

A SECOND CLASS OF ACETYLCHOLINESTERASE-DEFICIENT
MUTANTS OF THE NEMATODE
CAENORHABDITIS ELEGANS

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ABSTRACT

In JOHNSON *et al.* (1981), the *Caenorhabditis elegans* mutant strain PR1000, homozygous for the *ace-1* mutation *p1000*, is shown to be deficient in the class A subset of acetylcholinesterases, which comprises approximately one-half of the total *C. elegans* acetylcholinesterase activity. Beginning with this strain, we have isolated 487 new behavioral and morphological mutant strains. Two of these, independently derived, lack approximately 98% of the wild-type acetylcholinesterase activity and share the same specific uncoordinated phenotype; both move forward in a slow and uncoordinated manner, and when mechanically stimulated to induce reversal, both hypercontract and become temporarily paralyzed. In addition to the *ace-1* mutation, both strains also harbor recessive mutations in the same newly identified gene, *ace-2*, which maps to chromosome I and is therefore not linked to *ace-1*. Gene dosage experiments suggest that *ace-2* is a structural gene for the remaining class B acetylcholinesterases, which are not affected by *ace-1*.—The uncoordinated phenotype of the newly isolated, doubly mutant strains depends on both the *ace-1* and *ace-2* mutations; homozygosity for either mutation alone produces normally coordinated animals. This result implies functional overlap of the acetylcholinesterases controlled by *ace-1* and *ace-2*, perhaps at common synapses. Consistent with this, light microscopic histochemical staining of permeabilized whole mounts indicates some areas of possible spatial overlap of these acetylcholinesterases (nerve ring, longitudinal nerve cords). In addition, there is at least one area where only *ace-2*-controlled acetylcholinesterase activity appears (pharyngo-intestinal valve).

JOHNSON *et al.* (1981) describe the identification, in the nematode *Caenorhabditis elegans*, of a gene called *ace-1* that affects acetylcholinesterase activity. Of the five major separable forms of acetylcholinesterase previously identified in *C. elegans* (JOHNSON and RUSSELL, in press), the *ace-1* mutant allele *p1000* markedly reduces the activities of three forms, previously grouped together on kinetic grounds and called class A forms. Gene-dosage experiments suggested that *ace-1* is a structural gene for some component common to these

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three forms. The two major remaining forms, called class B forms, appeared unaffected by the *ace-1* mutation.

A surprising feature of the *ace-1* allele *p1000* is the fact that animals homozygous for this mutation, and thus lacking detectable class A acetylcholinesterase activity, are behaviorally and developmentally indistinguishable from wild type. Of the many possible implications such a result might have, several suggest a neurally and behaviorally relevant role for the remaining class B acetylcholinesterase forms. Therefore, it became of considerable interest to identify a gene or genes controlling these remaining forms.

As an initial strategy for identifying mutations in such genes, we decided that, while extreme mutations might well be lethal, less severe ones might be expected to produce recognizable behavioral defects; therefore, it seemed reasonable to screen for the desired mutants among strains selected for their uncoordinated behavior. To facilitate the screening procedure, we decided to use the existing *ace-1* mutation to eliminate class A acetylcholinesterases. This had two advantages. First, a mutation markedly reducing class B acetylcholinesterases should, in an *ace-1* mutant background, produce a marked drop in residual acetylcholinesterase activity levels that could be easily detected by straightforward enzymatic assay. Second, in an *ace-1* mutant background, mutations markedly reducing class B acetylcholinesterases might conceivably generate a behavioral defect that would be obscured if class A acetylcholinesterases were present and could compensate for the class B deficit. Against these advantages was the possibility that the *ace-1* mutant background might render many of the desired mutations lethal.

Below, we describe how 487 new behavioral and morphological mutant strains, isolated from the *ace-1* mutant strain, PR1000, have been screened for acetylcholinesterase activity to identify two strains with markedly reduced activity levels. We show that both strains harbor mutations in the same newly identified gene, called *ace-2*, that this gene is unlinked to *ace-1* and that it is most probably a structural gene for some component of class B acetylcholinesterases. We also describe genetic and histochemical experiments suggesting that class A and B acetylcholinesterase, controlled respectively by *ace-1* and *ace-2*, play some overlapping functional roles and may occupy some common anatomical sites.

MATERIALS AND METHODS

Chemicals: Sodium deoxycholate (DOC), bovine serum albumin (BSA), bovine catalase, Tris [(Tris hydroxymethyl) aminomethane], and Triton X-100 (TRX) were from Sigma Chemical Co. (St. Louis, MO). Ethyl methanesulfonate (EMS) was from Eastman Kodak (Rochester, NY). Aldicarb [2-methyl-2-(methylthio) propionaldehyde-O-(methyl carbamoyl) oxime: UC21149] was the kind of gift of Union Carbide, Agricultural Products Division, Salinas, CA. ([³H]-acetyl)-choline chloride was TRA277 from Amersham/Searle Corp., Arlington Heights, IL. All other chemicals were of standard reagent grade.

Media: NGM solid culture medium (BRENNER 1974) and S medium for liquid culture (SULSTON and BRENNER 1974) have been described previously.

Nematodes: Except for the preparation of extracts for biochemical characterization all nematodes were grown at 20° on NGM agar petri dishes with an *E. coli* strain OP50 as food source

(BRENNER 1974). Extracts were prepared from nematodes grown at 20° in S medium, with a concentrated *E. coli* strain NA22 suspension as food source (SULSTON and BRENNER 1974). The nematode strains used were:

- N2: wild type
- PR1000: *ace-1(p1000) X*
- GG xxx: strains isolated and described in this study.
- CB 61: *dpy-5(e61) I* (originally E61; BRENNER 1974)
- CB 128: *dpy-10(e128) II* (originally E128; BRENNER 1974)
- CB 364: *dpy-18(e364) III* (originally E364; BRENNER 1974)
- CB 1166: *dpy-4(e1166) IV* (originally E1166; BRENNER 1974)
- CB 224: *dpy-11(e224) V* (originally E224; BRENNER 1974)
- CB 950: *unc-75(e950) I* (originally E950; BRENNER 1974)

Strains have been numbered to conform with a recently agreed-upon nomenclature system for *C. elegans* (HORVITZ *et al.* 1979).

Synchronously grown animals were obtained by a plate-washing method based on the selective adhesion of eggs to the agar growth surface (BYERLY, CASSADA and RUSSELL 1976); populations hatching in a short (2 to 4 hr) interval were used.

Mutagenesis: A population of synchronous L1 juveniles, hatched during a 2-hr time period, was grown for 6 hr at 20° and then placed into 1% ethyl methanesulfonate (EMS) in S medium for 5 hr at room temperature. After washing with S medium, 10 mutagenized hermaphrodites were placed on each of 80 plates. From each plate, approximately 200 F₁ progeny were transferred with paper strips to fresh plates, allowed to lay eggs for 24 hr at room temperature, then gently washed off with sterile water, leaving the eggs to hatch. F₂ progeny were screened visually for morphological and behavioral alterations without prior selection.

