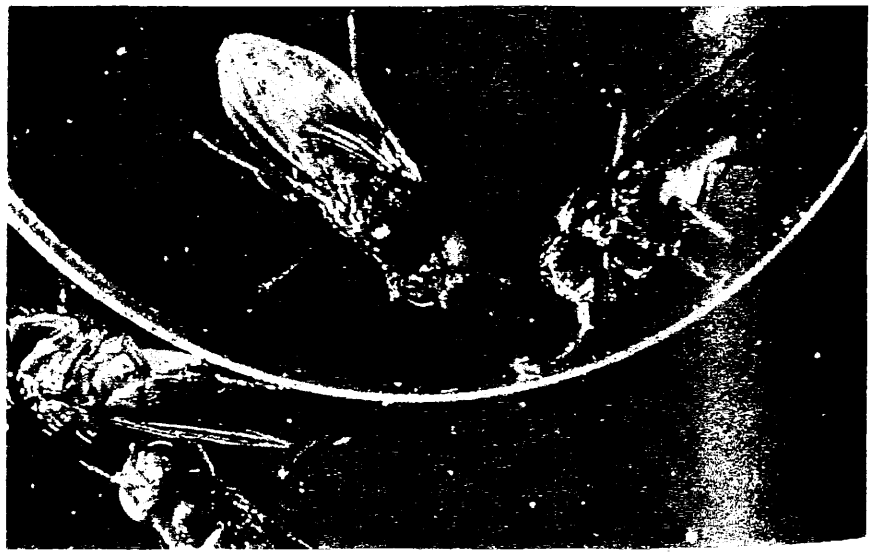
As for general locomotor activity, some mutants are *sluggish* and others, such as one found by William D. Kaplan at the City of Hope Medical Center, are *hyperkinetic*, consuming oxygen at an exaggerated rate and dying much earlier than normal flies. Whereas normal flies show strong negative geotaxis (a tendency to move upward against the force of gravity), *nonclimbing* mutants do not.



BEHAVIOR of a normal and of a mosaic fruit fly is demonstrated in an experiment photographed by F. W. Goro. Normal flies move toward light and upward against the force of gravity. A normal fly that is placed in a glass tube with a light at the top and photographed by successive stroboscopic flashes traces a line straight up the tube (*left*). A mosaic fly, with one good eye and one blind eye, also climbs straight up if there is no light, guided by its sense of gravity. If there is a light at the top of the tube, however, the mosaic fly traces a helical path (*right*), turning its bad eye toward the light in a vain effort to balance the light input to both eyes.



"WINGS-UP" FLIES are mutants that keep their wings straight up and cannot fly. Such behavior could be the result of flaws in wing structure, in musculature or in nerve function. Mosaic experiments in the author's laboratory have traced the defect to the muscle.

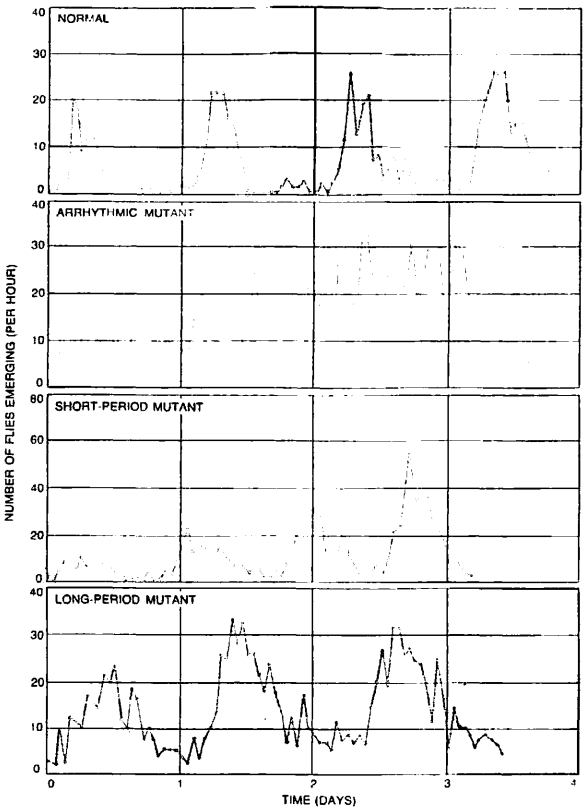


MOSAIC FLIES used for investigating behavior are gynandromorphs: partly male and partly female. The female parts are normal, the male parts mutant in one physical or behavioral trait or more. These flies have one normal red eye and one mutant white eye, and the male side of each fly also has the shorter wing that is normal for a male fly. The flies are about three millimeters long.

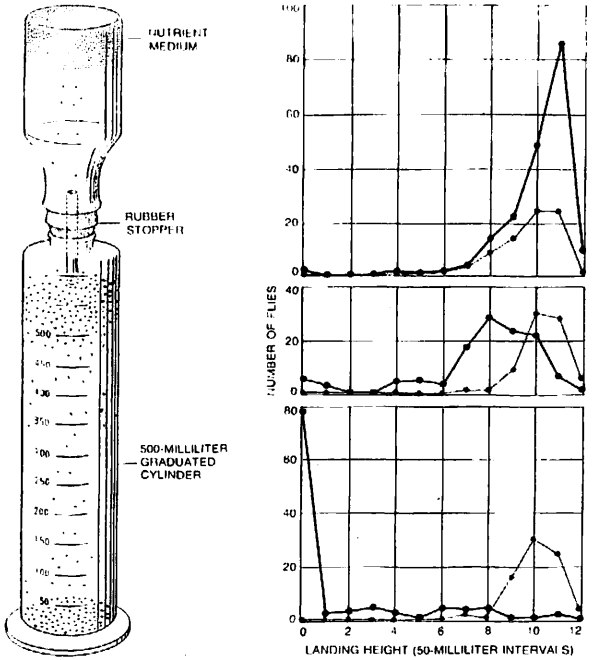
flightless flies do not fly even though they may have perfectly well developed wings and the male can raise his wing and vibrate it in approved fashion during courtship. Some individuals that appear to be quite normal may harbor hereditary idiosyncrasies that show up only under stress. Take the *easily shocked* mutants we have isolated, or the one called *tko*, found by Burke H. Judd and his collaborators at the University of Texas at Austin. When the mutant fly is subjected to a mechanical jolt, it has what looks like an epileptic seizure: it falls on its back, flails its legs and wings, coils its abdomen under and goes into a coma; after a few minutes it recovers and goes about its business as if nothing had happened. John R. Merriam and others working in our laboratory have found several different genes on the *X* chromosome that can produce this syndrome if they are mutated.

