Proc. Nat. Acad. Sci. USA
Vol. 68, No. 9, pp. 2112–2116, September 1971

Clock Mutants of *Drosophila melanogaster*

(eclosion/circadian/rhythms/X chromosome)

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Contributed by Seymour Benzer, July 2, 1971

ABSTRACT Three mutants have been isolated in which the normal 24-hour rhythm is drastically changed. One mutant is arrhythmic; another has a period of 19 hr; a third has a period of 28 hr. Both the eclosion rhythm of a population and the locomotor activity of individual flies are affected. All these mutations appear to involve the same functional gene on the X chromosome.

Rhythmic variations in behavior are displayed by many organisms, ranging from single cells to man (1). When the rhythm persists under constant conditions, and has a period of around one day, depending little on temperature, the rhythm is called circadian (2). Many experiments have attempted to probe the mechanism (3), but the nature of the underlying oscillation remains unknown (4). Perturbations by inhibitors of RNA or protein synthesis suggest that such molecules are involved (5–8). Biochemical systems that oscillate with much shorter periods have been demonstrated both *in vivo* and *in vitro* (9, 10), but their relation to circadian rhythms is not clear.

An approach that has been successful in unravelling mechanisms in some systems is the use of genetic alterations. Since the expression of a rhythm requires an integrated system, mutation of the genes responsible for development and function of the system could lead to abnormal rhythms. Various aspects of circadian rhythms have indeed been shown to be sensitive to genetic makeup (11–18). For genetic dissection of circadian rhythms in an organism having a nervous system, *Drosophila* offers certain advantages. Much is already known about the rhythm of eclosion (emergence of the adult fly from the pupa), and genetic methodology is readily available. This paper describes the first result of such an analysis.

MATERIALS AND METHODS

Isolation of mutants

D. melanogaster of the C-S (Canton-Special) wild strain was maintained on cornmeal medium. Mutagenesis by ethyl methane sulfonate was according to Lewis and Bacher (19), the treated males being mated to virgin attached-X females, so that each F_1 progeny male carried a treated X chromosome received from his father. Each male was mated individually to attached-X females, producing a stock of males bearing identical X chromosomes, plus normal-rhythm, attached-X females. The stocks were reared at constant temperature under LD 12:12 (12 hr of 50 foot-candles or more of white fluorescent light, 12 hr of darkness each day).

To detect X-linked rhythm mutants, the stocks were examined for ones in which males emerged abnormally. The normal females in each bottle served as an internal control, at least twice as many emerging during the light as during the dark period. In a few bottles, males emerged in approximately equal numbers during day and night. Each mutant candidate was examined in more detail by raising pupae in LD 12:12, then monitoring the adult eclosion rhythm in constant darkness. From a total of about 2000 F_1 males, three rhythm mutants were obtained.

Determination of eclosion and locomotor activity rhythms

Eclosion rhythms, free-running in constant darkness, were determined with automatic "bang boxes" (20), generously loaned by Dr. Colin Pittendrigh. Several hundred pupae, raised in LD 12:12, were transferred to the apparatus at the end of a light cycle. The apparatus was thereafter maintained in constant darkness. Fractions were collected every hour, yielding an eclosion profile.

Locomotor activity of individual adult flies was measured by monitoring their movement with infrared light, which does not affect the *Drosophila* clock (21). The devices, designed and built by Dr. Yoshiki Hotta, used a small incandescent lamp, a Wratten No. 87C filter transmitting only wavelengths greater than 800 μm, and a chamber (3 mm thick $\times$ 4 mm wide $\times$ 45 mm high) containing the fly, some food, and a cotton plug. Two silicon solar cells were arranged so that one received light transmitted through the upper third of the chamber, the other the lower third; they were wired so that the output voltage was zero when equal light fell on both cells. As the fly moved into or out of the area monitored by either solar cell, the resulting imbalance was converted to an all-or-none response registered on an event recorder. The sharpest rhythms were obtained with young flies (within one week of eclosion) previously exposed to at least three cycles of LD 12:12.