Genetic analysis: Standard techniques described by BRENNER (1974) were used for all genetic analyses. Segregation frequencies were determined by allowing adults to lay eggs for one or more successive 24 hr periods, then assaying all the individuals that hatched. Slow-growing individuals that may segregate from a heterozygote are not missed by this method, but the possible influence of parental age on recombination frequencies (ROSE and BAILLIE 1979) is not controlled.

Enzymatic assays: Acetylcholinesterase assays were performed by the radiometric, single-vial liquid extraction method of JOHNSON and RUSSELL (1975), modified only by the substitution of 10% butanol for 10% isoamyl alcohol in the organic scintillation phase, and sometimes by the use of [³H]-labelled acetylcholine, undiluted by unlabelled acetylcholine, at a final concentration of 1 × 10⁻⁶ M and specific activity of 250 mCi/mmol. In the latter case, as in the accompanying paper (JOHNSON *et al.* 1981), correction was made for substrate depletion. "Individual assays" were as described by JOHNSON *et al.* (1981). For measurement of DOC-resistant activity, assays were preceded by a 1-hr incubation in 0.4% DOC, but 0.2% DOC was present in the assay. For measurement of TRX-insensitive activity, 0.007% Triton X-100 was present during the assay. These conditions are based on the detergent-sensitive properties of separated *C. elegans* acetylcholinesterase forms (JOHNSON and RUSSELL, in press).

Bovine catalase, used as a sedimentation marker, was assayed by following the decrease in optical density at 240 nm, due to H₂O₂ depletion, at pH 7.0, 23°.

Behavioral assays: Individual assays for chemotaxis (WARD 1974), thertotaxis (HEDGECOCK and RUSSELL 1976) and osmotic avoidance (CULOTTI and RUSSELL 1978) have all been described. Response to mechanical stimulation was determined by tapping the tail or snout with a hand-held 25-gauge hypodermic needle.

Preparation of extracts: Liquid cultures of nematodes were grown in S medium with *E. coli* strain NA22 as food source. Animals were cleaned by centrifugation at 4°, followed by flotation on 35% sucrose (SULSTON and BRENNER 1974). After further washes with water to remove the sucrose, the animals were pelleted by centrifugation, frozen in liquid nitrogen and stored in liquid nitrogen or at -60°. Homogenization was carried out by powdering pellets frozen in liquid nitrogen with a cooled mortar and pestle (JOHNSON and RUSSELL, in press). All subsequent operations were performed at 0-4°. Freeze-powdered nematodes were extracted overnight in 2-3

volumes of 50 mM borate buffer, pH 8.8, sonicated and centrifuged at 20,000 rpm ($41,000 \times g$) in a Sorvall SE-12 rotor for 20 min. About 60% of the total activity was found in the low-speed supernatant, and approximately 80% of this activity was recovered in the supernatant following a high speed spin at 65,000 rpm in a Beckman Type 65 fixed-angle rotor. There was nearly 100% recovery of activity in all sucrose gradients.

Histochemical staining: Whole mounts of mutant and wild-type animals were stained for acetylcholinesterase activity with acetylthiocholine as substrate by the method of KARNOVSKY and RORS (1964), with the following modifications. Animals were washed from a growth plate with water, then treated with 1.5% glutaraldehyde in 100 mM maleate buffer, pH 6.0, for 1.5 hr at 5°. The glutaraldehyde was removed with several washes of maleate buffer. This was followed by a 3-min treatment with 95% acetone to permeabilize the cuticle. The acetone permeabilization was terminated by diluting and washing with excess maleate. A mixture containing 5 mg acetylthiocholine, 6.5 ml 100 mM maleate buffer, pH 6.0, 0.5 ml 100 mM sodium citrate, 1.0 ml 30 mM CuSO_4 , 1.0 ml H_2O and 1.0 ml 5 mM potassium ferrocyanide was then added to the animals and allowed to incubate at room temperature for several hr before mounting in water for light microscopy and photography.

RESULTS

Isolation of acetylcholinesterase double mutants: As explained above, in searching for new *C. elegans* mutants affected in class B acetylcholinesterases, we chose to begin with the strain PR1000, homozygous for the *ace-1* allele *p1000* and deficient in class A acetylcholinesterases. This strain, which has normal behavior, was mutagenized with EMS, as described in MATERIALS AND METHODS, and from F_2 progeny of 140 independent mutagenized lines, a total of 487 mutant derivatives representing a variety of morphological and behavioral phenotypes were isolated by visual screening. Clones of each were maintained at 16°.

Since we desired a reasonably accurate measure of acetylcholinesterase specific activity for each clone, we chose to use the "individual assay" described in JOHNSON *et al.* (1981), in which measured activity can be normalized to the counted number of worms producing the activity. In the hope of avoiding unnecessary age-synchronization steps, we first used the individual assay to measure acetylcholinesterase levels in synchronous population of N2 (wild type) and PR1000, grown at 20°. The results are shown in Figure 1. In both N2 and PR1000, there is a continuous rise in activity per worm from hatching to adulthood (50 hr), but thereafter, especially in PR1000, the activity level varies relatively little with age. We concluded therefore, that individual assays on unsynchronized adults would provide an acceptable and convenient method of screening the newly isolated mutant clones, at least for potential major changes in acetylcholinesterase specific activity.

For each mutant clone, the total acetylcholinesterase activity of five healthy adults was determined by individual assay. The results are summarized, in large part, in Figure 2, which includes data for the 341 clones assayed by a standard procedure, arrived at partway through the screen (5 adults per vial, assayed for 2 hours at room temperature (22–24°); the remaining 146 clones, because they were assayed for more than 2 hr, are not included in this summary). Five clones with abnormally high acetylcholinesterase levels are being studied further. The four strains with abnormally low levels—GG192, GG193, GG194 and GG195—

