# THE GENETICS OF LEVAMISOLE RESISTANCE IN THE NEMATODE *CAENORHABDITIS ELEGANS*

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ABSTRACT

We have characterized a small group of genes (13 loci) in the nematode *Caenorhabditis elegans* that, when mutated, confer resistance to the potent anthelmintic levamisole. Mutants at the 7 loci conferring the most extreme resistance generally possess almost identical visible and pharmacological phenotypes: uncoordinated motor behavior, most severe in early larval life, extreme resistance to cholinergic agonists and sensitivity to hypo-osmotic shock. Mutants with exceptional phenotypes suggest possible functions for several of the resistance loci. The most extreme mutants can readily be selected by their drug resistance (211 mutants, as many as 74 alleles of one gene). The more common resistance loci are likely to be unessential genes, while loci identified by only a few alleles may be essential genes or genes conferring resistance only when mutated in a special way. We propose that these mutants represent a favorable system for understanding how a small group of related genes function in a simple animal. The extreme drug resistance of these mutants makes them useful tools for the genetic manipulation of *C. elegans*. And, as the most resistant class of mutants might lack pharmacologically functional acetylcholine receptors (LEWIS *et al.* 1980), these mutants may also be of some neurobiological significance.

IN this paper, we describe a system in which mutants in a small set of related genes in the nematode *Caenorhabditis elegans* may be exhaustively obtained by drug-resistant selection. The process affected is a discrete and dispensable neurological function needed for coordinated motor behavior and proper osmoregulation. Because the selective agent, levamisole, is a stereospecific drug active at low concentration and suitable for tritium labeling, the affected gene products may be identifiable through a drug binding assay. The possibility (LEWIS *et al* 1980) that the resistance genes specify an acetylcholine receptor, an important regulator of ion flow and a participant in specific cell-cell contacts in nervous systems, makes the mutants especially interesting to study.

The more common levamisole-resistant mutants and a prototypical method for their selection were first described by BRENNER (1974). The mutants fall into three general classes: resistant *unc* mutants, pseudo-wild types and twitchers. Mutants in any one class are grossly phenotypically identical and can be reliably told apart only by complementation and linkage tests. Resistant *unc* mutants all have the same stereotyped mildly uncoordinated motor behavior as adults,

whether grown on regular or 1 mM levamisole plates. These mutants are extremely resistant upon naive presentation to levamisole or to cholinergic agonists mimicking the putative nematode neurotransmitter acetylcholine (LEWIS *et al.* 1980). The mutants are also sensitive to hypo-osmotic shock. Pseudowild types are mutants with wild-type osmotic sensitivity and reasonably wild-type motor behavior on regular plates, but are several times more resistant to levamisole and cholinergic agonists (LEWIS *et al.* 1980). When placed on 1 mM levamisole plates, pseudo-wild types initially contract, some mutants quite strongly, but after a number of hours gain the resistant *unc* phenotype, ostensibly because of a secondary cholinergic blocking action by levamisole. Twitchers are mutants with spasmodic muscle twitching superimposed on normal movement (BRENNER 1974), are partially resistant to levamisole and cholinergic agonists and, unlike resistant *unc* mutants and pseudo-wild types, are also resistant to the noncholinergic muscle agonist ouabain (LEWIS *et al.* 1980). The overlap in complementation groups and phenotypic traits between resistant *unc* mutants and pseudo-wild types indicate that these two classes probably correspond largely to mutants severely and partially deficient in the function fatally stimulated by levamisole, possibly a cholinergic muscle receptor, while the twitcher mutant type is likely to arise from partial deficiencies physiologically downstream from the levamisole-sensitive function (LEWIS *et al.* 1980). Our present work describes the isolation and genetic characterization of more than 250 of these mutants, with particular attention given to mutants of unusual phenotype of possible use in determining the function of individual resistance genes.

## MATERIALS AND METHODS

*Nematode strains:* General methods for the maintenance and genetic manipulation of *C. elegans* have been described by BRENNER (1974). X strains were isolated in our laboratory, and E strains originated from the M. R. C. Laboratory, Cambridge, England, and are largely those isolated and described by BRENNER (1974). Strains *unc-63(b404)* and *fer-?(b26ts)* were generously provided by D. HIRSH.

*Drugs and drug testing:* All drugs and their usage in the cut-worm pharmacological assay and their incorporation into growth media are as described in LEWIS *et al.* (1980).

*Isolation of resistant mutants:* Most mutants were isolated as follows: 20 adult wild-type hermaphrodites mutagenized with ethyl methanesulfonate (EMS; BRENNER 1974) were placed on a large (100 × 15 mm) Petri plate to be screened. Just as the plate was filled with second generation ($F_2$) larvae, we transferred the progeny to one side of a fresh plate containing 1 mM levamisole. After a few days, several resistant worms were picked from each plate, and one fertile strain was saved for further study.

In some instances worms and debris were also transferred by a paper strip from a levamisole-containing plate to regular medium. After a day or more of recovery at 16°, uncoordinated worms were picked and tested for immediate resistance to 1 mM levamisole. Any isolate failing to complement a strain picked in the initial screening of a selection plate was discarded. Most of the twitcher mutants and an *unc-68* allele were isolated in this way.

*Complementation:* Levamisole-resistant *uncs*, twitchers and *unc-68* were complemented simultaneously by scoring outcross males for visible phenotype and for resistance to muscle contraction within the first few minutes after transfer to 1 mM levamisole. Pseudo-wild type mutants were mated on regular medium. The following day, the mated parental hermaphrodites were transferred to 1 mM levamisole plates, and after several days the resistance of the outcross males was scored.

*Mapping:* Mutants were mapped at 20° as described by BRENNER (1974). Uncoordinated mutants were generally mapped by visible phenotype. Pseudo-wild type mutants, reliably distinguished from the wild type only by their levamisole resistance, were generally mapped by picking $F_2$ adult progeny to 1 mM levamisole and scoring resistance after several hours to overnight exposure to the drug.

The ability to select recombinants with levamisole proved to be a powerful genetic tool. Double mutants of a levamisole-resistant mutant with *unc* or *dpy* (dumpy) marker mutants could easily be constructed by picking adult $F_2$ homozygotes of the marker phenotype segregating from a *trans* double heterozygote. As many as 20 adults could be put on a small (60 × 15 mm) levamisole plate. The levamisole rapidly killed the parental animals and severely inhibited the growth of any nonresistant progeny hatching from the eggs of the dead animals. After a period of growth, a plate with the desired recombinant was usually detected simply by the quantity of worms it contained. Because the method probably selects among only the limited number of already fertilized eggs in the transferred animals and because there can be more than one recombination event per plate, it was considered only an approximate method of mapping.

More exact mapping with levamisole was done by selecting resistant recombinant progeny segregating at 20° from a *cis* double heterozygote of the resistance mutation and a marker mutation. *Cis* doubly heterozygous L3 to L4 stage hermaphrodite larvae produced by mating N2 males to a doubly homozygous hermaphrodite were placed 3 to an NG plate gridded with bacteria. After a day, the animals were transferred to another set of plates to avoid overlap of generations. Three days after the parental worms were first placed on a set of plates, 7 drops from a Pasteur pipette of 0.05 M levamisole in M9 buffer were distributed on each plate (final concentration ~ 1 mM). Two days after levamisole addition, a set of plates was screened for resistant recombinants. Putative recombinants were transferred to individual plates and their resistant recombinat identity confirmed by progeny testing. The total population of worms from which recombinants arose was estimated by counting all of the progeny of 5 *cis* double heterozygotes similarly transferred and extrapolating. Recombinants from doubles containing *dpy-5* could easily be recognized by their wild-type morphology. In selection experiments with *unc* marker mutations, the singly homozygous resistant recombinants were usually easily recognizable among the background of resistant double mutants because they were much larger and more coordinated. Control experiments with adults of levamisole-resistant *unc-38*, *unc-63* and *unc-74* demonstrated more than 99% survival (97% for *unc-38*) of animals treated with 0.05 M levamisole. The same procedures were used to pick levamisole-resistant recombinants in ordering uncoordinated and pseudo-wild type loci with respect to outside markers.

*Testing of chemosensory behavior:* Attraction to sodium chloride was determined by the orientation assay of WARD (1973), as modified by LEWIS and HODGKIN (1977), after adapting the worms to low ionic strength.

*Larval motor behavior:* Synchronously developing larvae of the wild-type and mutant strains hatched over a 1- to 2-hr period at 20° were obtained by the egg wash method (CASSADA and RUSSELL 1975; HIRSH, OPPENHEIM and KLASS 1976). The ability to move forward and backward was scored periodically by prodding 10 to 20 larvae with an eyelash or wire pick. The first larval molting period was gauged by observing plates at 100× under a compound microscope and scoring a similar number of larvae for the cessation of pharyngeal beating.

*Testing for hypo-osmotic shock:* Strains were picked from growth plates to 0.1 ml of 10 mM HEPES, pH 7.0, 0.25% Tween 20 (WARD 1973) in microtiter wells. Ten to 20 worms were transferred per well and observed periodically. Affected worms usually showed loss of coordination or paralysis within a few minutes and began recovering after 20 to 30 minutes. The fraction of worms of a strain sensitive to osmotic shock is given as the maximal percent showing loss of motility during the first 20 minutes in shock buffer.