Genetic mapping of rhythm mutants

The rhythm mutants had normal morphology, but their abnormal eclosion patterns could be used as markers in recombination experiments. Males bearing an X-linked rhythm mutation were crossed to females homozygous for the visible markers *yellow-2*, *scute*, *vermilion*, *forked*, and a wild-type allele of *yellow* located near the centromere. These markers had no effect upon rhythm. F_1 males, receiving their X chromosomes from their mothers, were all $y^2\ sc\ v\ f \cdot y^+$, and the females were all heterozygous, having the rhythm mutation on one X chromosome and $y^2\ sc\ v\ f \cdot y^+$ on the other. These males and females were mated to each other. F_2 males, receiving X chromosomes that had an opportunity to undergo recombination in their mothers, included various recombinants for the rhythm mutation and the morphological markers.

F_2 progeny were raised in LD 12:12 at 25°C, collected in the pupal stage, and transferred to "bang boxes," as above. The morphological phenotype of each male fly was scored by microscopic examination, and the eclosion profile was plotted for each parental and recombinant class.

Complementation tests on rhythm mutants

Flies heterozygous (in the *trans* arrangement) for two rhythm mutations were constructed as follows. Males bearing one of the mutations were crossed to females carrying the balancer X chromosome FM 7, which contains multiple inversions to suppress crossingover between the two X chromosomes, as well as the dominant marker *Bar* for identification (22). Virgin progeny females (mutant/FM 7) were crossed to males bearing the second rhythm mutation, and the double heterozygotes (identified by lack of the *Bar* marker) were selected. These were tested individually in the locomotor-activity meter. The same procedure was used for constructing flies heterozygous for rhythm mutations and various X-chromosome deletions.

RESULTS

Eclosion rhythms of normal and mutant strains

Fig. 1*A* shows the normal circadian rhythm of eclosion of adults. The data shown are for attached-X females (carrying the genetic markers *yellow* and *forked*), which were routinely used as internal controls in experiments involving mutants (see *Methods*). Their rhythm was indistinguishable from that of the C-S males from which the rhythm mutants were isolated. These eclosion peaks are somewhat broader than those reported for *D. pseudoobscura* (23). In *pseudoobscura*, the period of the eclosion rhythm has usually been determined with reference to the median point of each successive eclosion peak. For *melanogaster*, a more sharply definable point is the time at which the peak rises to half its maximum value. The average period for normal flies (Fig. 1*A*) is about 24 hr.

Figs. 1*B*, 1*C*, and 1*D* show the rhythms for males of three mutant types, each isolated by one-step mutation from the normal C-S strain. One mutant is essentially arrhythmic; another has a short period of about 19 hr; the third has a long period of about 28 hr. These profiles are reproducible in repeated runs for each strain and the properties of the mutants have been hereditarily transmitted over many generations.

Effect of temperature on the eclosion rhythms

Between 18°C and 25°C, the period of the eclosion rhythm of normal *D. melanogaster* remains constant to about 1 hr (the interval used in collecting fractions). The same is true for the short- and long-period mutants. The arrhythmic mutant remains arrhythmic in this temperature range.

Locomotor activity rhythm in individual flies

Eclosion occurs only once in a fly's lifetime; to study the clock that controls eclosion, one must observe an entire population. This raises a question for the apparently arrhythmic mutant: Is the absence of an eclosion rhythm due to lack of expression of the clock or simply desynchronization of the various individual flies? To answer this, it is necessary to assay some ongoing phenomenon in a single fly. We chose to measure locomotor activity, using the photoelectric device described in *Methods*. Earlier studies have demonstrated the existence of a rhythm of locomotor activity in *Drosophila* (24-26).

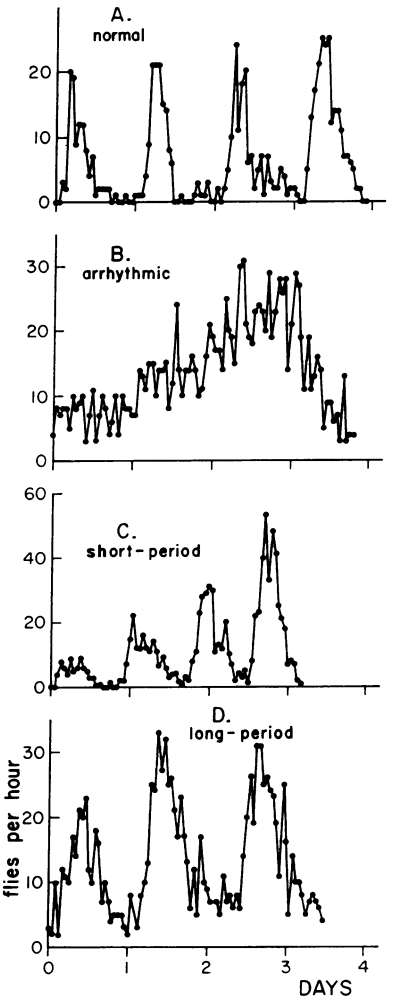

FIG. 1. Eclosion rhythms, in constant darkness, for populations of rhythmically normal and mutant flies, previously exposed to LD 12:12. T = 20°C.