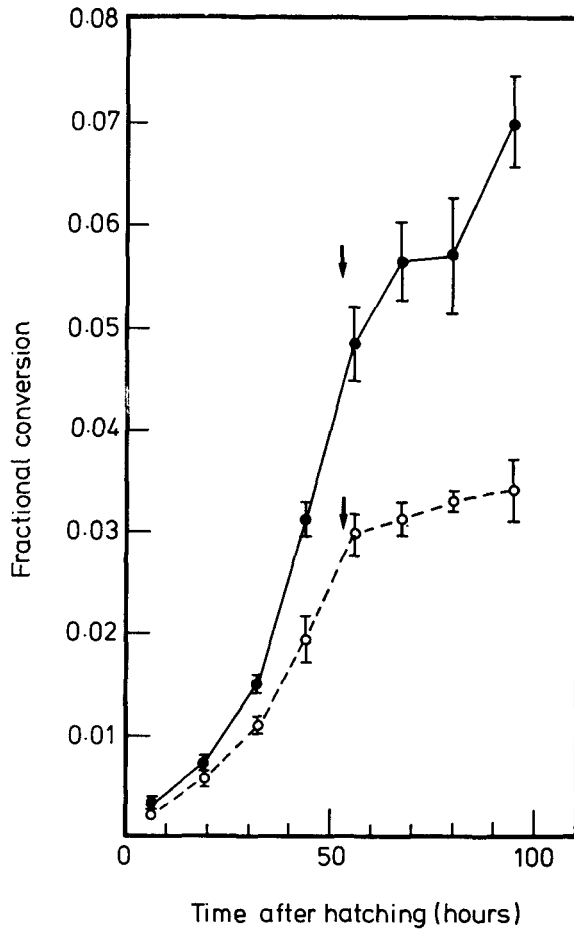


FIGURE 1.—Acetylcholinesterase levels during post-embryonic development of N2 (+) and PR1000 (*ace-1*). N2 and PR1000 juveniles, hatched synchronously as described in MATERIALS AND METHODS, were grown at 20°; at the indicated times, a counted number of individuals were removed and assayed at 23° by individual assay (JOHNSON *et al.* 1981) for 2 or 6 hours (depending on the number of individuals per vial). Arrows mark the beginning of egg laying. Activity is expressed as the corrected fraction of substrate converted (f_{corr}) per individual per hour. Error bars represent the standard errors for 6 assays. —●—●—, N2; ---○---○---, PR1000.

are described in more detail here. Strains GG192 and GG193 were not independently isolated and are probably siblings carrying identical mutations. The same is true for GG194 and GG195, but these were derived independently of GG192 and GG193. Only GG193 and GG194 have been characterized extensively.

Strains GG192, GG193, GG194 and GG195 all had approximately 2% of the wild-type levels of acetylcholinesterase activity (Table 1), and all had the same uncoordinated (*Unc*) phenotype. They all moved forward in a slow and somewhat uncoordinated manner, but were much more severely defective in reversal.

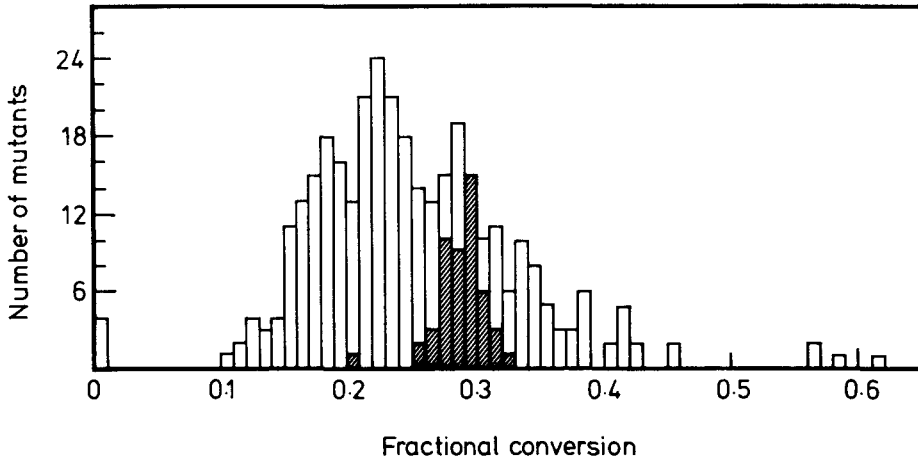


FIGURE 2.—Frequency histogram of mutant acetylcholinesterase activity levels. For each of 341 behavioral and morphological mutants derived from strain PR1000 (*ace-1*), 5 adult animals were collectively assayed for acetylcholinesterase activity in a 2 hour, 22–24° individual assay. Activity is plotted as the corrected fractional conversion of substrate, f_{corr} , and mutants are grouped in activity intervals of 0.01. Cross-hatched bars are for the parental strain PR1000.

TABLE 1

Levels and detergent sensitivities of acetylcholinesterase in certain strains

Strain	Acetylcholinesterase activity		
	Total	Resistant to DOC	Insensitive to TRX
N2 (wild type)	0.573 ± 0.040	0.282 ± 0.013	0.233 ± 0.010
PR1000 (<i>ace-1</i>)	0.273 ± 0.003	0.000 ± 0.000	0.215 ± 0.003
GG202 (<i>ace-2</i>)	0.194 ± 0.011	0.203 ± 0.023	0.024 ± 0.001
GG201 (<i>ace-2; ace-1</i>)	0.012 ± 0.003	—	—
GG194 (<i>ace-2; ace-1</i>)	0.014 ± 0.003	—	—

Animals of the indicated strain were grown for 70 hr at 20° from the time of hatching, transferred into 40 μ l of buffer in vials (5 per vial), freeze-thawed, incubated overnight at 25° and assayed, as described by JOHNSON *et al.* (1981). One hour before initiating the assay, each vial received 10 μ l of H₂O (for measurements of total activity), 10 μ l of 2% DOC (for measurement of DOC-resistant activity) or 10 μ l of 0.1% Triton X-100 (for measurements of TRX-insensitive activity). Assays were then carried out for 2 hr at 23°. The values reported are the means, \pm standard errors, of 10 assays each, expressed as the corrected fractional conversion of substrate, f_{corr} . Strains GG192, GG193, GG193* and GG195 gave results very close to those for GG201 and GG194 (data not shown).

When tapped on the snout they did not simply reverse the direction of sinusoidal wave propagation, as the wild type does, but instead became hypercontracted and temporarily paralyzed. The paralysis lasted for as long as a minute, depending on the number and severity of head taps used to induce it. Recovery of head movement usually preceded movement of the body. The hypercontracting Unc phenotype of all four strains was easy to distinguish from wild-type behavior, and was used for scoring mutant segregants in the genetic tests described below. (Other mutants with somewhat similar phenotypes have been found, but on close examination these phenotypes have always proved to be distinguishable

from that of GG192-195, and the corresponding mutant strains have not been acetylcholinesterase-deficient.)

Genetic characterization of double mutants: Strain GG193 was characterized most extensively. Since GG193 presumably still contained its initial *ace-1* mutation, in addition to a new mutation conferring its Unc phenotype, it was crossed to PR1000 (*ace-1*) males to examine the behavior of the new mutation in an *ace-1* mutant background. Among the F₁ progeny were a number of normally coordinated (non-Unc) animals, indicating that the new mutation was recessive. Because approximately half of these non-Unc progeny were males, the new mutation appeared to be autosomal. The non-Unc F₁ hermaphrodites were cloned, and their progeny were scored for the Unc phenotype, with the results shown in Table 2A. Very nearly one-fourth of these F₂ progeny were Unc, and all Uncs had the characteristic hypercontracting phenotype of GG193, suggesting that this Unc phenotype was produced either by a single new mutation or by closely linked mutations. To determine whether the Unc phenotype might have been separated from the acetylcholinesterase deficiency of GG193, 58 Unc and 50 non-Unc F₂ progeny were assayed for acetylcholinesterase by the individual assay. All 58 Unc animals were, like GG193, markedly deficient in acetylcholinesterase (Ace); whereas, all 50 non-Unc animals had significant levels of acetylcholinesterase activity (non-Ace), indicating that separation had not occurred. Thus in an *ace-1* background, both characteristics of GG193 (Unc and Ace phenotypes) were due either to the same single new mutation or to very closely linked new mutations. This new mutation (or set of mutations) will be referred to hereafter as the *ace-2* mutation(s).