*Mapping data:* Strains used in mapping were *unc-6(e78)*, *unc-11(e47)*, *unc-13(e450)*, *unc-21(e330)*, *unc-23(e324)*, *unc-26(e205)*, *unc-29(e1072)*, *unc-30(e191)*, *unc-38(x20)*, *unc-40 (e271)*, *unc-54(e190)*, *unc-57(e406)*, *unc-59(e261)*, *unc-63(x18)*, *unc-68(x14)*, *unc-74(x19)*, *lev-1(x21)*, *lev-1(x22)*, *lev-7(x13)*, *lev-8(x15)*, *lev-9(x16)*, *lev-10(x17)*, *lev-11(x12)*, *dpy-4*

*(e1166), dpy-5(e61), dpy-6(e14), dpy-10(e128), dpy-11(e224), dpy-13(e184), dpy-18(e364) and lon-2(e678).*

*unc-29* mapped somewhat closer to *dpy-5* than the 6.1% determined by BRENNER (1974). Segregants from *dpy-5 unc-29/++*:814 wild type, 23 Dpy, 16 Unc, 229 Dpy Unc, $p = 3.7\%$. Ordering: 3 of 10 Dpy and 5 of 10 Unc-29 recombinants segregated *unc-13*. *unc-21* failed to complement *unc-29* and is henceforth considered an *unc-29* allele.

unc-38, unc-63 *and* unc-74: Recombination distances from *dpy-5* are given in Table 2. Recombination distances from *unc-57*: Unc-57 segregants of + *unc-57*/resistant *unc* + were scored for levamisole-resistant progeny (Lev). For *unc-38*, 14 of 1016 *unc-57* hermaphrodites gave Lev progeny, $p = 0.69\%$; for *unc-63*, 19 of 1642, $p = 0.58\%$; for *unc-74*, 14 of 1284, $p = 0.55\%$. Ordering with ++ *unc-13 trans* to resistant *unc dpy-5* + : for *unc-38*, 6 of 6 Lev recombinants segregated *unc-13*; for *unc-63*, 6 of 6; for *unc-74*, 10 of 10. Ordering with ++ *dpy-5 trans* to resistant *unc unc-57* + : for *unc-38*, 0 of 6 Lev recombinants segregated *dpy-5*; for *unc-63*, 0 of 7; for *unc-74*, 8 of 8. Ordering with + + *dpy-5 trans* to *unc-11 unc-74* + : 0 of 9 Lev recombinants segregated *dpy-5*.

unc-40: Ordering with + *dpy-5* + *trans* to resistant *unc* + *unc-40* : for *unc-38*, 9 of 13 Lev recombinants segregated *dpy-5*; for *unc-63*, 4 of 5; for *unc-74*, 16 of 17.

unc-68: Segregants from *unc-68 dpy-11/++* : 885 wild type, 1 Dpy, 2 Unc, 207 Dpy Unc, $p = 0.3\%$. Five of 5 Dpy recombinants from ++ *unc-23/dpy-11 unc-68* + segregated *unc-23*.

*lev-1:* Segregants from *lev-1 dpy-13/++* : for *lev-1(x21)*, 836 wild type, 69 Lev, 63 Dpy, 207 Lev Dpy, $p = 11.7\%$; for *lev-1(x22)*, 807 wild type, 56 Lev, 65 Dpy, 222 Lev Dpy, $p = 11.1\%$. Lev recombinants from ++ *dpy-4/unc-26 lev-1* + : for *x21*, 9 of 10 failed to segregate *dpy*, 1 of 10 segregated neither *dpy* nor *unc-26*; for *x22*, 13 of 14 failed to segregate *dpy*, 3 of these also failed to segregate *unc-26*, the other 1 of 14 segregated *dpy* and *unc-26*. Lev recombinants from *unc-30* ++/+ *lev-1 dpy-4*: for *x21*, 0 of 25 segregated *unc-30*; for *x22*, 0 of 20 segregated *unc-30*, 4 of these also did not segregate *dpy-4*.

lev-7: Dpy segregants of *lev-7* +/+ *dpy* were scored for levamisole resistance. For *dpy-5 I*, 7 of 60 were Lev; for *dpy-10 II*, 19 of 82; for *dpy-18 III*, 16 of 59; for *dpy-13 IV*, 10 of 33; for *dpy-11 V*, 11 of 58. *lev-7* was not sex linked. Nonparalyzed segregants of *lev-7 unc-54/++*: 825 wild type, 134 Lev, $p \sim 24\%$. Segregants from *lev-7 lev-11/++*: 255 wild type, 52 Lev-7 44 Lev-11,, 50 Lev-7 Lev-11, $p \sim 28\%$.

lev-8: Wild-type males were mated to *lev-8* +/+ *unc-6 X* hermaphrodites and progeny males scored: 530 Lev, 44 wild type, 29 Lev Unc, 401 Unc, $p = 7.3\%$. Ordering: wild-type males were mated to *lon-2 unc-6* +/++ *lev-8 X* hermaphrodites and recombinant progeny males scored: 7 of 7 *lon-2* and 0 of 6 *unc-6* males were Lev.

lev-9: Unc segregants of *lev-9* +/+ *unc-6* were tested for levamisole resistance: 1 of 299 was Lev, $p = 0.2\%$. Ordering: 17 of 17 Lev recombinants from + *lev-9 unc-6/dpy-6* ++ segregated *dpy-6*.

lev-10: Nonparalyzed segregants from *lev-10 unc-54/++* were scored for levamisole resistance: 993 wild type, 31 Lev, $p = 4.7\%$. Zero of 13 Lev recombinants from + *lev-10 unc-54/ unc-59* ++ segregated *unc-59*.

lev-11: Segregants from *lev-10 lev-11/++*: 778 wild-type, 11 Lev-10, 26 Lev-11, 239 Lev-10 Lev-11, $p = 3.6\%$. Sixteen of 24 Lev-10 recombinants from *lev-10* + *unc-54/*+ *lev-11* + segregated *lev-11*.

## RESULTS AND DISCUSSION

### *Selection of levamisole-resistant mutants*

*The effect of levamisole on the wild-type worm:* We have studied the effect of levamisole on the wild-type *C. elegans* to design a good mutant selection procedure and to understand better the drug itself. We placed wild-type adult hermaphrodites on plates of growth media containing levamisole at 10-fold con-

centration increments from 1 μM to 1 mM. Levamisole has a noticeable contractile effect on intact wild type down to 10 μM concentration. One μM levamisole has no significant effect on the intact worm, but severely contracts cut worms with 25 min, suggesting the cuticle and/or metabolic degradation confer resistance to low levels of levamisole.

On 1 mM levamisole, adult worms show signs of muscle contraction within seconds, particularly at the tip of the head. Levamisole is thus distinguished in effect from cholinesterase inhibitors such as aldicarb and trichlorfon that at equimolar concentration take many minutes to affect the wild type and that contract the body before the head. Levamisole-poisoned adults crudely resemble levamisole-resistant *unc* mutants in motor behavior, which we interpret to mean that levamisole as an agonist interferes with the same function that is ablated to confer resistance. Within 30 min on 1 mM levamisole, adults relax into motionless rods. Adults removed from levamisole while still in the process of contraction usually recover, while relaxed worms are invariably dead. Scoring for relaxation is a reliable and rapid way of distinguishing even modestly levamisole-resistant strains from the wild type. Death may be caused by metabolic exhaustion or the crushing force of contraction.

Surprisingly, the younger the stage of the worm, the better it survives initial exposure to 1 mM levamisole. Almost all newly hatched first-stage juveniles survive and recover considerably from contraction (64 out of 76 survive to adulthood on levamisole). They still remain hypercontracted and grow up as stunted, dumpy, uncoordinated animals with gross disproportions and cuticular defects reminiscent of variable mutants (Brenner 1974). Such deformed animals removed from levamisole as adults never fully recover the wild-type morphology. Since these defects are not seen in any of the *unc* or more resistant pseudo-wild type mutants, levamisole probably secondarily induces these morphological and cuticular defects through its effect on muscle contraction. Neuromuscular abnormality may thus be a possible primary cause of morphological or cuticular defects in some mutants, *e.g.*, some dumpy mutants.

At 0.1 mM concentration, the biphasic mode of action of levamisole is more apparent. Most adults survive the strong initial contraction on levamisole, recover most of their body length and motor activity overnight, and over several days begin to resemble the levamisole-resistant *unc* mutants in motor behavior. This latter, progressive loss of levamisole response in the adult animal could be an age-related or drug-induced decline in the physical presence of the levamisole-sensitive function in the worm. Adults grown from larvae on 0.1 mM levamisole never outgrow the dumpiness and uncoordination induced by development on the drug.