Fig. 2*A* shows the activity, as registered on an event recorder, for a rhythmically-normal female (*yellow*, *forked*, attached-X). The fly was raised in LD 12:12, then placed in the monitoring device at the end of a light cycle. In these records, the offset of activity was typically more abrupt than the onset, so that the free-running period could be best determined by measurement of the average drift in time of offset per day. The rhythm shown in Fig. 2*A*, therefore, has a period of about 25 hr. For 8 females studied, the average period was 24.5 ± 0.4 hr. Fig. 2*B* shows the activity of a female homozygous for the arrhythmic mutation. The activity appears, by comparison, random in time. Thus, this mutation has indeed abolished the locomotor rhythm in individual flies. Four females studied gave similar results, with no evident periodicity.

Fig. 2*C* shows the activity for a homozygous short-period female. To better illustrate the short period, these records are displayed using a modulus of 19 hr. The locomotor activity rhythm for 5 short-period females was 19.5 ± 0.4 hr. Fig. 2*D* is for a homozygous long-period female, presented *modula* 28 hr. The average period for 4 females was 28.6 ± 0.5 hr.

Males of each mutant strain were also monitored (7 arrhythmic, 6 short-period, 4 long-period). The results were similar to those for females, giving average periods of 19.2 ±

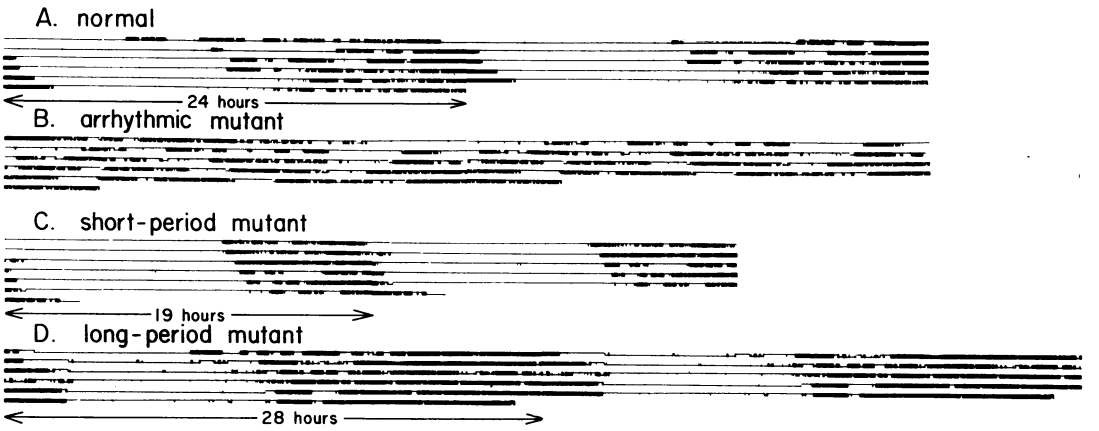

Fig. 2. Locomotor activity rhythms, monitored in infrared light, for individual rhythmically normal or mutant flies previously exposed to LD 12:12. Activity registered by event recorder. Records read from *left* to *right*, each new line representing the start of a successive interval. For visual continuity, each successive interval is also replotted to the right of the immediately preceding interval. The traces for normal and arrhythmic are plotted *modulo* 24 hr; for the short-period mutant *modulo* 19 hr is used; the long-period mutant is plotted *modulo* 28 hr. T = 25°C.

0.5 hr for the short-period mutant and 28.5 ± 0.5 hr for the long-period mutant, while no arrhythmic male showed any evident periodicity. Eight normal C-S males showed an average period of 23.8 ± 0.5 hr. Thus, in every case, the rhythm of ongoing locomotor activity in the adult corresponds to the rhythm of eclosion for the population.