To examine possible interactions between the *ace-1* background and the new *ace-2* mutation(s), GG193 hermaphrodites were crossed to N2 (wild-type) males. Again, non-Unc F₁ animals were observed. One non-Unc hermaphrodite was cloned and all of its F₂ progeny were scored for the Unc phenotype, as shown in Table 2B. Again, Unc and non-Unc F₂ progeny were obtained, and again the Unc animals (as judged by enzymatic assay of their progeny) were all Ace. In this case, however, the Unc animals were much less frequent among the F₂; they

TABLE 2

Phenotypic ratios of progeny segregating from certain hermaphrodites

Parents of hermaphrodite	Classes among progeny of hermaphrodite		Unc
			Total
PR1000 ♂ × GG193 ♀ (Unc, Ace)	non-Unc	Unc	0.278
	2235 (50/50 non-Ace)	655 (58/58 Ace)	
N2 ♂ × GG193 ♀ (Unc, Ace)	non-Unc	Unc	0.067
	375 (97/97 non-Ace)	27 (27/27 Ace)	

The origins of the strains used are given in the text. Capital-letter phenotype designations are: Unc, uncoordinated; Ace, less than 5% of wild-type levels of acetylcholinesterase; non-Ace, significant levels of acetylcholinesterase.

constituted only 27 out of a total of 402 F₂, a frequency of approximately 1/16. This low Unc segregation frequency suggested that the Unc phenotype of GG193 might depend not only on the new *ace-2* mutation(s), but also on the original *ace-1* mutation, as well.

Confirmation of this suggestion was sought as follows. One of the F₂ Unc segregants was selected for further work and called GG193*; this strain was apparently *ace-2*; *ace-1*, like its GG193 grandparent, but had had the opportunity to lose potential background genetic changes by virtue of its derivation from a backcross to wild type. Hermaphrodites of this strain were crossed to N2 males, and non-Unc F₁ hermaphrodites were picked and allowed to self-fertilize. As expected from Table 2B, most of the resulting F₂ progeny were non-Unc (1306/1395), and 180 of these non-Unc animals were further classified by two criteria. First, the frequency of Unc segregants in the F₃ generation was determined for each F₂. As Table 3 shows, non-Unc animals would be expected to segregate either no Uncs, 1/16 Uncs, or 1/4 Uncs, given that both *ace-1* and *ace-2* must be homozygous mutant to generate an Unc phenotype. As Table 3 also shows, these classes of non-Unc F₂ animals were indeed detected. Furthermore, the number of F₂ animals in each class was close to that expected from the assumption that *ace-1* and *ace-2* assort independently (as would be expected from their respective sex-linked and autosomal locations).

The second criterion for classifying the non-Unc F₂ animals was the detergent sensitivity of the acetylcholinesterase produced by their F₃ progeny. This classi-

TABLE 3
Segregation frequencies among F₂ progeny from cross of
N2 ♂ × GG193* ♀ (*ace-2*; *ace-1*)

Phenotype	F ₂ classes		Expected frequency among F ₂	Composition of F ₃ progeny	Number among 180 non-Unc F ₂ examined	
		Genotype			Expected	Observed
non-Unc	}	+ / + ; + / +	1/16	0 Uncs	84	83
		+ / + ; <i>ace-1</i> / +	1/8			
		+ / + ; <i>ace-1/ace-1</i>	1/16			
		<i>ace-2</i> / + ; + / +	1/8			
		<i>ace-2/ace-2</i> ; + / +	1/16			
	<i>ace-2</i> / + ; <i>ace-1</i> / +	1/4	1/16 Uncs	48	51	
}	<i>ace-2</i> / + ; <i>ace-1/ace-1</i>	1/8	1/4	1/4 Uncs	48	46
	<i>ace-2/ace-2</i> ; <i>ace-1</i> / +	1/8				
Unc		<i>ace-2/ace-2</i> ; <i>ace-1/ace-1</i>	1/16	all Uncs	—	—

Expectations are based on the assumption that GG193 (and its singly backcrossed derivative GG193*) is an *ace-2*; *ace-1* double mutant in which the *ace-1* and *ace-2* mutations are unlinked and the Unc phenotype depends on homozygosity for both mutations. The F₁ animals, identified as non-Unc ♀, were presumably *ace-2*/+; *ace-1*/+; as expected they segregated very nearly 1/16 Uncs (89/1395). For each non-Unc F₂, an average of 80 F₃ progeny were examined to determine the frequency of Uncs; division into the classes segregating 0 Uncs, 1/16 Uncs, and 1/4 Uncs was unambiguous.

fication was based on two observations. First, the class B acetylcholinesterases of *C. elegans* (which are intact in PR1000) are irreversibly inactivated by the ionic detergent sodium deoxycholate (DOC); whereas, two of the three class A acetylcholinesterases are quite resistant to DOC (JOHNSON and RUSSELL, in press; JOHNSON *et al.* 1981). Second, class A acetylcholinesterases are quite sensitive to inhibition by the non-ionic detergent Triton X-100 (TRX); whereas, class B acetylcholinesterases are much more resistant (JOHNSON and RUSSELL, in press). These complementary sensitivities permit the independent assay of class A or class B activity levels in the individual assay. The starting strain, PR1000, for example, exhibits DOC-sensitive and TRX-resistant activity (Table 1), as expected for a strain containing class B activity exclusively. Similarly, among the non-Unc F₂ segregants should be some *ace-1* (but not *ace-2*) homozygotes that should have, and should produce progeny having, acetylcholinesterase with a similar pattern of detergent sensitivity. Conversely, there should also be non-Unc F₂ segregants that are *ace-2* (but not *ace-1*) homozygotes, and these would be expected to have, and to produce F₃ progeny having the complementary pattern of detergent sensitivity (DOC-resistant and TRX-sensitive).

As Table 4 shows, when the detergent sensitivity criterion was used to classify the non-Unc F₂ animals, apparent *ace-1* and *ace-2* homozygotes were indeed found in very nearly the expected numbers. Furthermore, when these apparent homozygotes were further subdivided by the Unc segregation classification described above, all were found to segregate either no Uncs or 1/4 Uncs, as predicted. In addition, every non-Unc F₂ that segregated 1/4 Uncs was either an *ace-1* or an *ace-2* homozygote by the detergent sensitivity criterion.