In many organisms mutations have been discovered that are temperature-sensitive, that is, the abnormal trait is displayed only above or below a certain temperature. David Suzuki and his associates at the University of British Columbia discovered a behavioral *Drosophila* mutant of this type called *paralyzed:* when the temperature goes above 28 degrees Celsius (82 degrees Fahrenheit), it collapses, although normal flies are unaffected, when the temperature is lowered, the mutant promptly stands up and moves about normally. We have found other mutants, involving different genes, that become similarly paralyzed at other specific temperatures. In one of these, *comatose,* recovery is not instantaneous but may take many minutes or hours, depending on how long the mutant was exposed to high temperatures. Recent experiments by Obaid Siddiqi in our laboratory have shown that action potentials in some of the motor nerves are blocked until the fly recovers.

An important feature of behavior in a very wide range of organisms is an endogenous 24-hour cycle of activity. The fruit fly displays this "circadian" rhythm, and one can demonstrate the role of the genes in establishing it. A fly does well to emerge from the pupal stage around dawn, when the air is moist and cool and the creature has time to unfold its wings and harden its cuticle, or outer shell, before there is much risk of desiccation or from predators. (The name *Drosophila,* incidentally, means "lover of dew.") Eclosion from the pupa at the proper time is controlled by the circadian rhythm: most flies emerge during a few hours around dawn and those missing that interval tend to wait until dawn on the following day or on later days. This rhythm, which has been much studied by Colin S. Pittendrigh of Stanford University, persists even in constant darkness provided that the pupae have once been exposed to light; having been set, the internal clock keeps running. The clock continues to control the activity of the individual fly after eclosion, even if the fly is kept in the dark. By monitoring the fly's movement with a photocell sensitive to infrared radiation (which is invisible to the fly) one can observe that it begins to walk about at a certain time and does so for some 12 hours; then it becomes quiescent, as if it were asleep on its feet, for half a day. After that, at the same time as the first day's arousing or within an hour or so of it, activity begins anew. Ronald Konopka demonstrated the genetic control of this internal clock as a graduate student in our laboratory. By exposing normal flies to a mutagen he obtained mutants with abnormal rhythms or no rhythm at all. The *arrhythmic* flies may eclose at any time of day; if they are maintained in the dark



"BIOLOGICAL CLOCK" is an example of a behavioral mechanism that is genetically determined. It governs the periodicity of the time flies eclose from the pupa and also their daily cycle of activity as adults. The curves are for the eclosion of flies kept in total darkness. Normal flies emerge from the pups at a time corresponding to dawn; those that miss dawn on one day emerge 24 hours later (*top*). Mutants include arrhythmic flies, which emerge at arbitrary times in the course of the day, and flies with 19-hour and 28-hour cycles.



FLIGHT TESTER is a simple device for measuring the flying ability of normal and mutant flies. It is a 500-milliliter graduated cylinder, its inside wall coated with paraffin oil. Flies are dumped in at the top. They strike out horizontally as best they can, and so the level at which they hit the wall and become stuck in the oil film reflects their flying ability. The curves compare the performance of female control flies (*gray*) with that of males (*color*) that fly normally (*top*) or poorly (*middle*) and with male *flightless* mutants (*bottom*).

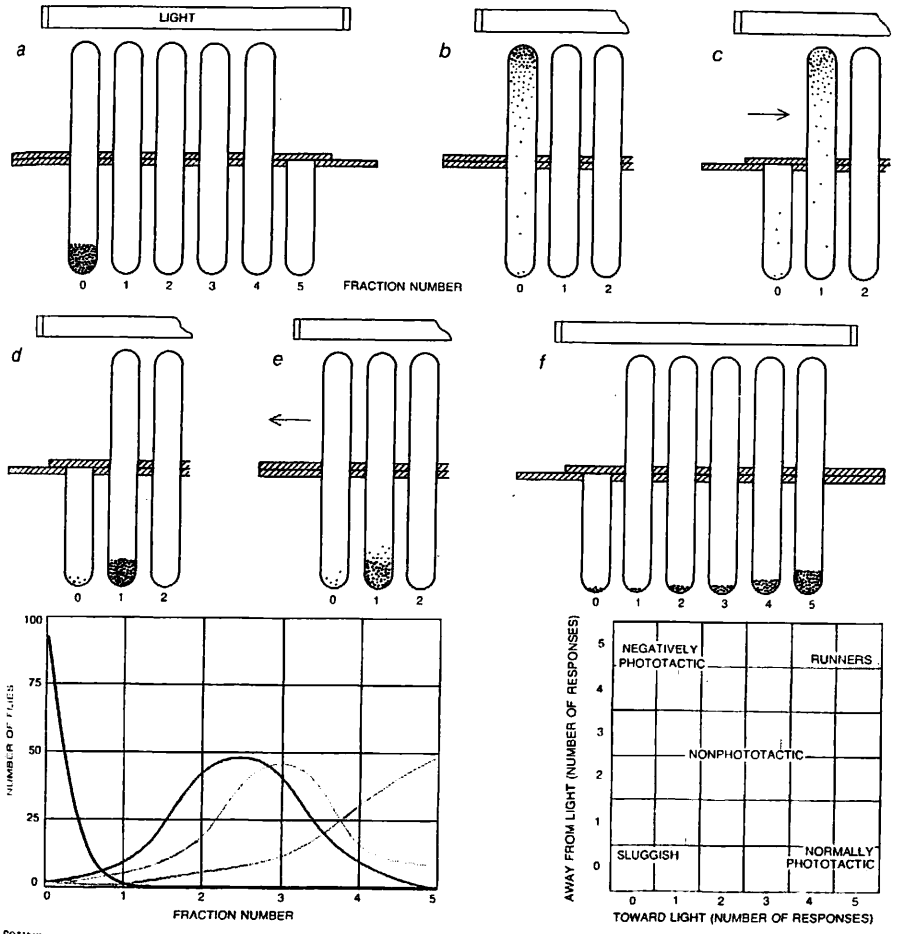
after emergence, they are insomniacs, moving about during random periods throughout the day. The *short-period* mutant runs on a 19-hour cycle and the *long-period* mutant on a 28-hour cycle. (May there not be some analogy between such flies and humans who are either cheerful early birds or slow-to-awaken night owls?)

Let me now use a defect in visual behavior to illustrate in some detail how we analyze behavior. The first problem is to quantitate behavior and to detect and isolate behavioral mutants. It is possible to handle large populations of flies, treating each individual much as a molecule of behavior and fractionating the group into normal and abnormal types. We begin, using the technique devised by Edward B. Lewis at Cal Tech, by feeding male flies sugar water to which has been added the mutagen ethyl methane sulfonate, an alkylating agent that induces mutations in the chromosomes of sperm cells. The progeny of mutagenized males are then fractionated by means of a kind of countercurrent distribution procedure [*see illustration on opposite page*], somewhat as one separates molecules into two liquid phases. Here the phases are light and darkness and the population is "chromatographed" in two dimensions on the basis of multiple trials for movement toward or away from light. Normal flies—and most of the progeny in our experiment—are phototactic, moving toward light but not away from it. Some mutants, however, do not move quickly in either direction; they are *sluggish* mutants. There are *runners*, which move vigorously both toward and away from light. A *negatively phototactic* mutant moves preferentially away from light. Finally, there are the *nonphototactic* mutants, which show a normal tendency to walk but no preference for light or darkness. They behave in light as normal flies behave in the dark, which suggests that they are blind.

