Caenorhabditis elegans Mutants Resistant to Inhibitors of Acetylcholinesterase

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ABSTRACT

We characterized 18 genes from *Caenorhabditis elegans* that, when mutated, confer recessive resistance to inhibitors of acetylcholinesterase. These include previously described genes as well as newly identified genes; they encode essential as well as nonessential functions. In the absence of acetylcholinesterase inhibitors, the different mutants display a wide range of behavioral deficits, from mild uncoordination to almost complete paralysis. Measurements of acetylcholine levels in these mutants suggest that some of the genes are involved in presynaptic functions.

TO understand the metabolism, release, and function of the neurotransmitter acetylcholine, we are pursuing genetic and molecular studies on synaptic transmission in the nematode *Caenorhabditis elegans*. We use *C. elegans* because of its simple nervous system and its ease of genetic and molecular analysis. One of our strategies involves the isolation and analysis of mutants that are resistant to inhibitors of the transmitter-hydrolyzing enzyme acetylcholinesterase (AChE).

There are published reports of *C. elegans* mutants resistant to AChE inhibitors (BRENNER 1974; CULOTTI and KLEIN 1983; RAND and RUSSELL 1984, 1985; HOSONO *et al.* 1989; HOSONO and KAMIYA 1991; NONET *et al.* 1993), as well as several unpublished studies (S. CARR and D. HIRSH, personal communication; J. CULOTTI, personal communication; K. PETERSEN and R. RUSSELL, personal communication). These studies have led to the identification of approximately a dozen genes that can mutate to produce resistance. We recently performed a large-scale isolation and characterization of mutants resistant to the AChE inhibitor aldicarb (A. ALFONSO, M. NGUYEN, C. D. JOHNSON and J. B. RAND, unpublished results), which led to the identification of several additional genes. In this article, we report the analysis of the phenotypes resulting from recessive mutations in all of the known resistance genes: developmental profiles, quantitative assessment of resistance to two different inhibitors, and biochemical assay of the level of acetylcholine. The phenotypic analysis provides insight into the nature of the functional disruptions associated with the mutants. Preliminary accounts of some of these results have appeared in abstract form (ALFONSO-PIZARRO *et al.* 1988; RAND *et al.* 1988, 1992).

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MATERIALS AND METHODS

Growth and culture: *C. elegans* was grown on NGM agar as described by BRENNER (1974), modified by the addition of streptomycin and mycostatin to reduce contamination and the use of the streptomycin-resistant bacterial strain OP50/1 (JOHNSON *et al.* 1988). Aldicarb, 2-methyl-2-[methylthio]propionaldehyde-O-[methylcarbamoyl]oxime (Figure 1), was obtained from Chem Service, Inc. (West Chester, PA) and was prepared as a 105 mM stock solution (1 g/50 ml) in 70% ethanol and added to the agar growth medium after autoclaving; the efficacy of the compound in the growth medium is not diminished for at least a month. Trichlorfon, dimethyl-[2,2,2-trichloro-1-hydroxyethyl]phosphonate (Figure 1), was obtained from Chem Service and was prepared as a 10-mM stock solution in 70% ethanol. Preliminary experiments indicate that the efficacy of trichlorfon decreases after 2 wk; all of the trichlorfon experiments were therefore performed with freshly prepared medium.

Mutations used: The wild-type Bristol strain N2 was used as the reference strain. The following resistance mutations were studied (for each gene, the reference allele was used): *cha-1(p1152)IV*; *ric-1(e239)III*; *ric-3(md158)IV*; *ric-4(md1088)V*, *snt-1(md125)II*; *unc-10(e102)X*; *unc-11(e47)I*; *unc-13(e51)I*; *unc-17(e245)IV*; *unc-18(e81)X*; *unc-32(e189)III*; *unc-36(e251)III*; *unc-41(e268)V*; *unc-63(x37)I* (see following paragraph); *unc-64(e246)III*; *unc-65(e351)V*; *unc-75(e950)I*; and *unc-104(e1265)II*. *ric-1* was formerly called *lan-5*. *snt-1* was formerly called *ric-2*. For mapping experiments, the following marker mutations were used: *daf-7(e1372)III*; *dpy-1(e1)III*; *dpy-5(e61)I*; *dpy-10(e128)II*; *dpy-11(e224)V*; *dpy-13(e184)IV*; *dpy-17(e164)III*; *dpy-18(e364)III*; *lon-1(e185)III*; *lon-3(e2175)V*; *sma-2(e502)III*; and *vab-2(e96)IV*.

We started these studies using the *e384* allele of *unc-63*, but in the course of our work, we found that strains containing *e384* (the nominal reference allele) also contained a closely linked *unc* mutation. We have identified this mutation as a weak allele of *unc-11*, which we have designated *ic9*. Thus, strain CB384 and many strains derived from it contain two linked aldicarb-resistance mutations. We therefore repeated all measurements using the *x37* allele of *unc-63*. To eliminate any possible future confusion, *x37* (LEWIS *et al.* 1980a) will

henceforth be designated as the *unc-63* reference allele (J. LEWIS and T. STIERNAGLE, personal communication).

Growth curves: Animals hatching in a 2-hr interval were transferred to fresh plates and grown at 20°. At the indicated times, a small number were eluted from the plates with M9 buffer and immediately heated to 60° for 15 min. This straightens the worms; they were then transferred to a glass slide and measured with a dissecting microscope equipped with an eyepiece reticle. Eight to 12 animals were measured at each time point.