Genetic mapping of rhythm mutants

To locate the mutant genes on the X chromosome, recombination was measured with respect to morphological markers with known position. Eclosion profiles were determined for various recombinant types and compared to the normal parental type. Fig 3 illustrates the method for the short-period mutant. The result is that recombinants lacking the portion of the marked chromosome carrying the genes for *yellow-2* and *scute* (and, hence, having obtained this portion from the rhythm-mutant chromosome) display the mutant period. The reciprocal recombinants (not shown) have a normal period. Thus, this rhythm mutation would appear to be located toward the left end of the X chromosome (the centromere being at the right end). The same procedure was also followed for the arrhythmic mutant; it also mapped to the same portion of the chromosome. The mapping was repeated for both mutants using the X-linked visible markers *white*, *singed*, and *miniature;* the results confirmed the assignment of both mutations to the left end of the X chromosome.

As a further check on all three rhythm mutants, recombinant males were recovered from crosses using the markers *white*, *singed*, *miniature*, or *yellow*, *white*, *split*. Each male was mated to virgin attached-X females to produce a stock of identical males, and the eclosion profile or locomotor rhythm of the stock was determined. The results in all 27 cases tested (9 for each rhythm mutant) were consistent with location of all three mutations to the left of *white*.

Complementation tests on rhythm mutants

The recombination experiments indicated similar positions on the X chromosome for the 3 rhythm mutations, raising the question whether these mutations represent changes in the same functional gene (cistron). This can be tested by constructing females bearing a different rhythm mutation on

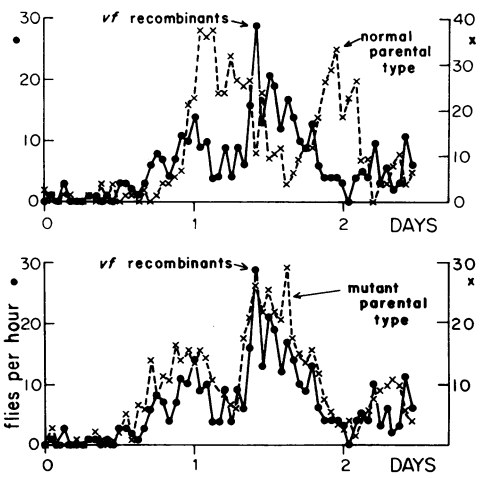

Fig. 3. Genetic recombination of the short-period gene with marker genes on the X chromosome. The eclosion profile is shown for one recombinant type emerging from the cross, compared to the normal and mutant parental types.

each of the two X chromosomes, and observing the resultant rhythms. This has been done for all combinations of the 3 rhythm mutant genes with each other and with the normal gene, measuring the activity rhythm on individual flies. Table 1 gives the results. Note the cases of heterozygotes with a mutant gene on one X chromosome and a normal gene on the other. For both the arrhythmic and the long-period mutants, the result is a rhythm with period close to normal. Thus, these mutant genes may be regarded as recessive to the normal one. In the case of the short-period mutant, however, the period of the heterozygote is intermediate between short and normal. This gene can, therefore, influence the rhythm even in the presence of a normal gene; it is only partially recessive. When the short-period mutant gene is opposed to the arrhythmic one, the rhythm displays a short period. Similarly, the arrhythmic gene is overshadowed by the long-period one. When the short-period and long-period mutants are tested together the result is a period close to