These results confirmed the identification of the apparent *ace-1* and *ace-2* homozygotes, thereby substantiating the suggestion that both the Unc and the Ace phenotype of GG193 depend on homozygosity for both the *ace-1* and the *ace-2* mutations. In view of the continued failure of the two GG193 phenotypes (Unc and Ace) to separate from one another, it was tentatively assumed that a single new *ace-2* mutation was involved in producing both, and this was given the name *g72*. One of the Unc F₂ segregants, now the result of two backcross cycles to wild type, was selected for further work and named GG201 (*ace-2*; *ace-1*). One apparent *ace-2* homozygote that segregated no Uncs in the F₃ generation was saved and called GG202 (*ace-2*).

Further proof that GG202 was indeed an *ace-2* homozygote was sought by attempting to reconstruct an *ace-2*; *ace-1* double mutant from GG202 and the original strain PR1000 (*ace-1*). Five spontaneously arising GG202 males were mated individually to *dpy-5*; *ace-1* hermaphrodites. Three to five non-Dpy progeny from each mating were cloned and checked for the segregation of Uncs in the F₂ generation. In all cases, the F₁ animals segregated Uncs, and at very nearly the expected frequency of 1/16. Moreover, all 140 Uncs segregating in the F₂ had the same hypercontracting phenotype as the original double mutant, and all 77 assayed were Ace, as well. Therefore, these Uncs were presumably the desired *ace-2*; *ace-1* double mutants. These results show that GG202 is indeed an *ace-2* homozygote and further support the idea that *ace-1* and *ace-2* must both

TABLE 4

Detergent sensitivity of acetylcholinesterase among F₂ progeny from cross of N2 ♂ × GG193 ♀ (ace-2; ace-1)*

Phenotype	F ₂ classes		Expected frequency among F ₂	Sensitivity of F ₃ progeny acetylcholinesterase to		Number among 184 non-Unc F ₂ examined	
	Genotype			TRX	DOC	Expected	Observed
non-Unc	+	+/+; ace-1/ace-1	1/16	R	S	37	31
		ace-2/+; ace-1/ace-1	1/8				
	ace-2/	+/+	1/16	S	R	37	31
		ace-2/ace-2; ace-1/+	1/8				
Unc	+	+/+; +/+	1/16	R	R	110	123
		+/+; ace-1/+	1/8				
	ace-2/+; +/+	1/8					
	ace-2/+; ace-1/+	1/4					
Unc	ace-1/ace-2; ace-1/ace-1		1/16	—	—	—	—

Same cross as in Table 3. For each of 184 non-Unc F₂s, 3 vials, each containing 5 F₃ progeny, were assayed by individual assay as described by JOHNSON *et al.* (1981). One hour before the start of the assay, one vial received 10 μ l H₂O (to measure total activity), a second received 10 μ l of 0.1% Triton X-100 (to measure TRX-insensitive activity) and the third received 10 μ l of 2% DOC (to measure DOC-resistant activity). Assays were for 2 hr at 23°. S means sensitive, R means resistant. For some of the genotypes whose F₃ progeny are described as having activity resistant to both detergents, it is expected that a small proportion of their progeny will not fit this description; however, the use of 5 F₃ progeny per assay effectively precludes the possibility that these might obscure the properties of their predominant, doubly resistant siblings. Distinctions between sensitivity and resistance were unambiguous. Expressed as a percentage of total activity, that measured in cases described as resistant averaged approximately 38% (TRX) or 54% (DOC); whereas, that measured in cases described as sensitive averaged approximately 3% (TRX) or 0.6% (DOC). As expected, no non-Unc F₂ clones produced activity sensitive to both detergents.

be homozygous for an animal to be uncoordinated and totally (>98%) acetylcholinesterase-deficient.

Strains GG194 and GG195, although derived independently from GG192 and GG193, showed the same hypercontracting phenotype. In addition, they also lacked more than 98% of the wild-type acetylcholinesterase activity, as shown by the individual assay. When GG194 hermaphrodites were crossed to N2 (wild-type) males, non-Unc F₁ progeny were found, and these segregated approximately 1/16 Uncs (52/980) in the F₂ generation. Moreover, all 52 of these Uncs were Ace; whereas, all 30 of their non-Unc siblings tested were non-Ace.

These similarities to the behavior of GG193 suggested that GG194 might also carry an *ace-2* mutant allele. To test for complementation, males of genotype *ace-2(g72)/+; ace-1/O* were constructed by crossing GG202 males with PR1000 hermaphrodites, as shown in Figure 3. When these were crossed to GG194 hermaphrodites and male progeny were examined, 36 out of 67 (very nearly the expected 1/2) were hypercontracting Uncs, indicating noncomplementation between the new mutation of GG194 and the *ace-2* allele *g72*. GG194, therefore,

TABLE 5

Levels and Triton X-100 sensitivities of acetylcholinesterase in uncoordinated mutants linked to ace-2

Strain	Acetylcholinesterase activity		
	Total	Insensitive to TRX	Percent TRX-insensitive
N2 (wild type)	1.661 ± 0.238	0.514 ± 0.025	31
GG202 (<i>ace-2</i>)	0.997 ± 0.075	0.080 ± 0.005	8
CB47 (<i>unc-11</i>)	1.503 ± 0.169	0.475 ± 0.018	32
CB1460 (<i>unc-89</i>)	1.768 ± 0.096	0.483 ± 0.024	27
CB936 (<i>unc-73</i>)	1.825 ± 0.186	0.555 ± 0.040	30
CB904 (<i>unc-38</i>)	1.532 ± 0.164	0.383 ± 0.021	25
CB883 (<i>unc-74</i>)	1.306 ± 0.170	0.474 ± 0.020	27
DR49 (<i>unc-35</i>)	1.993 ± 0.183	0.541 ± 0.083	27
CB384 (<i>unc-63</i>)	1.235 ± 0.023	0.449 ± 0.024	36
CB406 (<i>unc-57</i>)	1.578 ± 0.058	0.433 ± 0.020	27

Synchronously hatched animals of the indicated strain were grown at 20° until they were gravid, egg-laying adults, transferred into 50 µl of 10 mM borate buffer pH 8.8 in vials (5 adults per vial), freeze-thawed, incubated overnight at 25° and assayed as described by JOHNSON *et al.* (1981). One hour before initiating the assay, each vial received 10 µl of H₂O (for measurements of total activity), or 10 µl of 0.1% Triton X-100 (for measurements of TRX-insensitive activity). Assays were then carried out for 5 hr at 25° in a total volume of 110 µl. The values reported are the means, ± standard errors, of 3 to 5 assays each, expressed as the corrected fractional conversion of substrate, *f*_{corr}.

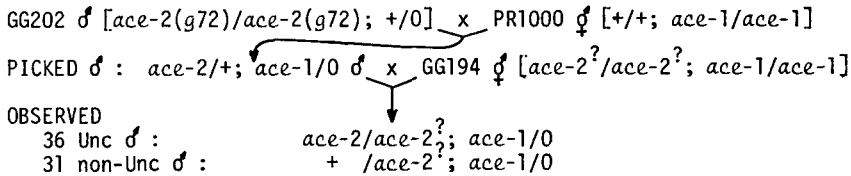


FIGURE 3.—Test for allelism between the new mutation of GG194 and the *ace-2* allele *g72* of GG202. The new mutation of GG194 is called *ace-2[?]*. The presence of approximately 50% Unc males in the final progeny indicates noncomplementation.

is also an *ace-2; ace-1* double mutant, and the *ace-2* mutation it carries is an independently derived allele, now called *g73*.