Quantitation of resistance: Animals hatching in a 2-hr interval were eluted from their plates with M9 buffer. An aliquot of the nematode suspension was counted, the volume was adjusted to 200 hatchees per 50 μl, and a 50-μl aliquot per plate was pipetted onto a set of drug-containing plates. For aldicarb, the concentrations used were 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.3 and 1.6 mM. For trichlorfon, the concentrations were 0, 0.02, 0.04, 0.06, 0.08, 0.10, 0.13 and 0.16 mM. Plates were maintained at 20° and screened once or twice a day; the number of eggs and hatchees on each plate was recorded. The "generation time" was defined (arbitrarily) as the time required for the 200 hatchees to grow and produce 200 new hatchees. We note that this approach does not distinguish between each of the original animals producing one offspring and only one of the original animals growing and producing 200 progeny. The growth rate was defined as the reciprocal of the generation time, and the "relative growth rate" as the ratio of the growth rate in the presence of the drug to the growth rate in the absence of the drug.

Genetic analysis: Newly identified *ric* genes were mapped and tested for genetic complementation with nearby loci using standard methods (HERMAN and HORVITZ 1980). Strains with appropriate markers were obtained from the Caenorhabditis Genetics Center (Columbia, MO).

Acetylcholine assays: Acetylcholine levels were measured by eluting one to three asynchronous plates of *C. elegans* (~5000–20,000 individuals) with M9 buffer, washing once with ice-cold M9, and resuspending in 1 N formic acid/acetone (85:15). After low-speed centrifugation (1000 rpm for 2 min in a clinical centrifuge), the supernatant was withdrawn, and aliquots (after lyophilization) were used for acetylcholine determinations by the method of MCCAMAN and STETZLER (1977). The pellets were then resuspended in 0.1 N NaOH, and aliquots were used to determine protein concentration, using the BCA method (Pierce Chemical Co.), with serum albumin as a standard.

RESULTS

Identification of aldicarb-resistant genes: When wild-type *C. elegans* are placed on AChE inhibitors such as aldicarb or trichlorfon (Figure 1), they quickly become hypercontracted and paralyzed and feeding, growth, and development soon cease. In the present study, resistance is defined as the ability to grow and reproduce at a level of drug that prevents the growth and reproduction of wild-type animals (RAND and RUSSELL 1985). There are alternate ways to define resistance, for example, the ability to move at a drug concentration that paralyzes wild-type animals or a decrease in the drug-induced hypercontraction of body-wall muscles (NONET *et al.* 1993). In general, mutant strains that are resistant by one of these criteria are also resistant by the others.

Some aldicarb-resistant mutations were identified by direct testing of previously described uncoordinated

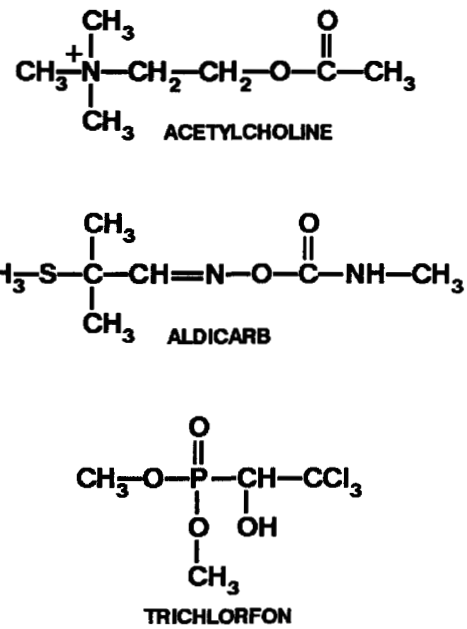

FIGURE 1.—Chemical structures of acetylcholine, aldicarb and trichlorfon.

(*unc*) mutants. In most cases, however, mutants were isolated on the basis of their drug resistance (ALFONSO *et al.*, unpublished data) and were then assigned to genes by standard genetic methods. Many of these genes had been previously identified; these include *cha-1* [the choline acetyltransferase (ChAT) structural gene], *snt-1* (the synaptotagmin structural gene), and all the genes with "*unc*" designations. Newly identified genes were designated "*ric*" for *r*esistance to *i*nhibitors of acetyl*c*holinesterase (Table 1).

Genetic mapping of new *ric* genes: Standard genetic techniques were used to determine the genetic map location for *ric-1*, *ric-3*, and *ric-4* (see MATERIALS AND METHODS). The data from these experiments are presented in Tables 2 and 3. Although the two-factor data with *dpy-1* suggest that *ric-1* is relatively close to *dpy-1*, two-factor data with three other markers (Table 2), as well as three-factor data (Table 3), indicate that *ric-1* is more centrally located on linkage group III. The genetic map showing the positions of all of the genes in the present study is given in Figure 2.

Behavior and development of Ric mutants: The phenotypes of animals homozygous for the reference allele of each of the aldicarb-resistant genes were examined (see MATERIALS AND METHODS). For some of these genes, reference alleles are known or believed to be null (*e.g.*, *snt-1*, *unc-13*, *unc-41*), whereas for others (*e.g.*, *cha-1*, *unc-17*, *unc-104*), null alleles are lethal and the reference alleles are known to retain some gene function (HALL and HEDGECOCK 1991; ALFONSO *et al.* 1993, 1994; NONET *et al.* 1993). Therefore, the phenotypic measurements of this latter class reflect residual gene activity.