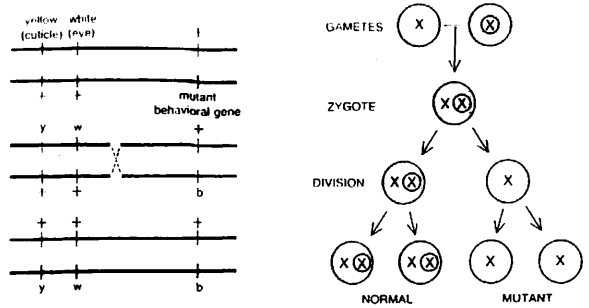
My colleague Yoshiki Hotta, who is now at the University of Tokyo, and I studied the electrical response of the nonphototactic flies' eyes. Similar mutant isolation and electrical studies have also been carried out by William L. Pak and his associates at Purdue University and by Martin Heisenberg at the Max Planck Institute for Biology at Tübingen, so that many mutants are now available, involving a series of different genes. The stimulus of a flash of light causes the photoreceptor cells of a normal fly's eye to emit a negative wave, which in turn triggers a positive spike from the next cells in the visual pathway; an electroretinogram, a record of this response, can be made rather easily with a simple wick electrode placed on the surface of the eye. In some nonphototactic mutants the photoreceptor cells respond but fail to trigger the second-order neurons; in other cases the primary receptor cells are affected so that there is no detectable signal from them even though they are anatomically largely normal. These mutants may be useful in understanding the primary transduction mechanism in the photoreceptor cells. Mutant material provides perturbations, in other words, that enable one to analyze normal function. When Hotta and I examined the eyes of some of the nonphototactic mutants, we found that the photoreceptor cells are normal in the young adult but that they degenerate with age. There are genetic conditions that produce this result in humans, and it may be that the fly's eye can provide a model system for studying certain kinds of blindness.

Now, if one knows that a certain behavior (nonphototactic, say) is produced by a single-gene mutation and that it seems to be explained by an anatomical fault (the degenerated receptors), one still cannot say with certainty what is the primary "focus" of that genetic alteration, that is, the site in the body at which the mutant gene exerts its primary effect. The site may be far from the affected organ. Certain cases of retinal degeneration in man, for example, are due not to any defect in the eye but to ineffective absorption of vitamin A from food in the intestine, as Peter Gouras of the National Institute of Neurological Diseases and Blindness has demonstrated. In order to trace the path from gene to behavior one must find the true focus at which the gene acts in the developing organism. How? A good way to troubleshoot in an electronic system—a stereophonic set with two identical channels, for example—is to interchange corresponding parts. That is in effect what we do with *Drosophila*. Rather than surgically transplanting organs from one fly to another, however, we use a genetic technique: we make mosaic flies, composite individuals in which some tissues are mutant and some have a normal genotype. Then we look to see just which part has to be mutant in order to account for the abnormal behavior.

One method of generating mosaics depends on a strain of flies in which there is an unstable ring-shaped *X* chromosome. Flies, like humans, have *X* and *Y* sex chromosomes; if a fertilized egg has two *X* chromosomes in its nucleus, it will normally develop into a female fly; an *XY* egg yields a male. In *Drosophila* it is the presence of two *X* chromosomes that makes a fly female; if there is only one *X*,

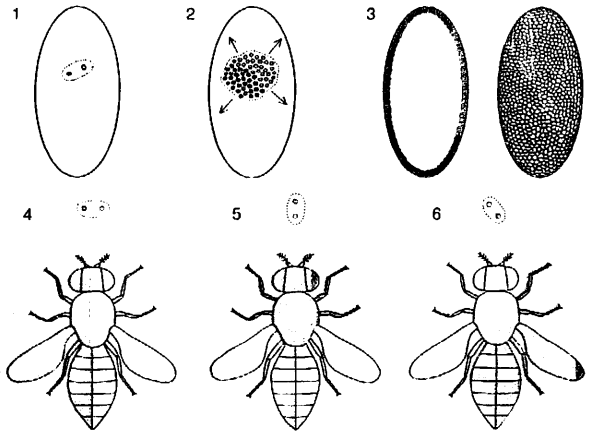


COUNTERCURRENT APPARATUS developed by the author can "fractionate" a population of flies as if they were molecules of behavior. The device consists of two sets of plastic tubes arranged in a plastic frame. Flies are put in Tube *0*; the device is held vertically and tapped to knock the flies to the bottom of the tube, and then the frame is laid flat and placed before a light at the far end of the tubes (*a*). Flies showing the phototactic response move toward the light, whereas others stay behind (*b*). After 15 seconds the top row of tubes is shifted to the right (*c*) and the responders are tapped down again (*d*), falling into Tube *1*. The upper frame is returned to the left (*e*), the frame is laid flat and again the responders move toward the light. The procedure is repeated five times in all. By then the best responders are in Tube *5*, the next best in Tube *4* and so on (*f*). The curves (*bottom left*) show typical results. Phototactic flies show two very distinct peaks depending on whether the light was at the opposite end of the tubes from the starting point (*color*) or at the starting end (*black*). Nonphototactic flies, however, yield about the same curve (*light color or gray*) regardless of the position of the light. In order to distinguish variation in motor activity from phototaxis, the separation is carried out first toward light and then, processing the flies in each tube again, away from light, yielding a two-dimensional "chromatogram" (*bottom right*).



PREPARATION OF MOSAIC FLIES depends on the recombination on one *X* chromosome of genes for mutant "marker" traits and for a behavioral mutation. Recombination occurs through the crossing-over of segments of two homologous chromosomes, as shown at left. Males with an *X* chromosome carrying the desired recombination (*black*) are mated with females carrying an unstable ring-shaped *X* chromosome (*top row at right*). Among the resulting zygotes, or fertilized eggs, will be some carrying the mutation-loaded *X* and a ring *X*. In the course of nuclear division the ring *X* is sometimes lost. Tissues that stem from that nucleus are male, and mutant. In tissues that retain the ring *X*, however, the mutant genes are masked by the genes on the ring *X*, and these tissues are female and normal.

the fly will be male. The ring *X* chromosome has the property that it may get lost during nuclear division in the developing egg. If we start with female eggs that have one normal *X* and one ring *X*, in a certain fraction of the embryos some of the nuclei formed on division lose the ring *X* and therefore have only one *X* chromosome left, and will therefore produce male tissues. This loss of the ring *X*, when it occurs, tends to happen at a very early stage in such a way as to produce about equal numbers of *XX* and *X* nuclei. The nuclei divide a few times in a cluster and then migrate to the surface of the egg to form the early embryonic stage called a blastula: a single layer of cells surrounding the yolk [*see bottom illustration on this page*]. The nuclei tend to retain their proximity to their neighbors in the cluster, so that the female (*XX*) cells populate one part of the blastoderm (the surface of the blastula) and the male cells cover the rest. It is a feature of *Drosophila* that the axis of the crucial first nuclear division is oriented arbitrarily with respect to the axes of the egg. The dividing line between the *XX* and *X* cells can therefore cut the blastoderm in different ways. Once the blastoderm is formed the site occupied by a cell largely determines its fate in the developing embryo, and so the adult gynandromorph, a male-female mosaic, can have a wide variety of arrangements of male and female parts depending on how the dividing line falls in each particular embryo. The division of parts often follows the intersegmental boundaries and the longitudinal midline of the fly's exoskeleton. The reason is that the exoskeleton is an assembly of many parts, each of which was formed independently during metamorphosis from an imaginal disk in the larva that was in turn derived from a specific area of the blastoderm [*see illustrations on opposite page*].