Brenner (1974) had used 0.1 mM d,l-tetramisole to select resistant mutants, roughly equivalent to 0.05 mM levamisole(l-tetramisole), the isomer in which most tetramisole activity resides (Raeymaekers, Roevens and Janssen 1967; Bullock, Hand and Waletsky 1968; Lewis *et al.* 1980). We found that 0.1 mM levamisole, while it caused marked contraction and uncoordination of the wild type, did not strongly select the most resistant mutants from the wild type

and more weakly resistant mutants. One mM levamisole markedly affected size, morphology and mobility of the wild type, while having slight effect on the most resistant mutants, *e.g.*, *unc-29(e1072)*, and was the concentration used by us in our selection.

*Mutant selection:* Initially, we blindly picked animals growing on 1 mM levamisole. We isolated 524 strains, 173 of which on retesting proved to be significantly more resistant than the wild type. We complemented all of those that were uncoordinated or showed little contraction on transfer from regular medium to 1 mM levamisole (92 resistant *unc* mutants, selection A, Table 1). In selection B, we deliberately looked for the largest, best moving animals on a selection plate. We isolated 132 resistant strains, actually discarding a number of pseudo-wild type strains, whose significance we did not appreciate. Extremely resistant strains were again complemented (109 resistant *unc* mutants, selection B, Table

TABLE 1

*Isolation of levamisole-resistant mutants by complementation group*

| | Selection A | Selection B | Selection C | Row total |
|---|---|---|---|---|
| Resistant *unc* mutants | | | | |
| *unc-29* | 34 | 36 | 4 | 74 |
| *unc-63* | 24 | 33 | 1 | 58 |
| *unc-38* | 22 | 22 | 0 | 44 |
| *unc-74* | 9 | 15 | 3 | 27 |
| *unc-50* | 1 | 2 | 2 | 5 |
| *lev-1* | 1 | 1 | 0 | 2 |
| *lev-7* | 1 | 0 | 0 | 1 |
| | 92 | 109 | 10 | 211 |
| Pseudo-wild types | | | | |
| *lev-1* | 4 | 9 | | 13 |
| *unc-29* | 1 | 1 | | 2 |
| *unc-63* | 0 | 1 | | 1 |
| *unc-38* | 0 | 3 | | 3 |
| *unc-74* | 1 | 0 | | 1 |
| *lev-8* | 0 | 1 | | 1 |
| *lev-9* | 2 | 1 | | 3 |
| *lev-10* | 1 | 0 | | 1 |
| Not identified | 4 | 0 | | 4 |
| Total complemented | 13 | 16 | | 29 |
| Total isolated | 80 | 19* | 1 | 100 |
| Twitchers | | | | |
| *unc-22* | 1 | 3 | 12 | 16 |
| *lev-11* | 0 | 1 | 0 | 1 |
| Anomalous *unc* | | | | |
| *unc-68* | 0 | 0 | 1 | 1 |

* In selection B, a conscious effort was made to avoid picking pseudo-wild types, which are less resistant to levamisole than are resistant *unc* mutants. Some potential pseudo-wild type isolates were also discarded without further characterization.

1). The similar frequencies at which complementation groups occur in selections A and B suggest that the isolation of the extreme resistance loci is not biased by our conscious selection of mutants, as in selection B ($P > 0.89$, $\chi^2$ test).

To avoid overlooking resistant mutants that might not grow or move well on 1 mM levamisole and might remain buried among the mass of sick and dead wild-type worms, worms from selection B plates were transferred to regular plates. After a recovery period, uncoordinated worms were picked and tested for immediate resistance to 1 mM levamisole (selection C, Table 1). With one exception, an *unc-68* mutant resistant only to contraction in its head region, all mutant types in selection C were the same as those found in selections A and B. The relative frequency of *unc-22* twitchers was greatly increased. *unc-22* twitchers are totally paralyzed and their growth much more inhibited than that of the wild type on 1 mM levamisole. Twitchers fare much better than the wild type on 0.1 mM levamisole and can be readily isolated at the lower drug concentration (BRENNER 1974). The muscle spasms of twitchers are greatly exacerbated on 1 mM levamisole compared to 0.1 mM drug or regular medium. Strains with the mildest spontaneous twitching grow better than the wild type or severe twitchers on 0.1 mM levamisole, suggesting that the twitching phenotype confers an advantageous partial resistance at lower levamisole concentration, but becomes a fatal liability if drug agonism pushes it too far. The two *unc-22* twitchers that grew well on 1 mM levamisole were shown by complementation and linkage tests also to contain other resistance alleles. A new twitcher locus, *lev-11*, was discovered comprising only two isolates, one previously picked by visible phenotype. Both isolates survive much better than the wild type and *unc-22* twitchers on 0.1 and 1 mM levamisole, perhaps partly because of their milder twitching phenotype.

The ease with which we found twitchers and resistant *unc* mutants in selection C suggests that we should have discovered any other common resistant mutant that might not have had enough motility or have grown well enough on levamisole to show up in selections A and B. For unknown reasons, selection C was enriched in *unc-50* mutants ($P < 0.02$, binomial test). Several strains having the degenerate, paralyzed phenotype of muscle protein mutants and contracting very slowly on levamisole were also discovered in selection C and discarded.

A mutant of anomalous phenotype was found in selection C. The mutant curiously has an uncoordinated motor behavior resembling that of the resistant *unc* mutants. Unlike the resistant *unc* mutants, resistance to levamisole and cholinergic agonists is confined to the head region and the head is also resistant to ouabain as well (LEWIS *et al.* 1980). The mutant does not survive on 1 mM levamisole (the wild types does). By complementation and linkage, the mutant was shown to be an *unc-68 V* allele (*x14*). The canonical *unc-68* mutant, E540, has similar motor behavior and pharmacological properties, as does a third non-complementing mutant *unc-68(x24)* picked for its resistant *unc*-like motor behavior. *unc-68* alleles occur frequently in BRENNER's (1974) collection of visible mutants, so that the rarity of this mutant type in our selection is probably due to its tissue-restricted resistance. Direct application of the selection C approach, *i.e.*,

picking putative $F_2$ mutant homozygotes from regular plates to levamisole and scoring for immediate resistance, might identify other such partially resistant mutant types.

The resistant mutants in selection A were isolated at 25° and those in selection B and C at 16°. Those isolated at one temperature were tested after at least one generation of growth at the opposite temperature for visible phenotype, immediate response to levamisole and viability on both plain and levamisole-containing plates. From comparison of the relative frequency of resistant *unc* mutants in selections A and B (Table 1), temperature of isolation does not seem to be an important variable. Ten mutants with some temperature-sensitive resistance were discovered. For none of these strains was the transition on temperature shift from wild-type sensitivity to full resistance. Transitions from wild type to pseudo-wild type resistance and from pseudo-wild type to extreme *unc* resistance were observed. Possibly, the difference in function between the wild type and the resistant *unc* mutants is too great to be easily bridged by a temperature-sensitive mutation. About 27 of the 154 strains isolated in selections B and C contained temperature-sensitive lethals when initially isolated. In every case, a viable resistance allele could be segregated from the locus giving *ts* lethality.

The growth selection method isolates resistant mutants in high frequency, as expected from the several thousand $F_1$ progeny produced per selection plate, an EMS- induced forward mutation rate of $5 \times 10^{-4}$ per locus (BRENNER 1974), and the five or so loci commonly conferring levamisole resistance. In selection B, a fertile levamisole-resistant *unc* or pseudo-wild type strain could be found on 123 of 145 selection plates started from 20 mutagenized hermaphrodites and on four of 15 plates started from two mutagenized hermaphrodites, indicating about two independent resistance mutations occurred per plate. The occurrence of more than one mutational event per plate was confirmed by showing that six of eight pairs of mutants originating from the same selection plate complemented. When spontaneous mutants were looked for, one resistant mutant was found among 35 plates, each started from 20 unmutagenized hermaphrodites. Levamisole resistance selection thus appears to be a convenient and sensitive way of determining the forward mutation rate of *C. elegans* under various conditions. Further testimony to the power of levamisole selection is our estimate that it took only several man-weeks to isolate our 328 resistant strains.

*Complementation:* Resistant *unc* mutants were generally complemented against the various *unc* resistance loci according to frequency of occurrence and were always shown to complement at least one other locus. Tester strains *unc-29 (e1072)*, *unc-63(x18)*, *unc-38(x20)*, *unc-74(x19)*, *unc-50(e306)*, *lev-1(x21)* and *lev-7(x13)* and all strains tested complemented each other cleanly and were recessive to the wild type with respect to motor behavior and immediate resistance to 1 mM levamisole. In most instances, failure to complement could be determined either from the uncoordinated motor behavior or the drug resistance of the outcross progeny males. In rare instances, either the uncoordination [*e.g.*, *unc-29 (x52)*] or drug resistance [*e.g.*, *unc-29(x27)*] of the strain to be complemented

was decidedly stronger and was also more strongly expressed in the progeny produced from the mating to the noncomplementing locus.