All of the mutants in the present study display some

TABLE 1

Genes conferring recessive aldicarb resistance

Gene	Reference allele	Null phenotype	Citations
cha-1	*p1152*	L1 lethal	RAND and RUSSELL (1984), RAND (1989), ALFONSO *et al.* (1994)
ric-1	*e239*		This study
ric-3	*md158*		This study
ric-4	*md1088*		This study
snt-1	*md125*	Viable Unc	NONET *et al.* (1993)
unc-10	*e102*		BRENNER (1974), RAND and RUSSELL (1985)
unc-11	*e47*		BRENNER (1974), RAND and RUSSELL (1985)
unc-13	*e51*	Viable Unc (?)	BRENNER (1974), RAND and RUSSELL (1985), MARUYAMA and BRENNER (1991)
unc-17	*e245*	L1 lethal	BRENNER (1974), ALFONSO *et al.* (1993)
unc-18	*e81*	Viable Unc (?)	BRENNER (1974), RAND and RUSSELL (1985), GENGYO-ANDO *et al.* (1993)
unc-32	*e189*		BRENNER (1974), RAND and RUSSELL (1985)
unc-36	*e251*		BRENNER (1974), RAND and RUSSELL (1985)
unc-41	*e268*	Viable Unc (?)	BRENNER (1974), HOSONO *et al.* (1989), this study
unc-63	*x37*		BRENNER (1974), LEWIS *et al.* (1980a), RAND and RUSSELL (1985)
unc-64	*e246*		BRENNER (1974), RAND and RUSSELL (1985)
unc-65	*e351*		BRENNER (1974), RAND and RUSSELL (1985)
unc-75	*e950*		BRENNER (1974), this study
unc-104	*e1265*	L1 lethal	OTSUKA *et al.* (1991), HALL and HEDGECOCK (1991), this study

The reference allele for each gene is not necessarily null. The bibliographic citations include the isolation of mutants, description of resistance, and molecular analysis of the gene. The designation "viable unc (?)" means that null mutants are believed to be viable, based on the phenotype in *trans* to a deficiency, isolation frequency, *etc.*, but a "guaranteed molecular null" mutation has not yet been described.

TABLE 2

Two-factor recombination data for new resistance genes

Genotype of heterozygous parent	Phenotype of selected self-progeny	Genotype of selected recombinants with respect to *trans* marker	Percent recombination
		A. Data for *ric-1* from *trans* heterozygotes	
ric-1 +/+ *dpy-17*[a]	Ric	19/319 *dpy*/+ 300/319 +/+	3.0 ± 1.5
ric-1 +/+ *lon-1*[b]	Ric	10/327 *lon*/+ 317/327 +/+	1.5 ± 1.0
ric-1 +/+ *dpy-18*	Ric	22/126 *dpy*/+ 104/126 +/+	8.7 ± 7.0
Genotype of heterozygous parent	Progeny with parental phenotype	Progeny with recombinant phenotype	Percent recombination
		B. Data from *cis* heterozygotes	
dpy1- ric-1/++	644 Dpy Ric[c]	86 Ric[d]	6.2 ± 1.9
ric-1 sma-2[e]/++	11,916 Total	37 Ric	0.6 ± 0.2
dpy-13 ric-3/++	675 Wt	16 Dpy	3.5 ± 1.8
dpy-11 ric-4/++	1659 Wt 525 Dpy Ric	30 Dpy 17 Ric	2.1 ± 0.7

Heterozygous hermaphrodites were constructed by standard methods, using the reference allele for each gene (given in MATERIALS AND METHODS). The Ric phenotype was scored by drug resistance for the *ric-1* crosses and was scored by uncoordinated behavior in the remaining crosses.

[a] The *dpy-17* chromosome was also marked with *unc-79(e1068)*.

[b] The *lon-1* chromosome was also marked with *mec-12(e1605)*.

[c] This number was based on an estimate of 750 total Ric (*i.e.*, Ric Dpy + Ric non-Dpy) animals.

[d] This number included 79 *dpy-1 ric-1*/+ *ric-1* and 7 + *ric-1*/+ *ric-1* animals, as determined by progeny analysis.

[e] The *ric-1 sma-2* chromosome was also marked with *unc-32(e189)*.

TABLE 3

Three-factor recombination data for new resistance genes

Genotype of heterozygous parent	Phenotype of selected recombinant self-progeny	Genotype of selected recombinants with respect to *trans* marker	Inferred gene order
dpy-1 ric-1/daf-7	Ric	14/18 *daf/+* 4/18 *daf/daf*	*daf-7 dpy-1 ric-1*
ric-1 sma2[a]/unc-36	Ric	16/37 *unc/+* 21/37 +/+	*ric-1 unc-36 sma-2*
dpy-13 ric-3/vab-2	Dpy	2/44 *vab/+* 42/44 +/+	*vab-2 dpy-13 ric-3*
dpy-11 ric-4/lon-3	Dpy Ric	5/5 *lon/+* 3/3 +/+	*dpy-11 ric-4 lon-3*

Heterozygous hermaphrodites were constructed by standard methods, using the reference allele for each gene (given in MATERIALS AND METHODS). The Ric phenotype was scored by drug-resistance for the *ric-1 sma-2* cross, and was scored by uncoordinated behavior in the remaining crosses. For three of the three-factor crosses, the marker *in trans* was not between the two *cis* markers. Nonetheless, given the known map positions of the markers (Figure 2) the results obtained are consistent with an unambiguous left-right order of the genes.