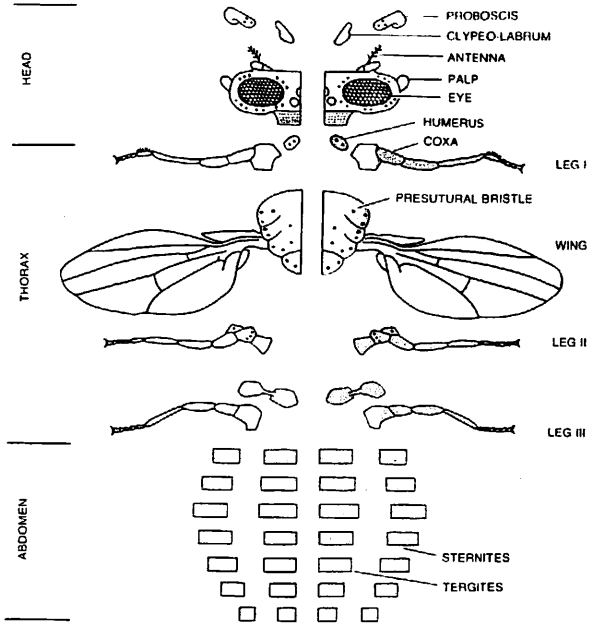
DEVELOPMENT OF A MOSAIC FLY proceeds from nuclear division (*shown in the illustration at top of page*), in which loss of the ring *X* occurs, producing an *XX* (*color*) nucleus and an *X* (*white*) nucleus (*1*). The nuclei divide a few times (*2*), then migrate to the surface of the egg and form a blastula: a single layer of cells, shown here in section and surface views (*3*). Note that female (*XX*) cells cover part of the surface and male (*X*) cells the other part. The arrangement of male (*white*) and female (*color*) parts in the adult fly depends on the way the boundary between the *XX* and *X* cells happened to cut the blastula, and that in turn depends on the orientation of the axis along which the original nucleus divides (*4–6*).

The reader will perceive that a mosaic fly is a system in which the effects of normal and of mutant genes can be distinguished in one animal. We use this system by arranging things so that both a behavioral gene and "marker" genes that produce anatomical anomalies are combined on the same *X* chromosome. This is done through the random workings of the phenomenon of recombination, in which segments of two chromosomes (in this case the *X*) "cross over" and exchange places with each other during cell division in the formation of the egg. In this way we can, for example, produce a strain of flies that are *nonphototactic* and also have white eyes (instead of the normal red) and a yellow body color. Then we breed males of this strain with females of the ring *X* strain. Some of the resulting embryos will have one ring *X* chromosome and one mutation-loaded *X* chromosome. In a fraction of these embryos the ring *X* (carrying normal genes) will be lost at an early nuclear division. The *XX* body parts of the resulting adult fly will have one *X* chromosome with normal genes and one with mutations; because both the behavioral and the anatomical genes in question are recessive (their effect is masked by the presence of a single normal gene) the mutations will not be expressed in those parts. In the body parts having lost the ring *X*, however, the single *X* chromosome will be the one carrying the mutations. And because it is all alone the mutations will be expressed. Examination of the fly identifies the parts that have normal color and those in which the mutant genes have been uncovered. We can select from among the randomly divided gynandromorphs individuals in which the dividing line falls in various ways: a normal head on a mutant body, a mutant head on a normal body, a mutant eye and a normal one and so on. And then we can pose the question we originally had in mind: What parts must be mutant for the mutant behavior to be expressed?

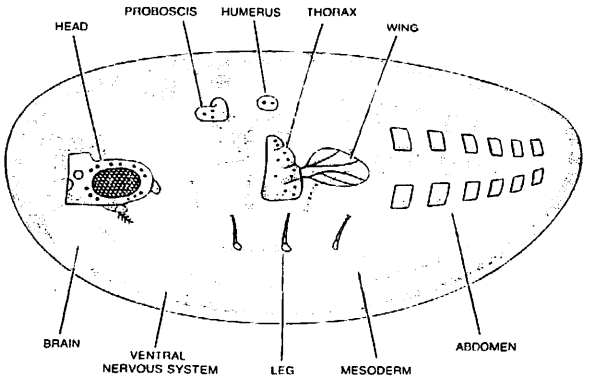
When Hotta and I did that with certain visually defective mutants, for instance ones that produce no receptor potential, we found that the electroretinogram of the mutant eye was always completely abnormal, whereas the normal eye functioned properly. Even in gynandromorphs in which everything was normal except for one eye, that eye showed a defective electroretinogram. This makes it clear that the defects in those mutants are not of the vitamin A type I mentioned above; the defect must be autonomous within the eye itself.

The behavior of flies with one good eye and one bad eye is quite striking. A normal fly placed in a vertical tube in the dark climbs more or less straight up, with gravity as its cue. If there is a light at the top of the tube, the fly still climbs straight up because phototaxis (which the fly achieves by moving so as to keep the light intensities on both eyes equal) is consistent in direction with the negative geotaxis. A mosaic fly with one good eye also climbs straight up in the dark, since its sense of gravity is unimpaired. If a light is turned on at the top, however, the fly tends to trace a helical path, turning its defective eye toward the light in a futile attempt to balance input signals. If the right eye is the bad one, the fly traces a right-handed helix; if the left eye is bad, the helix is left-handed. (Sometimes it is difficult to resist the temptation, out of nostalgia for the old molecular-biology days, to put in two flies and let them generate a double helix.)

In these mutants the primary focus of the *phototactic* defect is in the affected organ itself. More frequently, however, the focus is elsewhere. A good way to see how this situation is dealt with is to consider a *hyperkinetic* mutant that



ADULT FLY is an assembly of a large number of external body parts, each of which was formed independently from a primordial group of cells of the blastula. In a mosaic fly the boundary between male and female tissues tends to follow lines of division between discrete body parts. Here the main external parts are named; black dots are the major bristles.