Only a sample of the 99 pseudo-wild types isolated were complemented. Of 13 isolates from selection A and 16 isolates from selection B, a total of 25 were assigned to complementation groups (Table 1), three others being too leaky to identify and a fourth possibly identifying a 14th and yet-to-be-characterized resistance locus. The most frequent and most resistant pseudo-wild type alleles occur in *lev-1*. These alleles are at the same genetic locus producing rare *unc* alleles of *lev-1*, as determined by complementation and linkage tests. A slight ambiguity exists because both *unc* alleles of *lev-1* are semidominant in the growth test used for pseudo-wild type complementation. No other levamisole resistance alleles have significant dominance on 1 mM levamisole. *lev-1* pseudo-wild type alleles are the only *unc* or pseudo-wild type alleles clearly failing to complement the uncoordinated allele *lev-1(x21)* by a levamisole growth test. All *lev-1* pseudo-wild type alleles were subsequently identified by failure to complement the pseudo-wild type allele *lev-1(x22)*, which lacks the complication of semidominance. We estimate, making allowances for the fraction of pseudo-wild types complemented and the ones discarded in selection B, that *lev-1* pseudo-wild types occur as frequently as the most common *unc* resistance loci. *lev-1 (x22)* fails to complement *e211*, identifying the *tms-1* locus described by BRENNER (1974), a locus also occurring frequently in BRENNER's more limited study.

Most of the other pseudo-wild type alleles seem to be leaky alleles of *unc* resistance loci (Table 1). Pseudo-wild type alleles of *unc-50* and *lev-7* may occur at too low a frequency to be found in our limited sample. The leakiness of pseudo-wild type alleles is corroborated by several weakly temperature-sensitive isolates that are closer to a pseudo-wild type phenotype at 16° and more like a resistant *unc* in motor behavior and levamisole resistance at 25° (*e.g.*, X26, X30 and X39). Pseudo-wild type alleles *unc-29(x63) I*, *unc-63(x26) I* and *lev-1(x22) IV* were each shown to be on the same linkage group as the "tight" alleles of these loci. *unc-63* was shown to be about the same map distance from *dpy-5 I* as the canonical *unc-63* allele *x18*, less than one map unit ($P < 0.05$, Poisson). Furthermore, spontaneous and closely linked reversions of the *lev-1(x21)*, *lev-1(x61)* and *unc-29(e1072)* uncoordinated mutants convert these strains to the pseudo-wild type phenotype.

Three pseudo-wild type loci, *lev-8*, *lev-9* and *lev-10*, have been discovered for which there are no counterpart uncoordinated isolates. Their linkage positions on the sex chromosome and on LG I close to *unc-54* confirm their distinctness from the levamisole-resistant *unc* loci.

*Mapping:* Our map of the levamisole resistance loci is shown in Figure 1. We showed that *unc-38*, *unc-63* and *unc-74* all occur within about one map unit of each other on LG I, unusually close together considering the 100 or more units of the *C. elegans* map in which mutations are commonly located (Figure 2). MOERMAN and BAILLIE (1979) and R. WATERSTON (personal communication) have shown by intragenic recombination that the size of the *unc-22* and *unc-54* genes, respectively, is about 0.01 map units. The three resistant *unc* loci appear

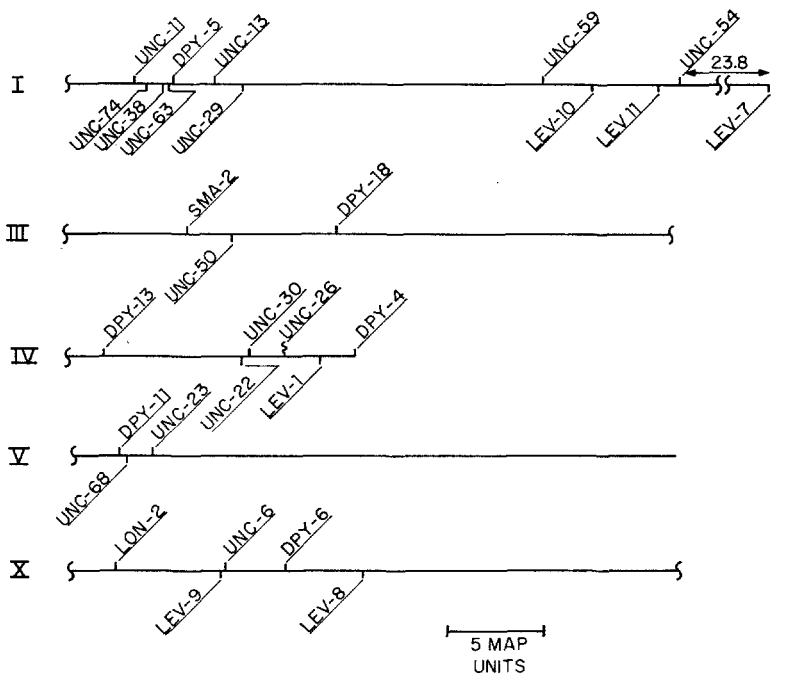


FIGURE 1.—Map of levamisole-resistance loci. The levamisole-resistance loci (below lines) were mapped with respect to nearby markers (above lines). The map positions of *unc-22* and *unc-50* are those determined by BRENNER (1974). The map position of *lev-7 I* was determined by its relative linkage to *dpy-5*, *unc-54* and *lev-11*. Linkage group II is not shown.

to be separated by distances greater than 0.1 map unit (Table 2). Two of the resistance loci are separated by *unc-57*, a locus having no special levamisole resistance properties and mapping roughly halfway between the two resistance loci. *unc-57*, in addition to its spastic uncoordinated phenotype, contracts when hit on the head. The contractile response is substantially blocked in double-mutants with the *unc-74*, *unc-38* and *unc-63* resistance loci (and also by combination with

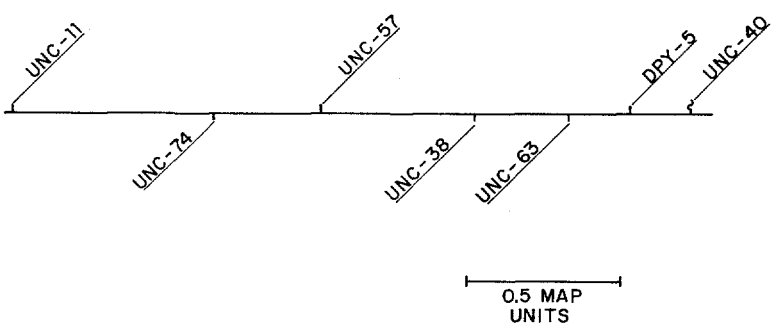


FIGURE 2.—High resolution map of the region of the *unc-74*, *unc-38* and *unc-63* loci. The resistance loci were mapped as described in MATERIALS AND METHODS. The order shown is unambiguous, except that *unc-38* and *unc-63* were not ordered relative to each other.

TABLE 2

*Linkage of* unc-74, unc-38, *and* unc-63 *to* dpy-5

| Locus | Mean offspring of untreated *unc dpy-5 cis* heterozygotes ± SEM | Estimated offspring selected against (no. of parents) | Resistant recombinants | Map units from *dpy-5* | 95% Confidence limits of map position |
|---|---|---|---|---|---|
| *unc-74* | 308 ± 16.1 | 20,328(66) | 137 | 1.36 | 1.68, 1.08 |
| *unc-38* | 275 ± 19.7 | 17,325(63) | 44 | 0.51 | 0.70, 0.36 |
| *unc-63* | 297 ± 22.5 | 19,899(67) | 20 | 0.20 | 0.31, 0.12 |

The map distances of *unc-74*, *unc-38* and *unc-63* from *dpy-5* were determined as described in MATERIALS AND METHODS. The 95% confidence limits of map position allow for the uncertainty in both the number of recombinants obtained and the estimate of total offspring selected against and were calculated by the method of fractional error.

the *unc-29* resistance locus; LEWIS *et al.* 1980). Although the three resistance loci and *unc-57* are apparently not contiguous genes, it will be interesting to see if their proximity has any developmental or functional signifiance.

*lev-11* maps close enough to the myosin gene *unc-54* (1.6 map units) to be a useful marker. Both *lev-11* alleles *x1* and *x12* are synthetic near-lethals when homozygous with *unc-54(e190)*, but R. WATERSTON (personal communication) has found this useful in balancing one *unc-54* allele over another for fine-structure mapping.

*lev-7*, identified by only one isolate, is weakly linked to *dpy-5* and roughly 25 map units from *unc-54* and *lev-11* (without correcting for multiple recombination events). *lev-7* thus appears to be the most distal marker known for the right half of LG I.

The *unc* allele *x21* and the pseudo-wild type allele *x22* were both shown to map to the same region of LG IV between *unc-26(e205)* and *dpy-4(e1166)* and at the same distance from *dpy-13(e184)*. The mapping supports the conclusion that *x21* and *x22* are in the same gene, for which there was some ambiguity in complementation. The apparent frequency of recombination between *unc-26* and both resistance alleles, easily more than 8 map units, is a discrepancy between the accepted map and our data ($P < 0.05$, binomial). Other mapping anomalies have been noted in this region (D. L. RIDDLE, personal communication) and further clarification would be desirable.

*Properties of levamisole-resistant mutants*

In a separate paper (LEWIS *et al* 1980), we presented the general pharmacological properties of levamisole-resistant mutants and our reasons for concluding that these mutants might affect the function of a cholinergic muscle receptor (resistant *unc* mutants and pseudo-wild types) or other processes of muscle contraction (twitchers). In this paper, we describe more fully other characteristics of these mutants, including phenotypic properties that distinguish the resistance loci from each other.