[a] The *ric-1 sma-2* chromosome in this cross was also marked with *unc-32(e189)*.

degree of uncoordinated behavior in the absence of AChE inhibitors, although the nature and severity of the behavioral impairment varies greatly. Some of the mutants are almost completely paralyzed (*e.g., unc-13, unc-18*), some are quite uncoordinated but relatively active (*e.g., snt-1, unc-41*), some are mildly uncoordinated but slow (*e.g., ric-4*), and some are active and only slightly uncoordinated (*e.g., ric-1*).

Growth curves for all of these mutants are given in Figure 3. For some of the strains, growth rate is significantly less than wild type, while others appear to grow at the normal rate. In addition, many, but not all, of the strains are significantly smaller as adults than wild type.

Drug resistance of Ric mutants: To obtain quantitative assessment of aldicarb resistance, the growth rate of each strain at different aldicarb concentrations was compared with the growth rate in the ab-

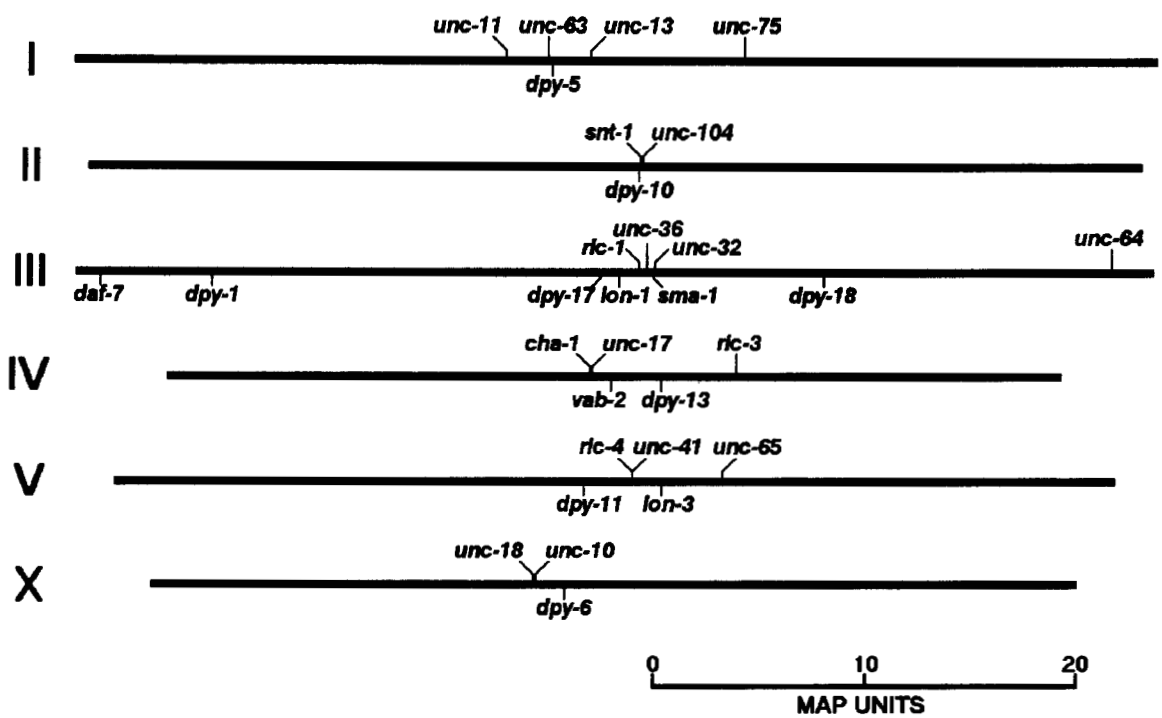

FIGURE 2.—Genetic map of *C. elegans*. The positions of each of the aldicarb-resistant loci are shown above the linkage groups; positions of marker genes are shown below the linkage groups. With the exception of the *ric* genes, map positions are based on the standard *C. elegans* genetic map (J. HODGKIN, R. DURBIN and M. O'CALLAGHAN, personal communication). The relative order of *ric-4* and *unc-41* has not yet been determined.