FANCIFUL DRAWING of the blastula shows how each adult body part came from a specific site on the blastula: left-side and right-side parts (or left and right halves of parts such as the head) from the left and right sides of the blastula respectively. The nervous system and the mesoderm (which gives rise to the muscles) have also been shown by embryologists to originate in specified regions of the blastula. It is clear that the probability that any two parts will have a different genotype (that is, that they will be on different sides of the mosaic boundary that cuts across the blastula) will depend on how far they are from each other on the blastoderm, the blastula surface. Conversely, the probability that two parts are of different genotypes should be a measure of their distance apart on the blastoderm.

was studied by Kaplan and Kazuo Ikeda. When such a fly is anesthetized with ether, it does not lie still but rather shakes all six of its legs vigorously. Kaplan and Ikeda found that flies that are mosaics for the gene shake some of their legs but not others and that the shaking usually correlates well with the leg's surface genotype as revealed by markers—but not always. (Suzuki and his colleagues found the same to be true of flies mosaic for the *paralyzed* mutation.) The point is that the markers are on the outside of the fly. The genotype of the surface is not necessarily the same as that of the underlying tissues, which arise from different regions of the embryo. And one might well expect that leg function would be controlled by nervous elements somewhere inside the fly's body that could have a different genotype from the leg surface. The problem is to find a way of relating internal behavioral foci to external landmarks. Hotta and I developed a method of mapping this relation by extending to behavior the idea of a "fate map," which was originally conceived by A. H. Sturtevant of Cal Tech.

Sturtevant was the genius who had earlier shown how to map the sequence of genes on the chromosome by measuring the frequency of recombination among genes. He had seen that the probability of crossing over would be greater the farther apart the genes were on the chromosome. In 1929 he proposed that one might map the blastoderm in an analogous way: the frequency with which any two parts of adult mosaics turned out to be of different genotypes could be related to the distance apart on the blastoderm of the sites that gave rise to those parts. One could look at a large group of mosaics, score structure *A* and structure *B* and record how often one was normal whereas the other was mutant, and vice versa. That frequency would represent the relative distance between their sites of origin on the blastoderm, and with enough such measurements one could in principle construct a two-dimensional map of the blastoderm. Sturtevant scored 379 mosaics of *Drosophila simulans*, put his data in a drawer and went on to something else. At Cal Tech 40 years later Merriam and Antonio Garcia-Bellido inherited those 379 yellowed sheets of paper, computed the information and found they could indeed make a self-consistent map.

When Hotta and I undertook to map behavior in *D. melanogaster*, we began by preparing our own fate map of the adult external body parts based on the scores for 703 mosaic flies [*see upper illustration on these two pages*]. Distances on the map are in "sturts," a unit Merriam, Hotta and I have proposed in memory of Sturtevant. One sturt is equivalent to a probability of 1 percent that the two structures will be of different genotypes.

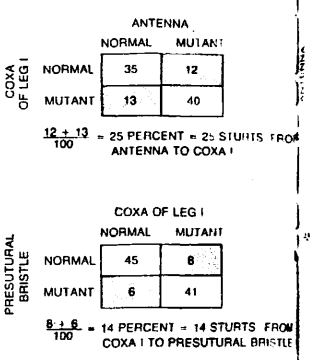
Now back to *hyperkinetic*. We produced 300 mosaic flies and scored each for a number of surface landmarks and for the coincidence of marker mutations at those landmarks with the shaking of each leg. We confirmed the observations of Ikeda and Kaplan that the behavior of each leg (whether it shakes or not) is independent of the behavior of the other legs and that the shaking behavior and the external genotype of a leg are frequently the same—but not always. The independent behavior of the legs indicated that each had a separate focus. For each leg we calculated the distance from the shaking focus to the leg itself and to a number of other landmarks [*see lower illustration on these two pages*] and thus determined a map location for each focus. They are near the corresponding legs but below them, in the region of the blastoderm that Donald F. Poulson of Yale University years ago identified by embryological studies as the origin of the ventral nervous system. This is consistent with electrophysiological evidence, obtained by Ikeda and Kaplan, that neurons in the thoracic ganglion of the ventral nervous system behave abnormally in these mutants.

Another degree of complexity is represented by a mutant we call *drop-dead*. These flies develop, walk, fly and otherwise behave normally for a day or two after eclosion. Suddenly, however, an individual fly becomes less active, walks in an uncoordinated manner, falls on its back and dies; the transition from apparently normal behavior to death takes only a few hours. The time of onset of the syndrome among a group of flies hatched together is quite variable; after the first two days the number of survivors in the group drops exponentially, with a half-life of about two days. It is as if some random event triggers a cataclysm. The gene has been identified as a recessive one on the *X* chromosome. Symptoms such as these could result from malfunction almost anywhere in the body of the fly, for example from a blockage of the gut, a general biochemical disturbance or a nerve disorder. In order to localize the focus we did an analysis of 403 mosaics in which the *XX* parts were normal and the *X* body parts expressed the *drop-dead* gene and surface-marker mutations, and we scored for *drop-dead* behavior and various landmarks.

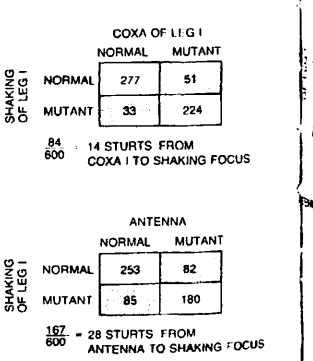
Drop-dead behavior, unlike shaking behavior, which could be scored separately for each leg, is an all-or-none property of the entire fly. First we did a rough analysis to determine whether the behavior was most closely related to the head, thorax or abdomen, considering only flies in which the surface of each of these structures was either completely mutant or completely normal. Among mosaics in which the entire head surface was normal almost all behaved normally, but six flies out of 97 died in the *drop-dead* manner; in the reciprocal class eight flies of 80 with mutant head surfaces lived. In other words, the focus was shown to be close to, but distinct from, the blastoderm site of origin of the head surface. Comparable analysis showed that the focus was substantially farther away from the thorax and farther still from the abdomen. Next we considered individuals with mosaic heads. The reader will recall that in certain visual mutants the visual defect was always observed in the eye on the mutant side of the head; flies with half-normal heads had normal vision in one eye. For *drop-dead*, on the other hand, of mosaics in which half of the head surface was mutant only about 17 percent dropped dead. All the rest survived.

Now, a given internal part should occur in normal or mutant form with equal probability, as the external parts in these mosaics did. On that reasoning, if there were a single focus inside the head of the fly, half of the bilateral-mosaic flies should have dropped dead. We formed the hypothesis, therefore, that there must be two foci, one on each side, and that they must interact. Both of them must be mutant for the syndrome to appear. In other words, a mutant focus must be "submissive" to a normal one. In that case, if an individual exhibits *drop-dead* behavior, both foci must be mutant, and if a fly survives, one focus may be normal or both of them may be.