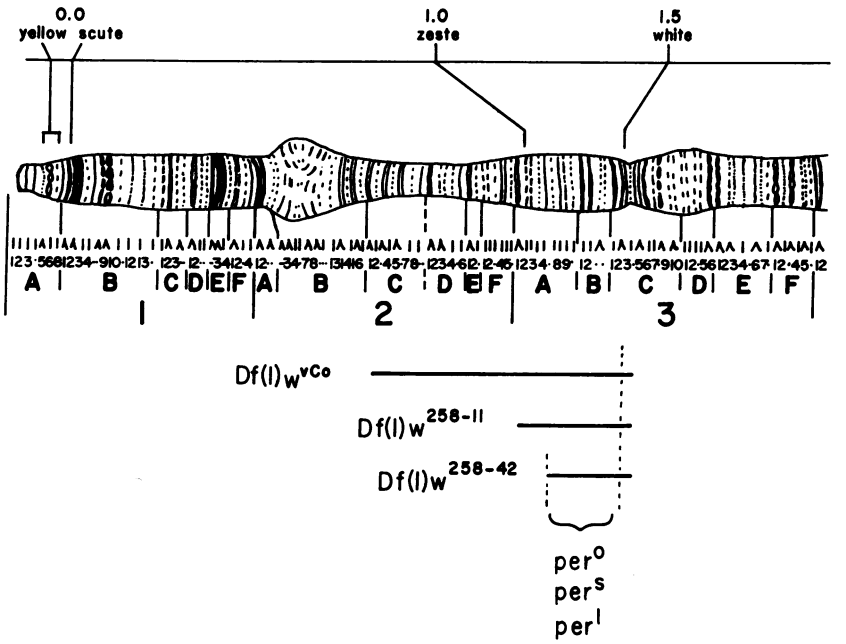

Fig. 4. Bridges' map (28) of the X chromosome of *Drosophila melanogaster*, showing about one-sixth of the chromosome at the end distal to the centromere. Each deficiency mutant lacks bands over the range indicated (9). per^{o} = arrhythmic mutant, per^{s} = short-period mutant, per^{l} = long-period mutant.

normal. This particular result does not provide a distinction between an additive effect or complementation of the two mutant genes to produce a normal period.

Complementation tests were also performed, using various deficiencies of the X chromosome, as illustrated in Fig. 4. All 3 rhythm mutations were tested in females heterozygous for the largest deficiency, w^{vCo}. The mutant phenotype was fully expressed in each case. Thus, this deficiency lacks the normal gene. The short-period mutant was similarly tested against the 2 shorter deletions shown. Again, this mutation was fully expressed; the heterozygotes had a short period. This result, combined with the fact that recombination experiments place the rhythm gene to the left of *white*, locates the gene within bands 3A6 to 3C2 (Fig. 4).

In sum, the results are consistent with the hypothesis that the arrhythmic mutant gene is simply inactive. Neither the short-period gene nor the long-period gene complements with the arrhythmic one to produce a normal rhythm. If all 3 mutations are, in fact, point mutations, they would appear to affect the same functional gene. However, it is not excluded that the arrhythmic mutation could be a deletion that overlaps the other two. Such a deletion could not be very large, since, unlike most known deficiencies, the arrhythmic mutant is fully viable and fertile, as are also the short- and long-period mutants.

DISCUSSION

As in many other aspects of behavior, alteration of a single gene can drastically change the properties of circadian rhythms. The different phenotypes of the three mutants described might be explained if, for instance, the long- and short-period mutants contain missense mutations producing alterations in quality or quantity of a gene product involved in the clock mechanism, while the arrhythmic mutant lacks the substance altogether. If this is so, the arrhythmic mutant may serve to identify the missing substance by comparison with normal flies.

TABLE 1. *Free-running period of locomotor activity*

Genotype				
First X chromosome	Second X chromosome	N	Period ± SD	Phenotype
normal (C-S)	normal (FM 7)	4	24.4 ± 0.5	normal
arrhythmic	arrhythmic	4	arrhythmic	arrhythmic
short-period	short-period	5	19.5 ± 0.4	short-period
long-period	long-period	4	28.6 ± 0.5	long-period
arrhythmic	normal (FM 7)	8	25.2 ± 0.4	~ normal
short-period	normal (FM 7)	5	21.9 ± 0.4	intermediate
long-period	normal (FM 7)	5	25.5 ± 0.5	~ normal
short-period	arrhythmic	6	19.5 ± 0.4	short-period
long-period	arrhythmic	5	30.6 ± 1.3	long-period
short-period	long-period	6	22.9 ± 0.4	~ normal

The fact that the *period* of the rhythm under constant conditions is altered implies that the mutations are affecting the basic oscillator. All three mutations affect both the pupal eclosion rhythm and the adult activity rhythm, as if a single clock system controls both. Since the rhythm that determines eclosion can be initiated and reset during the larval and pupal stages (27), it would appear that this clock system persists through metamorphosis. At the very least, both systems have some gene product in common. By measurement of the effect of the mutations on other rhythms (e.g., egg-laying, mating), it should be possible to determine whether rhythms having different phases are all coupled to the same basic oscillator.

The long- and short-period mutants retain a property of normal rhythms in that their periods change very little with

temperature. Thus, whatever mechanism is responsible for temperature compensation of the rhythms is still operative in these mutants.