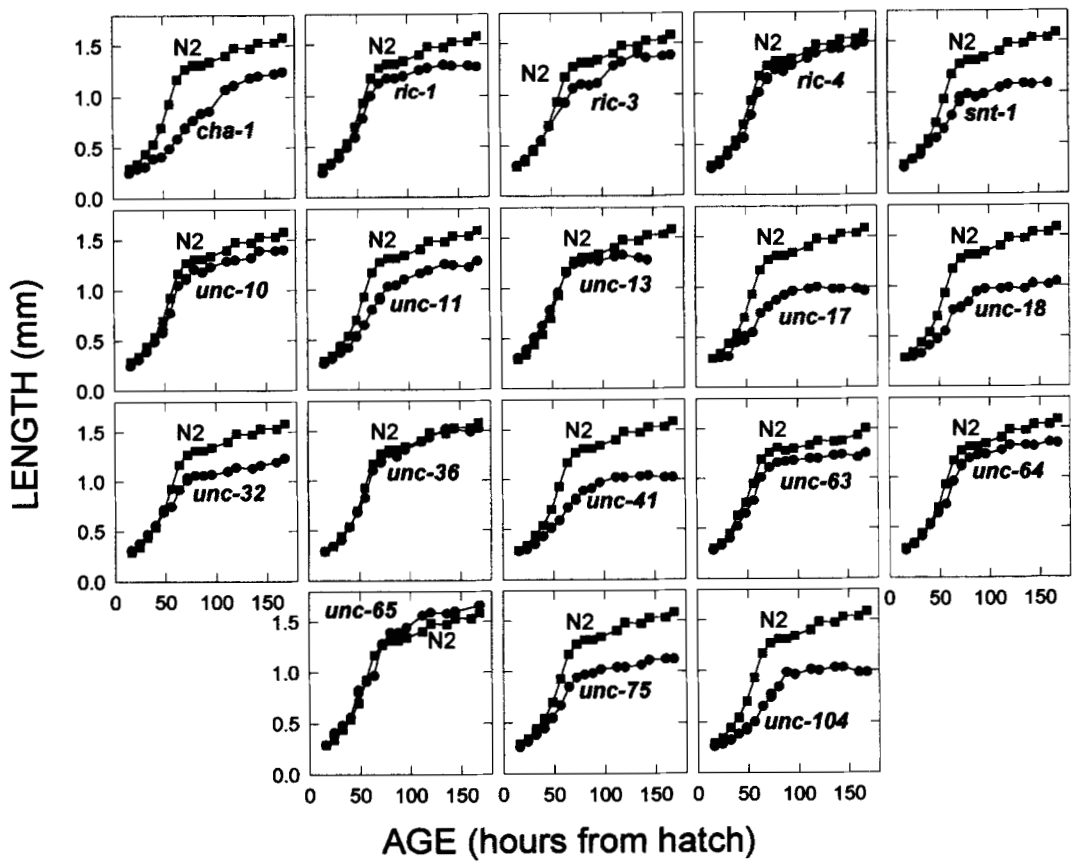

FIGURE 3.—Growth curves of wild-type N2 (■) and resistant mutants (●). The data for *unc-63(x37)* were obtained at a different time from the rest of the experiments; the wild-type data are from a concurrent control. The data for *snt-1* are from NONET *et al.* (1993).

sence of aldicarb (see MATERIALS AND METHODS). It was important to measure the relative growth rate of each strain on aldicarb because many of these strains grow slowly even in the absence of the drug (Figure 3). Figure 4 shows the effect of aldicarb on the growth rate of each resistant strains. Some of the strains, such as *cha-1, unc-17, ric-1,* and *snt-1,* were extremely resistant; their growth rate was essentially unaffected by aldicarb, even at the highest concentration (1.6 mM) tested. Nonetheless, the drug did have an effect on these animals: they were hypercontracted and their rate of locomotion was significantly decreased at high drug concentrations. We also noted that small quantities of aldicarb *stimulate* the growth of some of the highly resistant strains; this is demonstrated for *cha-1* in Figure 4 and was sometimes seen with *unc-17* (as noted by BRENNER 1974). Some strains, such as *unc-32, unc-36,* and *unc-65,* were only marginally resistant to aldicarb (Figure 4).

To determine whether this collection of mutants was resistant to a chemically different AChE inhibitor, we tested them for resistance to trichlorfon, an organophosphate pesticide. Trichlorfon is effective at a 10-fold lower concentration than aldicarb; however, with the exception of *unc-63(x37),* the resistance of each strain to trichlorfon (Figure 5) was correlated with its resistance to aldicarb (Figure 4). Even the *unc-63* animals did not display a very large discrepancy in their response to the two drugs: they were only weakly resistant to aldicarb but fully sensitive (or perhaps even slightly hypersensitive) to trichlorfon (Figure 4). Because the resistance conferred by mutations in the other 17 genes was independent of the type of AChE inhibitor (*i.e.,* carbamate or organophospate), we conclude that (for those genes) the mutant phenotype is derived from some alteration in cholinergic metabolism and/or function rather than from some effect specific to aldicarb, such as reduced permeability to the drug.

In general, there is little correlation between the degree of resistance of a given mutant (Figures 4 and 5) and its behavioral or growth impairment (Figure 3), for example, *ric-1* mutants are mildly uncoordinated but strongly resistant to aldicarb and *unc-36* mutants are strongly uncoordinated but only weakly drug resistant.

Acetylcholine assays: Acetylcholine was measured in extracts prepared from mixed (nonsynchronous) cultures (see MATERIALS AND METHODS); the data are presented in Table 4. Only the *cha-1* strain had reduced levels of acetylcholine, approximately half that of wild type. Six of the strains had normal amounts of acetylcholine, four strains had somewhat elevated acetylcholine (approximately twice wild type), and seven strains