*General properties of levamisole-resistant* unc *mutants:* Apart from their pharmacological phenotype, the most obvious thing wrong with resistant *unc* mutants

is a discrete, rather mild defect in their motor behavior as adults. Otherwise, the mutants, with exceptions to be noted, have good vitality. Like other EMS-derived strains not extensively backcrossed to the wild type, *unc* mutants generally grow slightly more slowly than the wild type and have somewhat fewer progeny, judging by the number of parental hermaphrodites that can be placed on a 100 mm Petri plate without overloading the plate with offspring (about 10 wild type *vs.* 15 to 20 mutant parents) and the number of days at 20° before most progeny mature (about three and one-half days for the wild type, slightly over four days for most mutants).

Mutants at all seven resistant *unc* loci (the complementation tester strains) are functional in several forms of sensory behavior: chemosensation, thermosensation, tactile response and male mating behavior. The chemosensory orientation response of the mutants to sodium chloride is strong, but somewhat less rapid and less efficient than that of the wild type. The mutants also tend to remain within the borders of bacterial lawns as does the wild type. The same representative mutants, like the wild type, flee a hot wire pick and startle when stroked with an eyelash. Homozygous male strains of these *unc* mutants and of all other resistance loci except twitchers are not difficult to maintain, showing that male sexual capability is not grossly impaired. Homozygous *unc* males, however, attempt copulation with less vigor and agility than wild-type males. Flexure of the male tail is more limited. These sensory behaviors *per se* are not essential to the viability of the wild type (LEWIS and HODGKIN 1977; DUSENBERY, SHERIDAN and RUSSELL 1975; HEDGECOCK and RUSSELL 1975; HODGKIN 1974) and are probably not strongly selected for. Therefore, the resistant *unc* genes are probably not critical to the sensory perception or central integration of the various stimuli. The lack of an effect is consistent with our hypothesis that dysfunction in levamisole-resistant *unc* mutants is chiefly confined to the muscles and that there is redundancy of nervous function (LEWIS *et al.* 1980). The mild reduction in chemosensory response and male sexual behavior is consistent with the loss of a noncritical component of these behaviors.

unc *motor behavior and its developmental aspects:* The defect in *unc* motor behavior is most severe in the anterior part of the adult hermaphrodite when going forward. The mutants tend to push their heads and the forward part of their bodies stiffly ahead of themselves by a slow, decayed sine wave growing out of the latter part of the body. The mutants move backward in a much better sine wave that includes the head, but is still less rapid and fluid than the wild type. The wild type moves forward and backward in a sine wave that freely traverses the length of the animal. When the most uncoordinated *unc* mutants are suspended in liquid (M9 buffer; BRENNER 1974), the stiff head vibrates like a tuning fork, *e.g.*, *unc-29 (e1072)*. The straightening and vibration are probably not caused by strong mutual opposition of anterior body muscles since that should result in observable contraction. Straightening is probably due to lack of muscle function and the inherent rigidity of the worm's body (LEWIS *et al.* 1980). A very small amount of vestigial motor activity could explain the vibration. We find that suspension in liquid is a quite sensitive assay for even weak motor activity. *unc-54* myosin mu-

tants grown on a bacterial surface are barely able to move against surface tension. Yet, when placed in liquid, they generate an obvious sine wave, albeit weaker than the wild type.

The severity of the defect in the anterior part of the body is not a result of the levamisole-sensitive function being confined to this region. Pieces of all parts of the wild-type body (head, tail, midsection, etc.) contract in 1 mM levamisole or carbachol and all parts of *unc-29(e1072)* show resistance. The phenotype suggests that the levamisole-sensitive function in the adult is present and used for both forward and backward motion in all regions of the motor system, but is critical only to forward motion in the anterior part of the body, otherwise being redundant, especially going backward.

In contrast, the entire motor behavior of the first-stage larva is critically dependent on the levamisole-sensitive function, especially for backward motion. Mutants at all seven resistant *unc* loci (complementation tester strains) as first-stage larvae cannot go backward and, when newly hatched, can barely move forward. During the first 10 hours of life at 20°, first-stage larval *unc* behavior going forward graduates into that of the adult. For all seven loci, the ability to go backward is gained in the second larval stage between about 20 to 22 hours post-hatching, after the first-stage mutant molt at 16 to 18 hours. Because of the small size of first-stage larvae (several hundred microns in length), one can readily test the distribution of sensitivity and resistance only on intact animals. As well as can be judged, the entire body of the newly hatched wild-type larva contracts, while the entire body of *unc-29(e1072)* is resistant.

Overall, then, the levamisole-sensitive function seems to be distributed throughout the body of the wild type both early and late in post-embryonic development and seems to be similarly uniformly missing from the mutant. The change in larval *unc* behavior is probably to be explained by the selective addition of another function(s) to parts of the larval motor system. Since our work (Lewis *et al.* 1980) suggested that the defect in resistant *unc* mutants might occur in cholinergic muscle receptor function, the improved phenotype could occur through the addition of new muscle synapses. New motorneurons and muscle cells are generated around the time of the first-stage larval molt when the complexity of the motor system is greatly increased (Sulston and Horvitz 1977), but the time when the new cells form functional synapses is not precisely known. The *unc* head improves little in forward motion and may only passively follow the body in backward motion. In agreement, the complete motor input for the first 16 muscle cells and part of the input for the next 16 muscle cells in the head region comes from the central nervous system of the worm (White *et al.* 1976), a part that at least in cell number does not change in larval development (Sulston and Horvitz 1977).

The resistant *unc* mutants are thus useful for revealing neurological changes that are behaviorally invisible in the wild type. Except for quiescent periods during molts, at all times the wild-type larva moves well forward and backward. With better synchronized larvae and more closely spaced observations, the transition in backward *unc* behavior may be found to be even sharper than observed.

Electron microscopical examination of *unc* larvae during the transition, and at intervals before, might correlate the transition with the formation of certain synapses. An *unc* mutant might be useful as a background strain with which to isolate or identify mutants deficient in the transition process.

*unc osmosensitivity:* Resistant *unc* mutants are generally more sensitive to hypo-osmotic shock than wild type. The sensitivity can be observed by transfer of mutants from growth plates to distilled water, but is clearest when worms are transferred to 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.0, 0.25% TWEEN 20. In this medium, 7% ± 6% (unbiased standard deviation) of 211 wild-type worms show loss of sinusoidal motion within 20 min of transfer (15 tests, 10 to 20 worms per test). Discounting resistant strains with more wild-type motor behavior (to be discussed), on average 79% ± 19% (unbiased standard deviation) of the 20 or more individuals tested for each of 20 backcrossed *unc* strains succumbed to osmotic shock. Hypo-osmotic sensitivity is not a general property of the progeny of mutagenized animals or of mutants with visible phenotypes. Only one of 20 strains started from randomly picked $F_2$ progeny of selfed EMS-mutagenized wild type was osmotically sensitive. Only two of the 55 strains tested having visibly mutant phenotypes were sensitive, and one of these [*unc-29(e1072)*] proved to be a levamisole-resistant *unc*.

The defect is not an absolute inability to osmoregulate, but apparently a defect in the rate of adjustment. For most strains tested, the number of individuals shocked peaks within the first 20 min, and some recovery takes place thereafter among those individuals escaping the greater traumas of osmotic stress, *e.g.*, explosion. The kinetic nature of the phenomenon may help explain why the phenotype is not 100% penetrant, although the characteristics of drug resistance and uncoordinated behavior are. Osmosensitivity, like the other two mutant traits, may be related to cholinergic dysfunction, in this instance in the nervous function of the osmoregulatory apparatus. The exploding phenotype of some mutants, *e.g.*, *unc-29(e1072)*, does not necessarily imply a structural defect, as even the wild type can be made to explode by pharmacological treatment with 3-quinuclidinol (LEWIS *et al.* 1980).

Osmotic shock might be a useful counterselection against the *unc* mutant phenotype. We have found that temperature, detergent and ionic composition affect the severity of shock, and we are presently optimizing conditions for counterselection. We have also shown that osmotic shock permeabilizes *unc-29 (e1072)* sufficiently to allow its use as a parent strain in a mass histochemical screening for enzyme-deficient mutants, *e.g.*, acid phosphatase mutants. A mutant would be rescuable from eggs in a nonstaining carcass.

*Differences in* unc *mutant phenotypes and their possible significance*: Relative differences in the expression of the uncoordinated, drug-resistant and osmosensitive phenotypes may be informative of different structural or regulatory properties encoded in the various levamisole-resistance loci. Osmosensitivity presumably originates in a different mutant tissue from that responsible for uncoordinated behavior and levamisole resistance, which is probably muscle (LEWIS *et al.* 1980). Uncoordinated behavior represents loss of endogenous function, while

levamisole resistance is failure to respond to a synthetic exogenous signal. Mutations affecting tissue-specific gene expression, the constitution and cellular placement required for endogenous function or the existence of a levamisole recognition site could selectively alter the relative severity of the three mutant phenotypes.