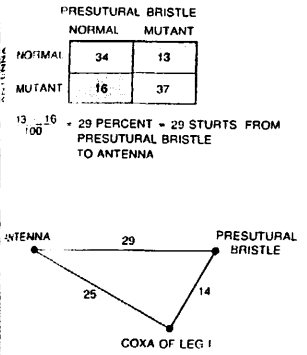
Mapping a bilateral pair of interacting foci calls for special analysis. By considering the various ways a mosaic dividing line could fall in relation to a pair of visible external landmarks (one on

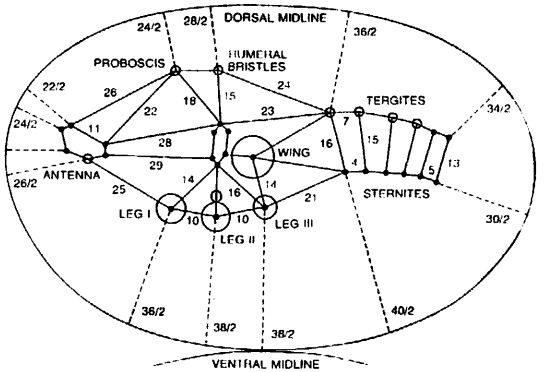


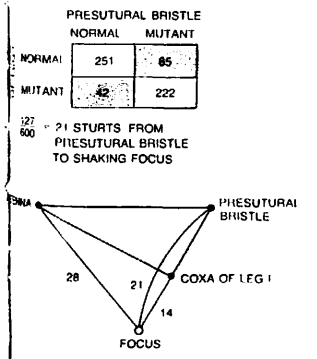
FATE MAP, a two-dimensional map of the blastoderm, is constructed by calculating the distances between the sites that gave rise to various parts. This is done by observing a large number of adult flies and recording the number of times each of two parts is mutant or normal. The numbers are entered in a matrix, as shown *(left)* for three pairs of parts. Instances in which one part is normal whereas the other part is mutant on the same fly *(colored boxes)* are totaled. That figure, divided by the total number of instances, gives the probability that the two parts are of different genotypes. And that probability is proportional to the distance between them, indicated in "sturts." Plotting the three distances triangulates the relative locations of the three sites. By thus scoring 703 mosaic flies for body parts, Yoshiki Hotta in the author's laboratory built up the fate map of external body parts *(right)*. Broken lines represent distances to the blastula midlines, obtained by dividing by two the distances between homologous parts on opposite sides of the fly.

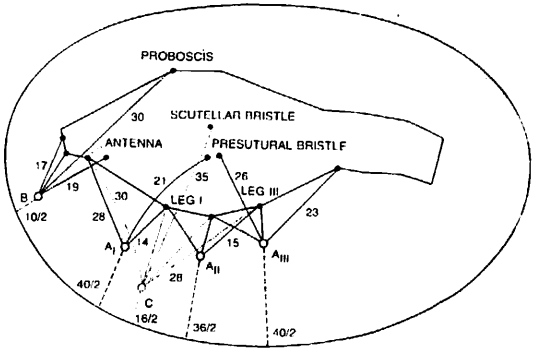


BEHAVIORAL FOCI, the sites at which a mutant gene exerts its effect on behavior, are plotted in the same way. The behavior in this example *(left)* is abnormal shaking of the legs under ether. The shaking is independent for each leg and here leg *I* has been scored for 300 flies—or 600 instances, since the data can be doubled to represent both sides of the fly. (The total of instances can be less than 600 because cases in which both the body part and the behavior are mosaic are eliminated.) Distances calculated *(colored lines)* triangulate the focus. In this way the foci for shaking behavior for each leg *(A)* are added to the map *(right)*. Foci for *drop-dead (B)* and *wings-up (C)* behavior are also found.









BRAINS OF DROP-DEAD MUTANTS that have reached the symptomatic stage show striking degeneration, as shown by photomicrograph (*left*) of a section of such a fly's head enlarged 300 diameters. The brain and the optic ganglia are full of holes. Sections fixed before a mutant has shown any symptoms, on the other hand, show no more degeneration than a section of a normal brain does (*right*).

FLIGHT MUSCLE OF WINGS-UP MUTANTS shows degeneration that seems to account for their behavior. Normal flight muscle, enlarged 30,000 diameters in an electron micrograph, has bundles of filaments crossed by straight, dense bands: the *Z* lines (*left*). In the muscle of flies heterozygous for the gene *wup B*, which hold their wings normally but cannot fly, the *Z* lines are irregular (*right*).

each side of the body) and a symmetrical pair of internal foci, one can set up equations based on the probability of each possible configuration. Using the observed data on how many mosaic flies showed the various combinations of mutant and normal external landmarks and mutant or normal behavior, it is possible to solve these equations for the map distance from each landmark to the corresponding focus and from one focus to the homologous focus on the other side of the embryo. The *drop-dead* foci turn out to be below the head-surface area of the blastoderm, in the area embryologists have assigned to the brain. Sure enough, when we examined the brain tissue of flies that had begun to exhibit the initial stages of *drop-dead* behavior, it showed striking signs of degeneration, whereas brain tissue fixed before the onset of symptoms appeared normal. As for mosaics whose head surfaces are half-normal, those that die show degeneration of the brain on both sides; the survivors' brains show no degeneration on either side, a finding consistent with the bilateral-submissive-focus hypothesis. It appears that the normal side of the brain supplies some factor that prevents the deterioration of the side with the mutant focus.

There is another kind of bilateral focus, "domineering" rather than submissive. An example is the mutant we call *wings-up*. There are two different genes, *wup A* and *wup B*, which produce very similar overt behavior: shortly after emergence each of these flies raises its wings straight up and keeps them there. It cannot fly, but otherwise it behaves normally. Is *wings-up* the result of a defect in the wing itself, in its articulation or in the muscles or neuromuscular junctions that control the wing, or of some "psychological quirk" in the central nervous system? The study of mosaic flies shows that the behavior is more often associated with a mutant thorax than with a mutant head or abdomen. The focus cannot be in the wings themselves or anywhere on the surface of the thorax, however, because in some mosaics the wings and the thorax surface are normal and yet the wings are held up and in other mosaics the wings and thorax display all the mutant markers and yet the fly flies. These observations suggest that some structure inside the thorax could be responsible.

Once again we look at the bilateral mosaics, those with one side of the thorax carrying mutant markers and the other side appearing normal. Unlike the *drop-dead* bilateral mosaics, most of which were normal, these bilaterals are primarily mutant; well over half of them hold both wings up. Both wings seem to act together; either both are held up or both are in the normal position. This suggests two interacting foci, one on each side, with the mutant focus domineering with respect to the normal one, that is, if either of the foci is mutant, or both are, then both wings will be up. Again we can set up equations based on the probability of the various mosaic configurations and solve to find the pertinent map distances. The focus comes out to be close to the ventral midline of the blastula. That is a region known to produce the mesoderm, the part of the developing embryo from which muscle tissue is derived, which suggested that a defect in the fly's thoracic muscle tissue could be responsible for *wings-up* behavior.