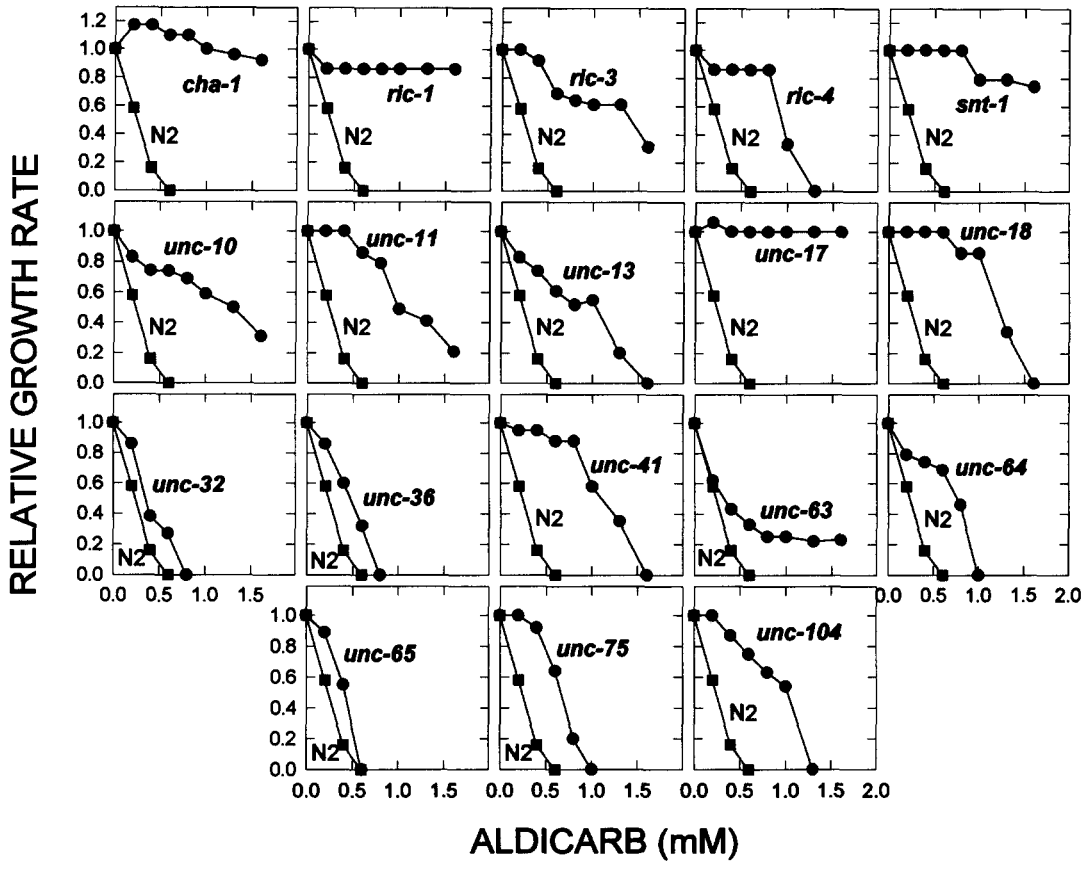

FIGURE 4.—Aldicarb resistance of wild-type N2 (■) and resistant mutants (●). The data for N2 are the mean of three experiments; the data for *unc-63* are the mean of two experiments. The data for *snt-1* are from NONET *et al.* (1993).

had substantially increased (two to three times wild type) acetylcholine.

In Table 4, we present all of the individual acetylcholine determinations as well as the mean and standard deviation to demonstrate the significant degree of scatter obtained in measuring some of the strains. Each of the replicate measurements was performed on a different batch of nematodes at a different time. All assays were performed in triplicate (at least) and in parallel with quadruplicate sets of standards. The substantial variation was not observed within a set of assays, rather it occurred between different samples of the same strain.

There are at least two possible sources of such variation: age structure and nutritional state. The assays were performed on asynchronous populations, and it is quite possible that acetylcholine levels depend on the age of the animal (the larval nervous system presumably represents a greater fraction of the total body mass than the adult nervous system); thus, the variability might reflect different population age structures. In addition, we note that different mutant strains have different patterns of food consumption: when the bacterial lawn is exhausted in one region, some uncoordinated animals have difficulty moving to the remaining food and are temporarily undernourished (or even starved). This could contribute to variation in the nutritional state of different animals within a population and perhaps to variations in acetylcholine synthesis.

DISCUSSION

This article describes recessive mutations that confer resistance to inhibitors of AChE (the "Ric" phenotype). Drug resistance is a powerful method for mutant selection, and mutations have now been identified in 18 different genes. Many of these genes had previously been identified by recessive mutations causing uncoordinated behavior (*unc* genes). Mutations in 11 of these genes have been reported to disrupt pharyngeal function (AVERY 1993). The description and characterization of three genes, *ric-1*, *ric-3*, and *ric-4*, are presented for the first time. Another newly identified gene, originally called *ric-2*, has been renamed *snt-1* and was described in detail by NONET et al. (1993). Isolation and analysis of dominant Ric mutants are in progress.

The cause of drug resistance: Genetic resistance to a toxic drug may be acquired through one of three mechanisms: mutations that alter the drug's target site, making it less sensitive; mutations that alter permeability to or metabolism of the drug, thereby decreasing the effective drug concentration at the sensitive site; and mutations leading to cellular or metabolic alter-