Mapping the gene ace-2. Possible linkage of *ace-2* to standard *dpy* markers on each of the five *C. elegans* autosomes (BRENNER 1974) was tested as follows. Males of genotype *dpy-x/+* were constructed for each of the five *dpy* markers, as shown in Figure 4A. Then, triple heterozygotes of genotype *ace-2 . . . +/+ . . . dpy-x; ace-1/+* were constructed by crossing these males with GG201 (*ace-2; ace-1*) hermaphrodites. Finally, these triple heterozygotes were allowed to self-fertilize, and their Dpy progeny were then scored for the hypercontracting phenotype characteristic of the *ace-2; ace-1* double homozygote. Only for *dpy-5 I* was there evidence for linkage (a frequency of much less than 1/16 Uncs among the Dpys). A rare recombination event between *ace-2* and *dpy-5* generated an *ace-2 dpy-5; ace-1* triple mutant, which was crossed with PR1000 males to pro-

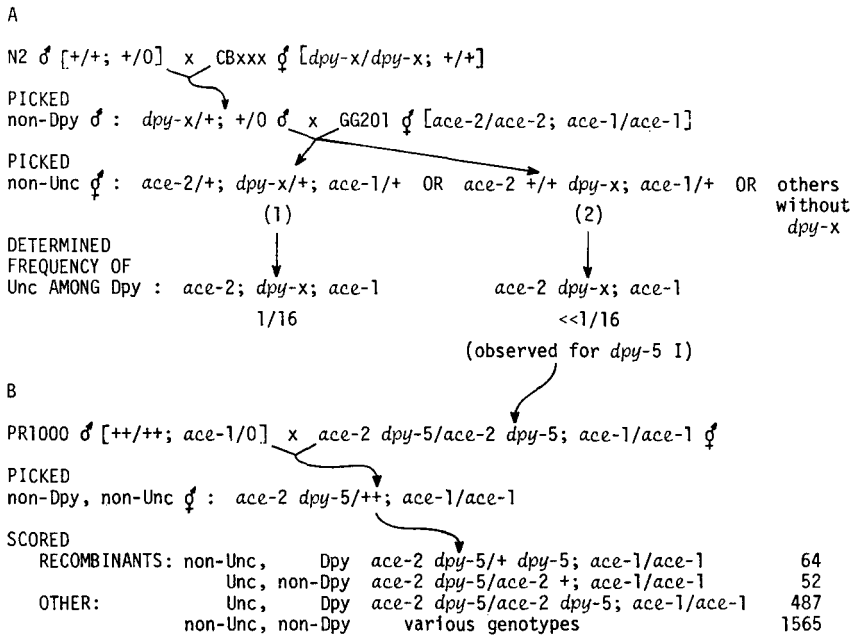


FIGURE 4.—Mapping the gene *ace-2*. (A) Chromosomal location. The symbol *dpy-X* represents one of five standard *dpy* markers for the five *C. elegans* autosomes (*dpy-5 I*, *dpy-10 II*, *dpy-18 III*, *dpy-4 IV*, and *dpy-11 V*). The result indicated by (1) is that expected for nonlinkage, that indicated by (2) is expected for linkage (as in fact observed for *dpy-5 I*). (B) Mapping on chromosome *I*. The scorable recombinants (non-Unc, Dpy and Unc, non-Dpy) are represented almost entirely by the indicated genotype (in which the chromosome opposite the recombinant one is the parental *ace-2 dpy-5*). Their cumulative frequency (116/2168) was used to calculate a map distance of 5.5 ± 0.9 centimorgans, using the formula given by BRENNER (1974).

duce animals of genotype *ace-2 dpy-5/+ +*; *ace-1*, as shown in Figure 4B. Among the self-progeny of these animals, the frequency of the recombinant types Unc, non-Dpy and non-Unc, Dpy was 116/2168, giving a map distance of 5.5 ± 0.9 centimorgans between *ace-2* and *dpy-5*.

A three-factor cross, using the righthand marker *unc-75*, was carried out to determine the direction of *ace-2* from *dpy-5*, as shown in Figure 5. Animals of genotype *ace-2 dpy-5 +/+ + unc-75* were constructed as shown and allowed to self-fertilize. One hundred Unc, non-Dpy progeny were picked and cloned; only 17 of these segregated Dpy progeny. The Dpy progeny from all 17 Uncs proved by enzymatic assay to be *ace-2* homozygotes, indicating that the *ace-2* locus is to the left of *dpy-5*. The approximate map position of the *ace-2* locus on chromosome *I* is shown in Figure 6.

The map data presented above are not sufficient to rule out the possibility that *g72* and *g73* are alleles of a previously identified *unc* gene located in the same region of chromosome *I*. Although the *ace-2* alleles described here produce an uncoordinated phenotype only in combination with an *ace-1* mutant allele, it is nevertheless possible that more severely defective alleles of *ace-2* might themselves produce an uncoordinated phenotype. The possibility that *g72* and *g73*

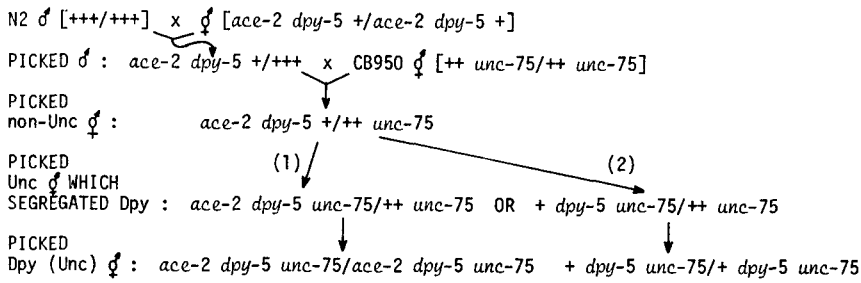


FIGURE 5.—Three-factor cross to order *ace-2*, *dpy-5* and *unc-75*. The starting *ace-2 dpy-5* strain was derived from the *ace-2 dpy-5*; *ace-1* strain whose origin is shown in Figure 4. In the penultimate generation, the genotype labelled (1) is the principal one expected if *ace-2* is to the left of *dpy-5*; it should segregate Dpy (Unc) animals, virtually all of which should be *ace-2* homozygotes. Conversely, the genotype labelled (2) is that expected for the opposite orientation, and should segregate Dpy (Unc) animals, virtually all of which should be *ace-2*(+) homozygotes.