The abnormality became obvious when we dissected the thorax. In the fly the raising and lowering of the wings in normal flight is accomplished by changes in the shape of the thorax, changes brought about by the alternate action of sets of vertical and horizontal muscles. Under the phase-contrast microscope these indirect flight muscles are seen to be highly abnormal in both *wings-up* mutants. Developmental studies show that in *wup A* the muscles form properly at first, then degenerate after the fly emerges. In the *wup B* mutant, on the other hand, the myoblasts that normally produce the muscles fuse properly but the muscle fibrils fail to appear. In both mutants the other muscles, such as those of the leg, appear to be quite normal, and the flies walk and climb perfectly well. In flies that are heterozygous for *wup A* (nonmosaic flies with one mutated and one normal gene) the muscles and flight behavior are normal, that is, the gene is completely recessive. In *wup B* heterozygotes, on the other hand, the wings are held in the normal position but the flies cannot fly. Electron microscopy shows that even in these heterozygotes the indirect flight muscles are defective: the microscopic filaments that constitute each muscle fibril are arranged correctly, but the Z line, a dense region that should run straight across the fibril, is often crooked and forked. Examination of the muscles in bilateral mosaics confirmed the impression that the *wup* foci are domineering. In every mosaic that had shown *wings-up* behavior one or more muscle fibers were degenerated or missing; no fibers were seriously deficient in any flies that had displayed normal behavior. The natural shape of the thorax apparently corresponds to the *wings-up* position, and the presence of defective muscles on either side is enough to make it impossible to change the shape of the thorax, locking the wings in the vertical position.

The mutants so far mapped provide examples involving the main components of behavior: sensory receptors, the nervous system and the muscles. For some of the mutants microscopic examination has revealed a conspicuous lesion of some kind in tissue. The obvious question is whether or not fate mapping is necessary; why do we not just look directly for abnormal tissue? One answer is that for many mutants we do not know where to begin to look, and it is helpful to narrow down the relevant region. Furthermore, in many cases no lesion may be visible, even in the electron microscope. More important, and worth reiterating, is the fact that the site of a lesion is not necessarily the primary focus. For example, an anomaly of muscle tissue may result from a defect in the function of nerves supplying the muscle. This possibility has been a lively issue in the study of diseases such as muscular dystrophy. Recently, by taking nerve and muscle tissues from a dystrophic mutant of the mouse and from its normal counterpart and growing them in tissue culture in all four combinations, the British workers Belinda Gallup and V. Dubowitz were able to show that the nerves are indeed at fault.

The mosaic technique in effect does the same kind of experiment in the intact animal. In the case of the *wings-up* mutant the primary focus cannot be in the nerves, since if that were so the focus would map to the area of the blastoderm destined to produce the nervous system, not to the mesoderm, where muscle tissue is formed. The *wings-up* mutants clearly have defects that originate in the muscles themselves.

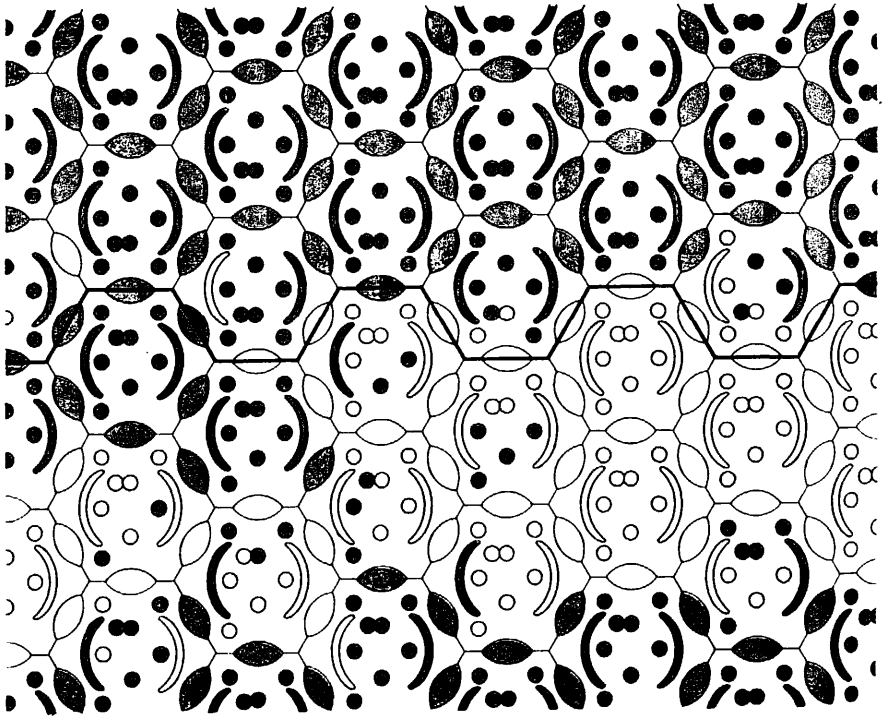
Another application of mosaics is in tagging cells with genetic labels to follow their development. The compound eye of *Drosophila* is a remarkable structure consisting of about 800 ommatidia: unit eyes containing eight receptor cells each. The arrangement of cells in an ommatidium is precise and repetitive; the eye is in effect a neurological crystal in which the unit cell contains eight neurons. Thomas E. Hanson, Donald F. Ready and I have been interested in how this structure is formed. Are the eight photoreceptor cells derived from one cell that undergoes three divisions to produce eight, or do cells come together to form the group irrespective of their lineage? This can be tested by examining the eyes of flies, mosaic for the *white* gene, in which the mosaic dividing line passes through the eye. By sectioning the eye and examining ommatidia near the border between white and red areas microscopically, it is possible to score the tiny pigment granules that are present in normal photoreceptor cells but absent in *white* mutant cells. The result is clear: A single ommatidium can contain a mixture of receptor cells of both genotypes. This proves that the eight cells cannot be derived from a single ancestral cell but have become associated in their special

group of eight irrespective of lineage. The same conclusion applies to the other cells in each ommatidium, such as the normally heavily pigmented cells that surround the receptors.

Not all cells have such convenient pigment markers. It would obviously be valuable to have a way of labeling all the internal tissues as being either mutant or normal, much as yellow color labels a landmark on the surface. This can now be done for many tissues by utilizing mutants that lack a specific enzyme. If a recessive enzyme-deficient mutant gene is recombined on the *X* chromosome along with the *yellow*, *white* and behavioral genes and mosaics are produced in the usual way, the male tissues of the mosaic will lack the enzyme. By making a frozen section of the fly and staining it for enzyme activity one can identify normal and mutant cells.