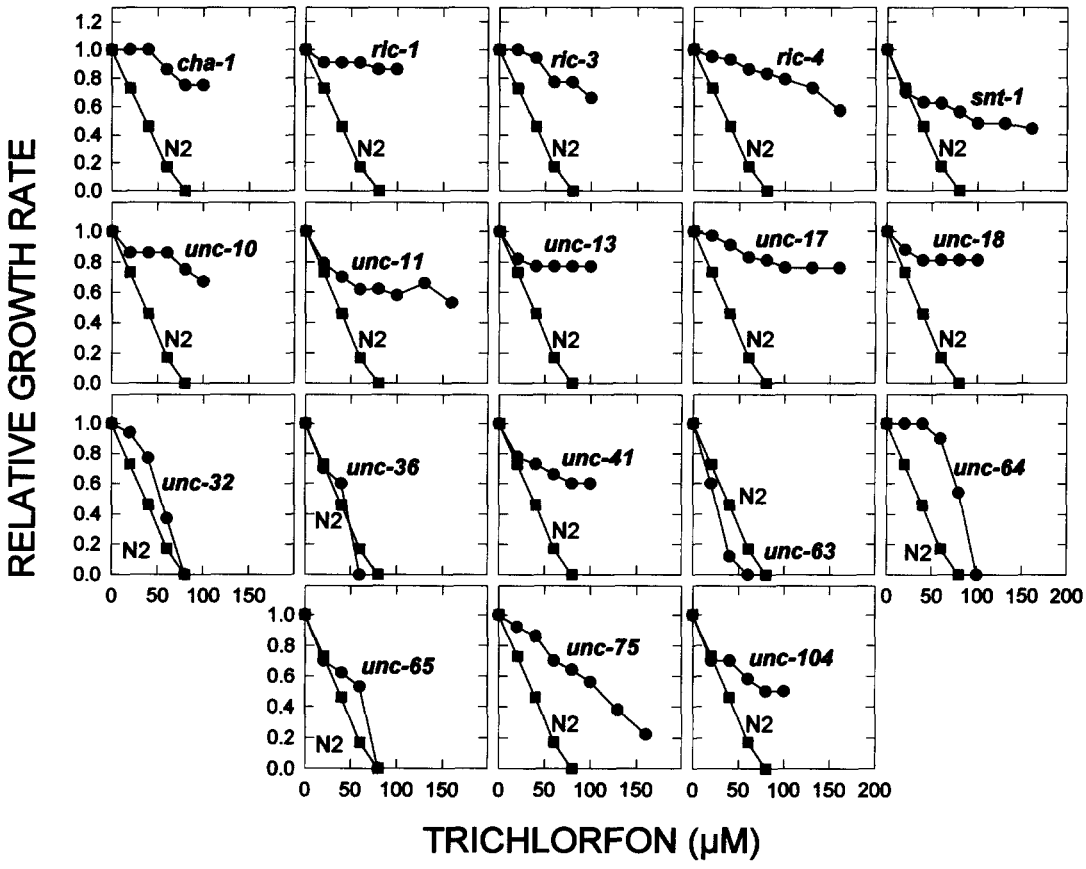

FIGURE 5.—Trichlorfon resistance of wild-type N2 (■) and resistant mutants (●). The data for N2 are the mean of four experiments; the data for *ric-4, snt-1, unc-11,* and *unc-75* are the means of three experiments; the data for *unc-17* and *unc-63* are the means of two experiments.

ations that can bypass or compensate for the action of the toxin. We believe that none of the genes characterized in the present study affect AChE, the target site of the toxin (mechanism 1). The map positions of the three AChE structural genes, *ace-1, ace-2,* and *ace-3,* are known and different from any of the genes listed in Table 1 (CULOTTI *et al.* 1981; JOHNSON *et al.* 1981, 1988). In addition, mutations of AChE are expected to confer dominant resistance; the mutations in the present study confer recessive resistance.

We have not yet examined whether any of the resistance mutants in *C. elegans* affect aldicarb uptake and/or metabolism (mechanism 2). However, all of the mutants in our study except *unc-63* had comparable resistance to two chemically different AChE inhibitors: aldicarb (a carbamate) and trichlorfon (an organophosphate). Thus, if the mechanism of resistance affected permeability or metabolism, it would need to affect these structurally different inhibitors in parallel, which seems unlikely. Thus, we believe that most of the resistance genes described in this study involve the third mechanism (metabolic or cellular compensation for AChE inhibition).

The site of drug resistance: Acetylcholine is synthesized by ChAT and transported into synaptic vesicles, where it is stored until it is released. After release, it stimulates postsynaptic receptors until its action is terminated by hydrolysis by AChE. Inhibition of AChE is presumed to lead to a toxic overstimulation of acetylcholine receptors, causing paralysis and death. The active toxin is thus not the AChE inhibitor but rather acetylcholine itself. A mutation that reduces the buildup of acetylcholine is expected to provide some protection from an AChE inhibitor. Failure to synthesize acetylcholine is one obvious way to prevent the buildup of acetylcholine, and we have found that mutants of ChAT (*cha-1*) are strongly resistant to AChE inhibitors (RAND and RUSSELL 1984). Similarly, we expect that mutational defects in other aspects of acetylcholine synthesis, packaging or release would also lead to drug resistance. Other mechanisms of drug resistance might include alterations of acetylcholine receptors or of postsynaptic elements involved in signal transduction.