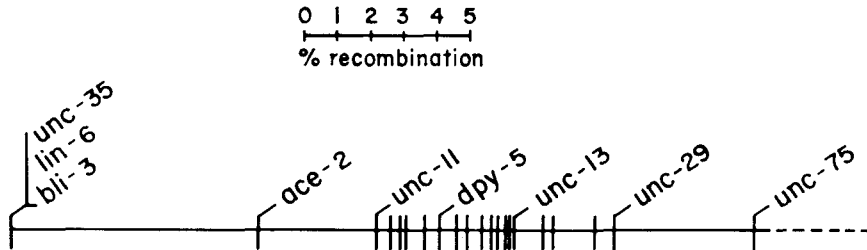


FIGURE 6.—Partial map of *C. elegans* chromosome I. Only the left half is shown, and only certain mapped genes are labelled. Other mapped genes are indicated by vertical lines only to show that *ace-2* maps to the left of the prominent gene cluster. The map is derived from a compilation of data from several laboratories by R. K. HERMAN (personal communication), to whom we are grateful.

are alleles of a previously identified *unc* gene has therefore been examined in several ways. For example, we would expect homozygotes of equivalent *unc* alleles to be missing at least as much class B, TRX-resistant acetylcholinesterase activity as homozygotes of the *ace-2* alleles. Strains homozygous for representative alleles of all of the closely-linked *unc* genes (*unc-11*, *unc-63*, *unc-57*, *unc-74*, *unc-38*, *unc-73*, *unc-89* and *unc-35*) were therefore tested by the individual assay for TRX-resistant acetylcholinesterase activity, with the results shown in Table 5. All of the *unc* strains had levels of TRX-resistant acetylcholinesterase activity similar to that of the wild-type control.

In addition, all of these strains, including N2, PR1000 and GG202, were tested for their developmental sensitivity to Aldicarb, to which all of the *unc* mutants exhibited the same developmental response as wild type (*i.e.*, growth and reproduction at 50 μ M and 100 μ M, but no growth or reproduction at 200 μ M and 400 μ M). GG202, however, was much more sensitive to this inhibitor (*i.e.*, no growth or reproduction at any of the inhibitor concentrations tested). Finally, males heterozygous for *g72* and each of the representative *unc* alleles were constructed and found to exhibit wild-type coordination and to have wild-type levels of class

B, TRX-resistant acetylcholinesterase activity. *ace-2* is therefore a newly identified gene and is not the same as any of the known *unc* genes on chromosome I. Further mapping studies using a number of duplications and deficiencies of this region of chromosome I (A. ROSE, personal communication) are underway.

Enzymatic deficiency of ace-2 mutants: Two observations suggested that *ace-2* mutations had a selective effect on the class B acetylcholinesterases of *C. elegans*. First, in the initially isolated *ace-2 ace-1* double mutants, the newly arising *ace-2* mutation almost completely eliminated the acetylcholinesterase activity present in the original *ace-1* mutant strain PR1000 (Table 1), and this activity was previously shown to belong to class B only (JOHNSON *et al.* 1981). Second, when animals homozygous for *ace-2* alone were obtained, their acetylcholinesterase activity showed a pattern of detergent sensitivity characteristic of the class A forms, with no indication of class B activity (Table 4). To examine this point further, we analyzed the easily solubilized acetylcholinesterase activity in extracts of N2 (wild type), PR1000 (*ace-1*) and GG202 (*ace-2*) in parallel, by a combination of sucrose gradient sedimentation and detergent sensitivity. Figure 7 shows the results.

In N2 there are two major sedimentation peaks, at about 13s and 5s, corresponding respectively to form IV and forms IA and IB (see JOHNSON and RUSSELL, in press). In addition there is a small, more rapidly sedimenting ($\sim 7s$) shoulder on the 5s peak, corresponding to form II (which extracts quite inefficiently without detergent; JOHNSON and RUSSELL, in press). As expected, form IV is resistant to DOC and sensitive to TRX. Conversely, the 5s peak is sensitive to DOC and largely resistant to TRX, as expected if it were to contain predominantly form IB with a minority of form IA (see JOHNSON and RUSSELL in press).

In PR1000, in confirmation of the accompanying paper (JOHNSON *et al.* 1981), the 13s peak is missing. In addition the ($\sim 7s$) shoulder is more pronounced (and thereby reveals clearly its resistance to TRX), and the 5s peak, although still sensitive to DOC, is more fully resistant to TRX, as expected if it contained only form IB and not form IA.

In GG202 the 13s peak is present and, as in N2, resistant to DOC. There is also a 5s peak, but this is much reduced and, more significantly, is now totally sensitive to TRX, as if it contained only form IA and not IB. In addition, there is no peak of TRX-resistant ($\sim 7s$) activity (form II).

These results demonstrate the complementarity of the *ace-1* and *ace-2* mutations and are consistent with the notion that the *ace-2* allele *g72* selectively eliminates the class B acetylcholinesterase forms IB and II.

Gene dosage experiments: Since the *ace-2* mutation *g72* appeared to affect class B acetylcholinesterase forms selectively, it was of interest to determine whether *ace-2* might be a structural gene for these forms. Table 6A presents individual assay data for animals carrying 0, 1 or 2 copies of the *ace-2*(+) allele in a homozygous *ace-1* background. Clearly, the amount of residual acetylcholinesterase activity is very nearly proportional to the number of *ace-2*(+) gene copies present, suggesting that *ace-2* is indeed most probably a structural gene for the class B acetylcholinesterase forms.