The severity of all three mutant characteristics is generally correlated for mutants of all seven *unc* resistance loci. Of 94 unbackcrossed strains screened for all three characteristics, the phenotypic properties of 80 strains were well correlated, 78 strains being severely mutant. Almost all the observed relative phenotypic variability occurs in mutants of the *unc-29*, *unc-63* and *unc-38* loci (81 mutants tested). The uniformly extreme phenotype of *unc-74*, *unc-50*, *lev-1* and *lev-7* mutants may be due only to the more limited number of these *unc* mutants tested (13 mutants). For 12 of the 94 mutants screened, osmosensitivity is poorly correlated with drug resistance and uncoordinated behavior. Six of these strains are *unc-29* mutants.

We were more concerned with strains differing in the expression of drug-resistant and uncoordinated phenotypes. We have compared these properties in 214 mutant strains, mostly our 211 resistant *unc* mutants (Table 3). The properties of strains described by name were studied after resegregating the mutant from a backcross to the wild type and showing that at least 10 to 15 resegregated homozygotes possessed the same properties as the original isolate.

TABLE 3

*Uncoordinated and drug-resistant phenotypes of levamisole-resistant mutants*

| Phenotype | Complementation group *unc-29* | *unc-63* | *unc-38* | | Other loci |
|---|---|---|---|---|---|
| 1. Fully uncoordinated, drug-resistant phenotype | 67 | 53 | 14 | 33 | |
| 2. Uncoordinated phenotype, head susceptible | 1 | 3 | 25 | 1 | —(*unc-50*) |
| 3. More wild type motor behavior, strongly resistant | 5 | 1 | 5* | 0 | |
| 4. Uncoordinated phenotype, head resistant, body susceptible, does not survive on levamisole | 0 | 0 | 0 | 1 | —(*unc-68*) |
| 5. Wild type motor behavior, head resistant, body susceptible survives on levamisole | 0 | 0 | 0 | 1 | —(*lev-8*) |
| 6. Uncoordinated phenotype, poor resistance | 0 | 1† | 0 | 0 | |
| 7. Weak uncoordinated phenotype, poor resistance | 1 | 1 | 0 | 1 | |

The table includes mutants of good uncoordinated and/or levamisole-resistant phenotypes found in selections A, B and C. The designation "other loci" includes *unc-74*, *unc-50*. *lev-1*, *lev-7* and where noted, *unc-68* and *lev-8*. Except for *lev-8*, pseudo-wild types and twitchers are not listed.

* Four of these mutants have heads susceptible to levamisole.

† *unc-63(b404)* isolated by D. HIRSH.

The degree of uncoordinated behavior and levamisole resistance corresponds well for 200 of the 214 strains in Table 3, 197 strains being strongly uncoordinated and levamisole resistant (rows 1 and 2). Most of the variability again resides in *unc-29*, *unc-38* and *unc-63* strains. Strains with uncoordinated behavior not in the range between the wild type and the usual resistant *unc* behavior were always found to contain an extraneous mutation. Since our isolation method favored levamisole resistance, the range of resistance phenotypes may be more limited than that obtainable if mutants were isolated by uncoordinated behavior.

*Very resistant mutants showing more nearly wild-type motor behavior*: Five of 74 *unc-29*, one of 58 *unc-63* and five of 44 *unc-38* mutants show more nearly wild-type motor behavior, although contracting little on one mM levamisole plates as intact worms (row 3, Table 3; Table 4). Two of the *unc-29*, the *unc-63* mutant and three of the *unc-38* strains show almost wild-type osmosensitivity. The mean osmosensitivity of all the resistant, more nearly wild-type behaving strains is 30% ± 25% (unbiased standard deviation), which is very significantly different from the 79% mean osmosensitivity for 20 other segregated resistant *unc* strains ($P \ll 0.001$, Student's $t$ test). When these worms were tested as cut worms, two of the *unc-29*, the *unc-63* mutant and two of the *unc-38* mutants contracted within five minutes in 1 mM levamisole, being several times more resistant than the wild type. The mutants also contracted strongly in 1 mM carbachol. For these mutants, the threshold for levamisole response has apparently been raised, so that the concentration entering the intact worm is not enough to have an effect. Presumably, the threshold for endogenous function is still low enough that the worms have nearly wild-type motor behavior. One of these mutants *unc-29(x39)*, despite its immediate resistance, grows as a spastic,

TABLE 4

*Very resistant mutants showing a more nearly wild-type motor behavior*

| Strain | Osmotic sensitivity, no. sensitive of total | Comments |
|---|---|---|
| Wild type | 1/20 | Contracts within 1 minute in 1 mM levamisole and within 3 minutes in 1 mM carbachol when cut. |
| *unc-29(x30)* | 11/20 | These strains are resistant to 1 mM levamisole as intact worms and are very wild type in movement, but contract within five minutes as cut worms in 1 mM levamisole or carbachol (except that *x30* takes 10 to 20 minutes to contract in carbachol). |
| *unc-29(x39)* | 12/20 | |
| *unc-38(x41)* | 1/20 | |
| *unc-38(x58)* | 1/20 | |
| *unc-63(x26)* | 6/20 | |
| *unc-29(x27)* | 2/20 | Resistant to 1 mM levamisole as both intact and cut worms, bodies contract moderately within first 5 minutes in 1 mM carbachol, less wild type but move freely even when poked. |
| *unc-29(x44)* | 1/20 | |
| *unc-29(x46)* | 12/20 | |
| *unc-38(x43)* | 13/20 | Resistant to 1 mM levamisole as whole and cut worms and to 1 mM carbachol for over 20 minutes when cut, susceptible in head region, motor behavior least like wild type, especially when poked. |
| *unc-38(x45)* | 1/20 | |
| *unc-38(x48)* | 6/20 | |

partially contracted worm on 1 mM levamisole at 15°[1]. The other three *unc-29* mutants and three *unc-38* mutants contract very little as cut worms in 1 mM levamisole. The *unc-29* mutants partially contract in the first few minutes in 1 mM carbachol, while the unc-38 mutants are very resistant (except in their heads, as will be discussed). The motor behavior of the *unc-29* mutants is more nearly wild type and more rapid compared to the *unc-38* mutants, especially when poked.

The more wild-type resistant *unc-29* and *unc-38* mutants might be explained if these genes encoded structural proteins of the levamisole-sensitive function and were selectively altered in levamisole response. These rare alterations are hard to explain if the *unc-29* and *unc-38* products only indirectly promote the levamisole-sensitive function or are physiologically downstream from it. The latter three *unc-29* mutants are the most resistant to levamisole that retain an appreciable wild-type behavior, wild-type osmotic resistance (for X27 and X44) and some response to carbachol. Carbachol is an analog of acetylcholine, a probable endogenous signal in the levamisole-sensitive pathway (LEWIS *et al.* 1980). We think that the *unc-29* phenotype is most like that expected from site-specific changes in the protein binding levamisole (evidence for such a protein is discussed in LEWIS *et al.* 1980). Due to its small size and the phenotype of resistance, levamisole is unlikely to bind more than one nematode protein. The occurrence of more wild-type resistant mutants in *unc-38* suggests a physical interface between the *unc-29* and *unc-38* functions. Alteration of the interface by *unc-38* mutation might make the levamisole response communicated by the *unc-29* peptide less effective than the endogenous signal, or the interface itself could be the binding site for levamisole.

The *unc-63* mutant is one of the least levamisole-resistant in the class of more wild-type mutants. It is the only one of 58 *unc-63* mutants showing the phenotype, while five of 44 *unc-38* mutants are more wild type.

*Head-body differences in* unc-38 *and* lev-8 *mutants*: *unc-38* mutants exhibit a unique variation of the resistant *unc* phenotype. The heads of most *unc-38* mutants initially contract on 1 mM levamisole, although their bodies are strongly resistant. The resistance of the head is pseudo-wild type, as the heads recover on continued exposure and do not have any of the developmental defects caused by growth of the wild type on levamisole. The mutants were tested without knowledge of their complementation group. Twenty-nine of 44 *unc-38* mutants and five of 165 mutants at other loci had susceptible heads, but very resistant bodies. When the *unc-38* mutants X20, X43 and X45 were cut at midsection in 1 mM levamisole or carbachol, their heads rapidly contract, but their bodies do not. Since cutting makes the body of the wild type contract before the head, the *unc-38* phenotype is probably derived from tissue-specific resistance, rather than from differences in drug accessibility in the intact worm.

The pseudo-wild type mutant *lev-8(x15)* has a complementary phenotype of levamisole resistance. The body contracts, but the head does not. The phenotype

[1] We found a mutant in BRENNER's collection (BRENNER 1974) somewhat similar to *unc-29(x39)*. *unc-38(e264)* is uncoordinated and has good immediate 1 mM levamisole resistance, but growth in the presence of levamisole is strongly inhibited. This mutant also accumulates eggs.

is temperature sensitive. At both 15° and 25°, the bodies of adult hermaphrodites are susceptible, but at 25° worms recover well overnight. At 15°, most *lev-8* individuals are still paralyzed the next day and slowly recover over the next few days. Growth on levamisole demonstrates the temperature sensitivity and head-body differences more dramatically. At 25° on 1 mM levamisole, *lev-8* grows like other levamisole-treated pseudo-wild type mutants with resistant *unc* motor behavior, but is spastic in its body movement. At 15° *lev-8* has a hypercontracted, grossly shortened body with a head of normal length having no developmental defects. Animals grown at one temperature and placed as adults on 1 mM levamisole at the other temperature recover at the rate expected for the new temperature. Newly hatched larvae shifted in temperature and placed on levamisole plates grow up with the phenotype expected for the new temperature.