It is striking that the first three rhythm mutants isolated, having very different phenotypes, all affect the same functional gene, since one might expect that many genes play a role. Isolation of additional mutants on the X chromosome, as well as the autosomes, may turn up other relevant genes.

The anatomical site of action of a rhythm mutation can be investigated by using genetic mosaics, i.e., flies composed of mutant and nonmutant parts, and determining which parts control the rhythm of the composite fly. Preliminary results indicate that the rhythm corresponds to the genotype of the head.

This work was supported by a grant from the National Science Foundation to S.B. and a National Science Foundation predoctoral fellowship to R.K. We thank Dr. Colin Pittendrigh for enlightening discussions and for the loan of several bang boxes, Dr. Yoshiki Hotta for design and construction of the activity detectors, and Barbara Stewart for help with the mapping experiments.

1. Sollberger, A., *Biological Rhythm Research* (Elsevier Publishing Company, New York, 1965).
2. Halberg, F., *Z. Vitamin.-, Hormon.- Fermentforsch.*, **10**, 225 (1959).
3. For a review, see Bünning, E., *The Physiological Clock* (Springer-Verlag New York, Inc., New York, 1967).
4. Anon., *Nature New Biol.*, **231**, 97 (1971).
5. Karakashian, M. W., and J. W. Hastings, *Proc. Nat. Acad. Sci. USA*, **48**, 2130 (1962).
6. Strumwasser, F., *Invertebrate Nervous Systems*, ed. C.A.G. Wiersma (U. of Chicago Press, Chicago, 1967), p. 291.
7. Feldman, J. F., *Proc. Nat. Acad. Sci. USA*, **57**, 1080 (1967).
8. Van Den Driessche, T., *Biochim. Biophys. Acta*, **126**, 456 (1966).
9. Pye, K., and B. Chance, *Proc. Nat. Acad. Sci. USA*, **55**, 888 (1966).
10. Chance, B., R. W. Estabrook, and A. Ghosh, *Proc. Nat. Acad. Sci. USA*, **51**, 1244 (1964).
11. Pittendrigh, C. S., *Proc. Nat. Acad. Sci. USA*, **58**, 1762 (1967).
12. Bünning, E., *Jahrb. Wiss. Bot.*, **77**, 283 (1932).
13. Rensing, L., *Zool. Anz. (Suppl.)*, **32**, 298 (1969).
14. Karakashian, M., *J. Cell Physiol.*, **71**, 197 (1968).
15. Pittendrigh, C. S., V. G. Bruce, N. S. Rosensweig, and M. L. Rubin, *Nature*, **184**, 169 (1959).
16. Sussman, A. S., R. J. Lowry, and T. Durkee, *Amer. J. Botany*, **51**, 243 (1964).
17. Sargent, M. L., W. R. Briggs, and D. O. Woodward, *Plant Physiol.*, **41**, 1343 (1966).
18. Feldman, J. F., *Proceedings of the International Symposium on Biochronology* (National Academy of Sciences, Washington, D.C., in press).
19. Lewis, E. B., and F. Bacher, *Drosophila Information Service*, **43**, 193 (1968).
20. Zimmerman, W. F., C. S. Pittendrigh, and T. Pavlidis, *J. Insect Physiol.*, **14**, 669 (1968).
21. Frank, K. D., and W. F. Zimmerman, *Science*, **163**, 688 (1969).
22. Merriam, J., *Drosophila Information Service*, **44**, 101 (1961).
23. Pittendrigh, C. S., *Z. Pflanzenphysiol.*, **54**, 275 (1966).
24. Roberts, S., *Science*, **124**, 172 (1956).
25. Medioni, J., *Nachr. Akad. Wiss. Göttingen, Math.-Phys. Kl.*, p 117 (1967).
26. Hardeland, R., and G. Strange, *J. Insect Physiol.*, **17**, 427 (1971).
27. Pittendrigh, C. S., and S. Skopik, *Proc. Nat. Acad. Sci. USA*, **65**, 500 (1970).
28. Bridges, C. B., *J. Hered.*, **29**, 11 (1938).
29. Lindsley, D. L., and E. H. Grell, *Genetic Variations of Drosophila melanogaster*, Carnegie Inst. of Wash. Publ. No. 627 (1967).