The measurements of acetylcholine levels can help identify site(s) of action of mutant gene products. A defect in the synthesis of acetylcholine is expected to decrease total acetylcholine, whereas defects in steps after the synthesis but before the release of acetylcholine (*e.g.,* transport into vesicles, vesicle translocation,

TABLE 4

Acetylcholine levels in aldicarb-resistant mutants

Strain	Acetylcholine	Individual measurements
N2	4.1 ± 1.6	1.7, 3.0, 3.2, 3.7, 4.0, 4.3, 4.6, 5.8, 7.0
cha-1(p1152)	1.9 ± 0.8	1.2, 1.3, 2.3, 2.9
unc-65(e351)	3.7 ± 0.7	3.2, 3.3, 3.5, 4.8
unc-10(e102)	4.1 ± 0.5	3.7, 3.8, 3.8, 4.5, 4.9
unc-63(x37)	4.6 ± 1.9	2.8, 4.4, 6.6
unc-32(e189)	5.0 ± 2.2	2.5, 4.2, 5.4, 7.8
unc-36(e251)	5.2 ± 1.2	4.1, 4.9, 5.0, 6.9
ric-3(md158)	5.6 ± 2.8	1.6, 6.2, 6.4, 8.1
unc-75(e950)	7.3 ± 2.0	5.7, 6.0, 7.3, 10.0
ric-4(md1088)	8.0 ± 4.9	2.4, 6.2, 9.5, 14.0
unc-64(e246)	8.0 ± 6.0	4.2, 5.0, 5.8, 17.0
ric-1(e239)	8.3 ± 4.9	2.3, 6.8, 8.3, 8.3, 16.0
unc-104(el265)	8.9 ± 0.7	8.0, 8.7, 9.3, 9.5
unc-18(e81)	9.5 ± 2.4	7.4, 7.5, 11.0, 12.0
unc-17(e245)	10.2 ± 7.9	4.7, 5.9, 6.7, 6.8, 7.0, 9.2, 12.0, 29
snt-1(md125)	11.0 ± 4.5	4.2, 10.0, 11.0, 15.0, 15.0
unc-11(e47)	11.0 ± 2.8	8.0, 9.1, 10.0, 14.0, 14.0
unc-41(e268)	12.1 ± 8.0	5.2, 5.3, 18.0, 20.0
unc-13(e51)	12.3 ± 2.8	9.1, 12.0, 12.0, 16.0

Acetylcholine is presented as means ± SD, in units of $\times 10^{-7}$ μmol/μg of protein. Also presented are all of the individual measurements used to obtain the mean. The large scatter in some of the determinations is not understood. Strains are grouped approximately into those with low ACh (*cha-1*), those with normal amounts of ACh, those with slightly elevated ACh, and those with significantly increased (two to three times N2) ACh. The data for *snt-1* are from NONET *et al.* (1993).

docking, exocytosis) are expected to generate an accumulation of unreleased (and unreleasable?) acetylcholine. Finally, a defect in the response to acetylcholine (*e.g.*, receptor function), is expected to have no effect on the level of acetylcholine.

As described above, mutations in only one gene, *cha-1*, lead to decreased acetylcholine; this may therefore represent the only gene involved in acetylcholine synthesis. Mutations in 11 genes lead to elevated acetylcholine; these genes may control presynaptic functions. Finally, mutations in six genes do not affect acetylcholine and may represent postsynaptic defects. Although the scatter present in some of the assays means that a few of these assignments should be considered tentative, to the extent that gene products have been identified for some of these genes (see following section), these assignments appear to be correct.

Acetylcholine levels have previously been reported for seven of the mutants described above. Although the values we report are considerably higher than the values originally published by HOSONO *et al.* (1989), they are in good agreement with a subsequent study by HOSONO and KAMIYA (1991). The only significant discrepancy between our acetylcholine data and those of HOSONO and KAMIYA (1991) is for *unc-63*, and this may be due to the presence of the *unc-11(ic9)* mutation in the CB384 *unc-63* strain that they used (see MATERIALS AND METHODS).

Molecular analysis of the resistance genes: Cloning and sequence have been reported for six of the genes described above; five of the six are clearly involved in presynaptic function. *cha-1* encodes ChAT, the acetylcholine synthetic enzyme (ALFONSO *et al.* 1994), and *unc-17* encodes the synaptic vesicle acetylcholine transporter (ALFONSO *et al.* 1993); these two genes are expected to be specific for cholinergic neurons. *unc-104* encodes a kinesin-like protein required for the axonal transport of synaptic vesicles to synaptic regions (HALL and HEDGECOCK 1991; OTSUKA *et al.* 1991), and *snt-1* encodes the synaptic vesicle protein synaptotagmin (NONET *et al.* 1993); these two genes are presumably required for synaptic function in all neurons. A mammalian homolog of *unc-18*, called "Munc-18" (HATA *et al.* 1993) or "n-Sec1" (PEVSNER *et al.* 1994), has recently been reported. The Munc-18/n-Sec1 protein was found to bind stably to the presynaptic protein syntaxin, suggesting that the *C. elegans unc-18* gene product (GENGYO-ANDO *et al.* 1993) is also important for presynaptic function. Finally, the *unc-13* gene product is a fundamentally unfamiliar protein, although it has a domain with homology to the regulatory region of protein kinase C (MARUYAMA and BRENNER 1991; AHMED *et al.* 1992).

Although molecular analysis has not yet been reported for any of the putative postsynaptic genes, there is genetic, biochemical and pharmacological evidence that one of these genes, *unc-63*, is involved in postsynaptic function. *unc-63* mutants are resistant to the postsynaptic acetylcholine agonist levamisole (LEWIS *et al.* 1980b), and homogenates of *unc-63* mutants have al-

tered binding of the cholinergic ligand *meta*-aminolevamisole (LEWIS *et al.* 1987). Together, these data suggest that the *unc-63* gene product is required for proper assembly and/or function of a postsynaptic acetylcholine receptor.

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