*unc-38* and *lev-8* might encode isozymic forms of the same function. The distribution of resistance in *unc-38* and *lev-8* mutants indicates that their respective functions have complementary, but overlapping, distributions in the head and the body. The double mutant *unc-38(x20) lev-8(x15)* is resistant throughout its head and body. Alternatively, *lev-8* could represent a regulatory or processing element required by the levamisole-sensitive function in the head, but not in the body of the worm. Although we have 44 mutants of *unc-38*, *x15* is the only one of *lev-8* identified. Perhaps *lev-8* is an essential function or otherwise does not usually confer much resistance when mutated.

unc-63 *variants*: Several unusual strains of widely divergent phenotype are known for *unc-63*. The most interesting is B404, isolated by D. HIRSH as a mutant partially resistant to the cholinesterase inhibitor trichlorfon. We find that *b404* fails to complement *unc-63(x18)* and maps within 0.8 units of *dpy-5 I* ($P < 0.05$). The mutant has mild resistant *unc* motor behavior. Adult hermaphrodites contract on 1 mM levamisole, survive, but remain partially contracted even when grown on the drug. No other visibly uncoordinated resistant strain behaves so. Even pseudo-wild types recover much more completely. As a cut worm, B404 is leaky in its resistance to levamisole and to cholinergic agonists, but is relatively more resistant than other worms tested to the effects of esterase inhibitors alone (aldicarb, trichlorfon, eserine), which depend on endogenous acetylcholine release, than to the exogenous effects of levamisole, carbachol or acetylcholine plus an esterase inhibitor. Shown in Table 5 are data on the wild type, *lev-1(x22)* (one of the most resistant pseudo-wild types), B404, X18 and X37 (a moderately leaky and a tight *unc-63* mutant). B404 might be a candidate for a mutant in which the levamisole-sensitive function is made, but not properly constituted for endogenous function. The *unc-63(b404)* phenotype and our failure to obtain a similar mutant among more than 200 uncoordinated mutants (58 *unc-63* isolates) clearly show that levamisole is an inappropriate selective agent for such weakly resistant, uncoordinated mutants. Selection for resistance to a low dose of a cholinesterase inhibitor may be the most straightforward method of isolating other mutants like *unc-63(b404)*.

Three *unc-63* mutants, X33, X40, and X59, have an *unc-38*-like phenotype. They are uncoordinated (especially X59), but contract in the head region on

TABLE 5

*The effect of levamisole and cholinergic agonists on B404 and some other levamisole-resistant strains*

| Strain | Time for contraction, minutes Agonist Lev | Car | Ald | Ac & Ald | Tcf | Ac & Tcf | Es | Ac & Es |
|---|---|---|---|---|---|---|---|---|
| Wild type | 0.3 | 3 | 14 | 5 | 22 | 6 | 13 | 4 |
| *lev-1(x22)* | 2 | 4 | 25 | 7 | 40 | 7 | — | — |
| *unc-63(b404)* | 1 | 6 | 63 | 8 | 120 | 8 | >120 | 6 |
| *unc-63(x18)* | >120 | 20 | >120 | 7 | >120 | 8 | 120 | 8 |
| *unc-63(x37)* | >120 | 56 | >120 | >120 | — | — | — | — |

Ten cut worms of each strain were tested in the indicated agonists at 1 mM concentrations, except aldicarb was used at 2 mM concentration. X22, unlike the wild type, X22 or B404, does not contract very severely in acetylcholine plus an esterase inhibitor. The time at which it is maximally contracted in acetylcholine is shown. The error in the assay is 22% ± 16% (unbiased standard deviation), calculated as the range divided by the mean for 11 duplicate determinations of the wild type and X18, which gave finite contraction times. Abbreviations: Lev, levamisole; Car, carbachol; Ald, aldicarb; Ac, acetylcholine; Tcf, trichlorfon; Es, eserine.

levamisole. When cut at midsection in 1 mM levamisole or carbachol, the heads of these mutants contract strongly, while the body contracts weakly (carbachol) or not at all (levamisole) during the first few minutes in drug solution. These mutants complement *unc-38(x20)*.

*unc-63(26)* identifies a fifth variant previously discussed as being very resistant, while having more nearly wild type motor behavior.

The variety of qualitative differences seen in *unc-63* variants is most likely to be explained by mutation of a structural element, rather than by indirect effects, which would more likely cause simple quantitative differences in levamisole sensitivity. The lack of very resistant mutants with nearly wild type motor behavior would imply that *unc-63* is not a structural unit participating directly in the response to levamisole, as hypothesized for the *unc-29* and *unc-38* functions. It is possible to imagine explanations for the B404 mutant and the mutants with head-body differences in which at least part of the function that *unc-63* subserves is as a structural glue or positioning factor.

*Temperature-sensitive mutants of* unc-63 *and* unc-29: The proposed structural roles of the *unc-29*, *unc-38*, *lev-8* and *unc-63* gene products could be confirmed with the proper temperature-sensitive mutants. A mutant whose adult phenotype could be changed within minutes by temperature shift would surely be affected in a structural element. The most likely examples of such mutants that we have been able to find for *unc-29*, *unc-63* and *lev-8* (already described) undergo some shift in a matter of hours. The more nearly wild-type behaving alleles *unc-29(x30)* and *unc-63(x26)* are partially resistant when grown at 15° and fully resistant at 25°. When grown at 15° and transferred to 25° as adults, individuals of either strain show increased resistance within five hr and full resistance by 17 hr. Worms of either strain grown at 25° and shifted to 15° are still intermediate between the 25° and 15° phenotypes 48 hr after the downshift. The temperature shift results for *unc-29*, *unc-63* and *lev-8* are consistent with

structural roles for these gene products, but could also be explained by indirect effects if the levamisole-sensitive function were rapidly turning over in the adult nematode.

lev-1: We do not think *lev-1* has a direct role in the levamisole-sensitive function. Most *lev-1* alleles are pseudo-wild type, the phenotype associated with partial function (LEWIS *et al.* 1980). *lev-1* pseudo-wild type alleles are estimated to occur roughly at the forward mutation rate of an "average" *C. elegans* gene, suggesting that *lev-1* can be freely mutated without completely destroying the levamisole-sensitive function. The two rare resistant *unc* isolates of *lev-1*, X21 and X61, are semidominant in conferring resistance. The semidominance implies that the *unc* phenotype of these mutants results not from a complete lack of *lev-1* function, but from a negative interaction of these particular mutant gene products with other wild-type functions. Semidominance strictly because of limiting gene dosage is unlikely since alleles of other *unc* loci are more strongly resistant and uncoordinated as homozygotes and not semidominant as heterozygotes, *e.g.*, *unc-29(e1072)*, *unc-38(x20)*.

*Essentiality of resistance loci*: Essentiality of function breaks down into two questions: (1) Are the functions of the individual resistance loci essential to life or motility, and (2) is the levamisole-sensitive function (the physiological process) to which they contribute essential?

The principal evidence on the essentiality of individual resistance loci is their apparent forward mutation rate. Homozygously viable mutations in *unc-29*, *unc-38*, *unc-63*, *unc-74* and *lev-1* can be obtained at a rate consistent with free mutation at these loci, implying nonessentiality. *unc-50* and *lev-7* are isolated at a much lower rate, suggesting essential function. There is only one isolate each of *unc-74* and *unc-50* and none of *lev-7* in the large set of visible mutants isolated by BRENNER (1974). Three of six *unc-50* strains, E306, X35 and X47, are severely debilitated when raised at 25°. *lev-7(x13)* raised at 25° has the unhealthy tendency to accumulate eggs. The twitcher *unc-22* is one of the most commonly occurring visible mutants (BRENNER 1974), and *unc-22* is probably a nonessential gene. Our two isolates of *lev-11* are the only homozygous viable alleles known. P. ANDERSON (personal communication) has isolated a lethal allele of *lev-11*, *e1724*.

In a trial experiment, we have selected new alleles of *unc-29* as heterozygotes by mating mutagenized males to a triple homozygote containing the marker *dpy-5(e61) I* linked to *unc-29(e1072)* with the unlinked *ts* spermless mutation *fer-?(b26)* used to prevent resistant self-cross progeny. Mating 50 EMS-mutagenized males to several hundred *ts* spermless triple mutants per small plate and selecting against nonresistant outcross animals by adding 0.05 M levamisole as in our mapping experiments, a new resistant mutant appears obtainable on at least every other selection plate. The experiments must be done using a marker closer than *dpy-5* and a less extreme allele than *e1072* to draw any proper conclusions about the essentiality of *unc-29*. Nevertheless, our technique seems the method of choice for further study of essentiality and other properties of individual resistance loci. Weakly resistant pseudo-wild type loci like *lev-9* and *lev-10*

might create resistance by an indirect effect on the levamisole-sensitive function. If there is a more extreme mutant phenotype for these genes, it may be discoverable through precomplementtaion.