In order to apply this method in the nervous system one needs to have an enzyme that is normally present there in a large enough concentration to show up in the staining procedure and a mutant that lacks the enzyme, and the lack should have a negligible effect on the behavior under study. Finally, the gene in question should be on the *X* chromosome. Douglas R. Kankel and Jeffrey Hall in our laboratory have developed several such mutants, including one with an acid-phosphatase-deficient gene found by Ross J. MacIntyre of Cornell University. By scoring the internal tissues they have constructed a fate map of the internal organs of the kind made earlier for surface structures. We are now adapting the staining method for electron microscopy in order to work at the level of the individual cell.

The staining procedure has demonstrated graphically that the photoreceptor cells of the eye come from a different area of the blastoderm than do the neurons of the lamina, to which they project. In the adult fly the two groups of cells are in close apposition, but the former arise in the eye whereas the latter come from the brain. The distance between them on the fate map, determined by Kankel, is about 12 sturts, so that a considerable number of mosaic flies have a normal retina and a mutant lamina or vice versa. This makes it possible to distinguish between presynaptic and postsynaptic defects in mutants with blocks in the visual pathway. In the nonphototactic mutants Hotta and I analyzed in mosaics, the defect in the electroretinogram was always associated with the eye. In contrast, a mutant with a similar electroretinogram abnormality that was studied by Linda Hall and Suzuki showed, in some mosaics, a normal trace for a mutant eye—and vice versa. Fate mapping placed the focus in precisely the region corresponding to the lamina. What appeared to be similar malfunctions in two mutants were thus shown to be different, due in one case to a presynaptic block and in the other case to a postsynaptic one.



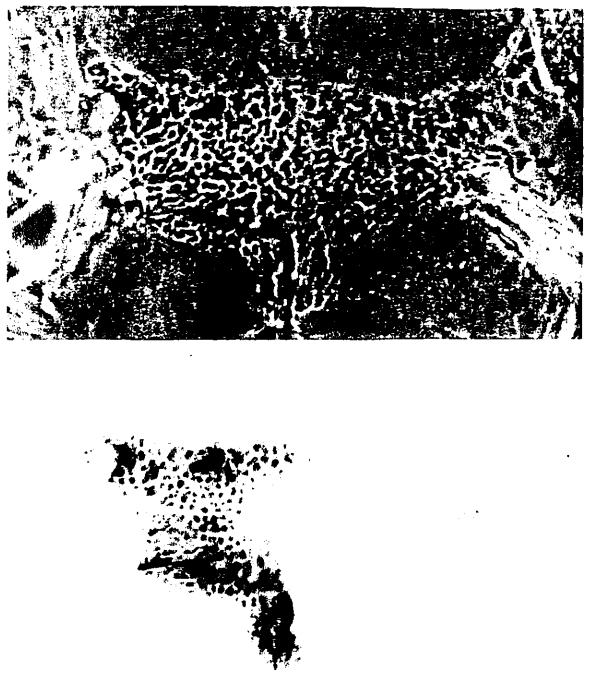
MOSAIC EYE contains a patch of cells that carry the *white* gene and therefore lack the normal red pigment. The fly's compound eye is an array of hexagonal ommatidia, each containing eight photoreceptor cells (*circles*) and two primary pigment cells (*crescents*) and surrounded by six shared secondary pigment cells (*ovals*). The fact that a single ommatidium can have *white* and normal gene types shows its cells are not necessarily descended from a common ancestral cell. Nor is the mirror-image symmetry about the equator (*heavy line*) the result of two cell lines: mutant cells appear on both sides. Drawing is based on observations by Donald Ready.

Much of what has been done so far involves relatively simple aspects of behavior chosen to establish the general methodology of mutants and mosaic analysis. Can the methodology be applied to more elaborate and interesting behavior such as circadian rhythm, sexual courtship and learning? Some beginnings have been made on all of these. By making flies that are mutant for normal and mutant rhythms, Konopka has shown that the internal clock is most closely associated with the head. Looking at flies with mosaic heads, he found that some exhibited the normal rhythm and others the mutant rhythm but that a few flies exhibited a peculiar rhythm that appears to be a sum of the two, as if each side of the brain were producing its rhythm independently and the fly responded to both of them. By applying the available cell-staining techniques it may be possible to identify the cells that control the clock.

Sexual courtship is a higher form of behavior, since it consists of a series of fixed action patterns, each step of which makes the next step more likely. The sex mosaics we have generated lend themselves beautifully to the analysis of sexual behavior. A mosaic fly can be put with normal females and its ability to perform the typical male courtship steps can be observed. Hotta, Hall and I found that the first steps (orientation toward the female and vibrating of the wings) map to the brain. This is of particular interest because the wings are vibrated by motor-nerve impulses from the thoracic ganglion; even a female ganglion will produce the vibration "song" typical of the male if directed to do so by a male brain. It would appear that the thoracic ganglion in a female must "know" the male courtship song even though she does not normally emit it. This is consistent with recent experiments by Ronald Hoy and Robert Paul at the State University of New York at Stony Brook, in which they showed that hybrid cricket females responded better to the songs of hybrid males than to males of either of the two parental species.

Sexual behavior in *Drosophila*, although complex, is a stereotyped series of instinctive actions that are performed correctly by a fly raised in isolation and without previous sexual experience. Other forms of behavior such as phototaxis also appear to be already programmed into the fly when it ecloses. Whether a fruit fly can learn has long been debated; various claims have been made and later shown to be incorrect. Recently William G. Quinn, Jr., and William A. Harris in our laboratory have shown in carefully controlled experiments that the fly can learn to avoid specific odors or colors of light that are associated with a negative reinforcement such as electric shock. This opens the door to genetic analysis of learning behavior through mutations that block it.

In tackling the complex problems of behavior the gene provides, in effect, a microsurgical tool with which to produce very specific blocks in a behavioral pathway. With temperature-dependent mutations the blocks can be turned on and off at will. Individual cells of the nervous system can be labeled genetically and their lineage can be followed during development. Genetic mosaics offer the equivalent of exquisitely fine grafting of normal and mutant parts, with the entire structure remaining intact. What we are doing in mosaic mapping is in effect "unrolling" the fantastically complex adult fly, in which sense organs, nerve cells and muscles are completely interwoven, backward in development, back in time to the blastoderm, a stage at which the different structures have not yet come together. Filling the gaps between the one-dimensional gene, the two-dimensional blastoderm, the three-dimensional organism and its multidimensional behavior is a challenge for the future.



DIRECT SCORING OF NORMAL AND MUTANT CELLS within the nervous system is possible with a staining method developed by Douglas Kankel and Jeffrey Hall in the author's laboratory. Mosaics are produced in which mutant cells are deficient in the enzyme acid phosphatase. When the proper stain is applied to a section of nerve tissue, normal cells stain brown and mutant cells are unstained. Here a section of the thoracic ganglion, thus stained, is shown in phase-contrast (*top*) and bright field (*bottom*) photomicrographs. In the bottom picture normal cells are marked by the stain, delineating the mosaic boundary.