Suppression of *unc-29(e1072)* by *sup-5(e1464)* also suggests that the *unc-29* function is not essential. *sup-5* appears to be a weak informational suppressor, possibly of null alleles (WATERSTON and BRENNER 1978). Alleles of some genes are well suppressed, possibly because function is required in only catalytic amounts, while alleles of myosin and paramyosin mutants are poorly suppressed, probably because these structural muscle proteins are needed in stoichiometric amounts and *sup-5* restores no more than 5 to 10% of the protein missing from null alleles (WATERSTON and BRENNER 1978).

An *unc-29(e1072) sup-5(e1464)* double homozygote was constructed by the method of WATERSTON and BRENNER (1978), using *sma-2(e502)*. The double-mutant is slightly more wild type and exhibits spastic paralysis in the first hour or so on 1 mM levamisole. The mutant does not contract as either a whole or cut worm in 1 mM levamisole, but contracts moderately in the first few minutes in 1 mM carbachol, compared to the thickening if the unsuppressed *unc-29* mutant. The phenotypes of six other *unc-29* mutants as intact worms were not affected by *sup-5*. The weak suppression of *e1702* is consistent with the proposed structural function of *unc-29* and with the possibility that *e1072*, of strong mutant phenotype, is a null allele.

It might be advantageous to search for mutations that improve the suppression of *e1072* by *sup-5*. Such mutations could conceivably operate by either improving the suppression mechanism itself or increasing the level of *unc-29* expression on which suppression operates. The latter type of mutation, when placed in a wild-type background, might be an overproducer of the *unc-29* function.

The levamisole-sensitive function itself is probably dispensable. Nonessentiality of a putative structural locus like *unc-29* would imply this. Pharmacologically, there is also little function left in the most resistant *unc* mutants. The best mutants are more than a 1000-fold less sensitive to levamisole than wild type (LEWIS *et al.* 1980). Mutants of obvious partial function, the pseudo-wild types, gain the resistant *unc* phenotype when placed on levamisole, but resistant *unc* mutants show no further change in phenotype, suggesting that no more function is lost through the blocking effect of levamisole (LEWIS *et al.* 1980). We have also constructed double homozygotes of *unc-29(e1072) I* with *lev-1(x21) IV* and *unc-50(e306) III* and verified the genotypes by complementation. If leakiness were important to the remaining phenotype of the single mutants, the double mutants might be more severely debilitated. They are not.

*Other mutant types*: Mutants hypersensitive to levamisole and revertants of the resistant *unc* phenotype would be useful because a subset of such mutants might be overproducers of the levamisole-sensitive function or its constituent parts. We have isolated prototypical examples of such mutants.

Mutants hypersensitive to levamisole might be those with cuticular or metabolic defects allowing a higher internal drug concentration or those more sensitive to a given drug concentration, possibly because of increased amounts of the

levamisole-sensitive function. By screening for initial behavior on 0.05 mM levamisole, we have found a mutant, *unc-?(x54)*, which as an adult becomes paralyzed several times faster than the wild type and, unlike the wild-type adult, does not recover. Growth of the mutant on 0.05 mM levamisole is severely inhibited. Very stunted dumpy larvae grow up and die somewhere between the last larval stage (L4) and early adulthood. On regular plates, X54 is mildly dumpy and uncoordinated and shows variable cuticular defects in its head and at its vulva. Regardless of whether its complex phenotype is neurological or cuticular in origin, X54 should be useful for devising a mass screening method for hypersensitive mutants.

Reversion is a classical method of isolating overproducers and of further defining interactions among a set of loci. We have obtained about 10 revertants of resistant *unc* mutants caused by both closely linked and extragenic mutations. The closely linked revertants regain wild-type motor activity and some or all of their levamisole sensitivity. The extragenic revertants move in a more wild-type way, but are still resistant to levamisole and cholinergic drugs. For example, *unc-29(e1072) sup-?* (strain ZZ1000) as a cut worm is as resistant as *unc-29(e1072)*, not contracting after two hr in either 1 mM levamisole or carbachol. ZZ1000, examined as a first-stage larva, was found to be only slightly improved over the resistant *unc* larval phenotype. Its more nearly wild type behavior was gained during the second larval stage (17 to 19½ hours post-hatching). The closely linked reversions are likely to be intragenic. We think that the extragenic revertants as exemplified by ZZ1000 are likely to be compensatory changes beyond the levamisole-sensitive function, perhaps installing a function gained during larval development at sites where the levamisole-sensitive function is required. True reversion to levamisole sensitivity may be favored by selecting against either the early larval or the osmosensitive phenotype of resistant *unc* mutants, neither of which seems much affected by an extragenic revertant like ZZ1000.

*The epistatic effect of twitchers on some dumpy mutants:* By careful measurement, we have found that double mutants of the twitcher alleles *lev-11(x12)* and *unc-22(x25)* with *dpy-5(e61)*, *dpy-13(e184)*, *dpy-11(e224)* and *dpy-13(e184)* are up to 22% longer and/or less dumpy than the single *dpy* homozygotes. Comparison was made by determining the lengths and length-to-width ratios of synchronously grown animals at four and five days after hatching at 20°. We had observed that a low dose ($10^{-5}$ M) of a muscle hypercontracting agent like levamisole could produce an excellent synthetic dumpy phenotype in the wild type. The twitcher phenotype, a partial block in muscle contraction (LEWIS *et al.* 1980), rescues the wild type from levamisole (BRENNER 1974). Hence, a *dpy* mutant partially rescuable by a twitcher could result from muscle hypercontraction. Since measurement was tedious and confirmed judgment by eye, we simply constructed the double mutants of *lev-11(x12)* with the remainder of the first 20 *dpy* genes and made a side-by-side comparison with the parental dumpy strains. Any *dpy* muscle hypercontraction might also be lessened by blocking input or by detroying muscle structure; thus, we tested the effect of *unc-29(e1072)*

and the myosin mutant *unc-54(e190)* (EPSTEIN, WATERSON and BRENNER 1974) on the *dpy* alleles. Mutants that *lev-11* appeared to make longer or less dumpy also included *dpy-7(e88)*, *dpy-8(e130)*, *dpy-16(e225)*, *dpy-18 (e364)* and *dpy-19(e1259)*. Mutants that were not noticeably affected included *dpy1(e1)*, *dpy-2(e187)*, *dpy-3(e27)*, *dpy-4(e1166)*, *dpy-6(e14)*, *dpy-9(e12)*, *dpy-10(e128)*, *dpy-12(e182)*, *dpy-14(e188)*, *dpy-15(e24)*, *dpy-17(e164)* and *dpy-20(e1282)*. *unc-29(e1072)* and *unc-54(e190)* had no unambiguous suppressive effect on any of the 20 *dpy* mutants tested [but the double-mutant of *e190* with *dpy-10(e128)* is a synthetic lethal and that with *dpy-14(e188)* apparently so, since we were unable to construct it].

The failure of *unc-54* to suppress noticeably the *dpy* alleles affected by *lev-11* casts doubt on the muscle hypercontraction hypothesis. However, referring to our levamisole analogy, we note that the behavior of the two twitcher mutants and the *unc-54* mutant differs. Twitchers survive much better than the wild type on 0.1 mM levamisole, while *unc-54(e190)* dies on 0.05 mM levamisole. We have found a homozygous cold-sensitive, dumpy, paralyzed mutant *unc-?(x51)*, suggesting that some genetic neuromuscular abnormalities can cause dumpiness. Since a mutant greatly overproducing the levamisole-sensitive function might have a dumpy, hypercontracted appearance from excess function and be suppressible by resistant *unc* or twitcher alleles, it may be worthwhile to continue testing the epistatic effects of levamisole resistance loci on newly discovered dumpy mutants.

*Outlook:* The levamisole resistance loci appear to be a small set of related higher eukaryotic genes suitable for exhaustive genetic analysis. The principal virtue of the mutants is their ready selectability: individual resistant animals can easily and rapidly be distinguished from nonresistant animals. Levamisole resistance might become a general nematode genetic tool, allowing selection of rare somatic mosaics or the generation of deletions in various chromosomal regions. We expect such deletions, which might be generated by our precomplementation technique, will be useful in a classical and recombinant DNA fine-structure analysis of the resistance loci themselves. Whether or not the levamisole-sensitive function comprises a nematode acetylcholine receptor or any other molecule of the nematode nervous system, it is likely to be present in very small amounts, perhaps one part in $10^6$ or less of total protein. Given the amount of DNA that may be deletable to either side of a nonessential resistance locus, it may be easier to clone a resistance gene from DNA failing to hybridize to a deletion mutant than it might be to find the protein product *in vitro*. With a cloned gene, it might become possible to identify the RNA and protein gene products and thus take advantage of the especially powerful genetics of levamisole resistance for functional analysis of a set of eukaryotic genes.

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