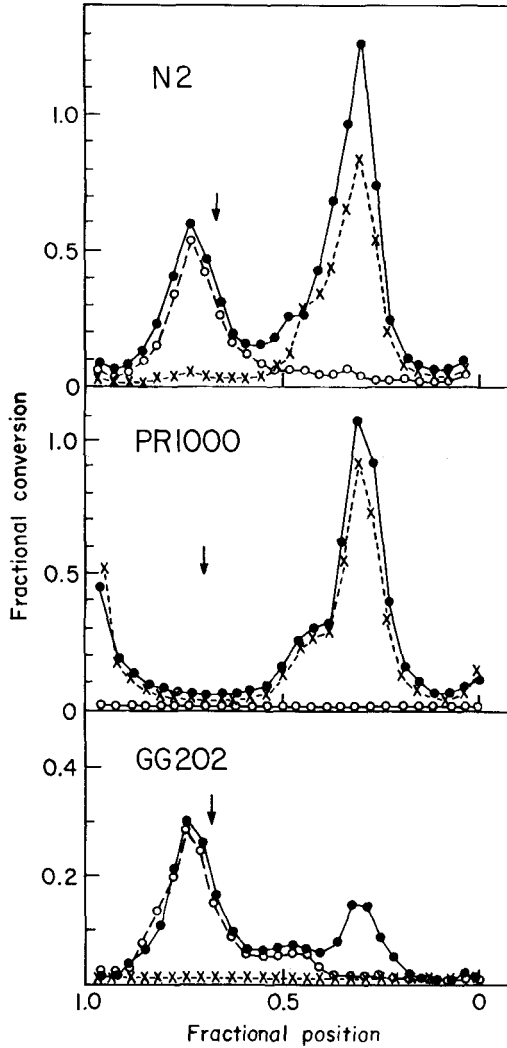


FIGURE 7.—Sucrose gradient sedimentation patterns and detergent sensitivities of soluble acetylcholinesterase from N2, PR1000 and GG202. Approximately 0.5–1.0 g wet weight of nematodes (asynchronous populations) were freeze-powdered 3 times, resuspended in 2 volumes of 50 mM borate buffer, pH 8.8, and incubated for 20 hr. at 5°. Extracts were then sonicated briefly and spun for 20 min at 48,000 \times g, and aliquots of the supernatants were loaded onto 5 ml, 5–20% sucrose gradients containing 10 mM borate buffer, pH 8.8, plus 1 mg/ml BSA. After centrifugation for 6.5 hr at 65,000 rpm (SW65 rotor, Beckman L265B ultracentrifuge), fractions were collected by pumping from the bottom of the gradient (fractional position 1.0) and assayed either without detergent treatment (—●—●—); after 1 hr treatment at 23° with 0.4% DOC, followed by 0.2% in the assay (—○—○—); or with 0.007% TRX in the assay (—×—×—). Acetylcholinesterase activity is expressed as the corrected fractional conversion of substrate, f_{corr} . Arrows mark the position of bovine catalase (11.3s), used as a sedimentation marker.

TABLE 6

Dependence of acetylcholinesterase levels on dosage of genes ace-1(+) and ace-2(+)

Genotype	Strain number	Number of <i>ace-1</i> (+) Gene copies	Number of <i>ace-2</i> (+) Gene copies	Acetylcholinesterase activity	
				Total	Percent of case with 2 copies
<i>ace-2/ace-2; ace-1/ace-1</i>	GG201	0	0	0.003 ± 0.001	1.7 ± 0.6
A. <i>ace-2/ + ; ace-1/ace-1</i>		0	1	0.080 ± 0.010	47.0 ± 5.9
<i>+ / + ; ace-1/ace-1</i>	PR1000	0	2	0.170 ± 0.010	100
<i>ace-2/ace-2; ace-1/ace-1</i>	GG201	0	0	0.004 ± 0.001	1.4 ± 0.4
B. <i>ace-2/ace-2; ace-1/ +</i>		1	0	0.160 ± 0.010	57.0 ± 3.6
<i>ace-2/ace-2; + / +</i>	GG202	2	0	0.280 ± 0.010	100

Animals of the indicated genotype were grown for 72 hr at 20° from the time of egg laying, then transferred into vials (5 per vial) and assayed for 1 hr by the individual assay method, as described by JOHNSON *et al.* (1981). The values reported are the means, ± standard errors for 6 assays each, expressed as the corrected fractional conversion of substrate, f_{corr} .

The availability of the *ace-2* mutations also made possible a complementary gene dosage study of *ace-1* in a homozygous *ace-2* background. As Table 6B shows, the results confirm those of JOHNSON *et al.* (1981) and extend them to suggest that *ace-1* is a structural gene not just for the DOC-resistant forms III and IV, but for all class A forms.

Behavioral analysis: Like the *ace-1* mutant strain PR1000 described in JOHNSON *et al.* (1981), the *ace-2* mutant strain GG202 could not be distinguished from wild type by locomotion, growth rate or fecundity under ordinary culture conditions. Even the backcrossed *ace-1 ace-2* double mutant strain GG201, although clearly uncoordinated, exhibited nearly normal growth rate and fecundity under these conditions. Attempts to find extreme conditions that might reveal behavioral or development differences in each single mutant strains have so far, in a limited set of tests, proven unsuccessful.

Individuals of each single mutant strain were tested for their ability to follow isotherms in a radial thermal gradient (HEDGECOCK and RUSSELL 1976), for their ability to orient and migrate up gradients of NaCl (WARD 1974; DUSENBERY, SHERIDAN and RUSSELL 1975) and for their ability to avoid high osmotic strength conditions (CULOTTI and RUSSELL 1978). Both single mutant strains were normal for each of these sensory behaviors. The double mutant strain GG201, although it moves more slowly than wild type or either single mutant, was also able to orient and move up a gradient of NaCl. The double mutant was not tested for thermotaxis or the osmotic response because it moves too slowly to give reliable results in these assays. Both single mutant and the double mutant were normally sensitive to light touch.

Males of both single mutant strains can mate successfully, but their mating behavior has not been quantitatively studied; like other uncoordinated males, those of the double mutant strain do not mate effectively and may not be able to mate at all (not tested).

Because the acetylcholinesterase inhibitor Aldicarb had been shown *in vitro*

to have an approximately 10-fold greater inactivation rate against Class A forms of acetylcholinesterase than against class B forms (JOHNSON and RUSSELL, in press), we tested animals of wild type, both single mutant strains and the double-mutant strain on plates containing a series of Aldicarb concentrations. These concentrations extended downwards from 1 mM, at which wild-type movement, growth and reproduction are blocked. At a concentration of 50 μ M we found that wild type (N2) and the *ace-1* mutant strain PR1000, while noticeably uncoordinated because of the Aldicarb, could nonetheless grow and reproduce successfully; at this same concentration, however, both the *ace-2* mutant strain GG202 and the double-mutant strains GG201 and GG194 were blocked in development shortly after hatching from the egg. To date, this increased Aldicarb sensitivity has been the only *ace-2*-dependent phenotype that can be scored visually on live animals.

Histochemical staining: Figure 8A shows an N2 juvenile animal, chosen to illustrate most of the kinds of staining observed when whole mounts are permeabilized and stained for acetylcholinesterase, as described in MATERIALS AND METHODS. Intense staining was seen in the area of the nerve ring and ventral ganglion, in the pharyngeal-intestinal valve and in the region of the depressor ani muscle of the tail. Somewhat less intense staining could be seen in the ventral nerve cord and in a variety of nonneuronal nuclei in the body and the head. (It is currently unclear whether or not the staining of nonneuronal nuclei is artifactual and, if not, what its significance might be.) Not present in this example, but often observed in older animals, was staining in the dorsal nerve cord resembling that seen in the ventral nerve cord. Also not present in this example, but sometimes observed in younger animals, was staining in the pre-anal ganglion.

The different aspects of this staining pattern had different age dependences and different degrees of reproducibility. The area of the nerve ring and ventral ganglion was the most reproducibly stained, being visible in virtually all treated animals, regardless of age. The nerve ring was also the first structure in which staining could be observed when the progress of staining was followed over time, suggesting that it may have the highest concentration of acetylcholinesterase activity. The pharyngeal-intestinal valve, although somewhat smaller and usually somewhat less intensely stained than the nerve ring, was nonetheless stained in almost all animals. Staining in the other structures was both more variable and more age-dependent. The body and head nuclei stained well in young juveniles, but very poorly (if at all) in older juveniles and adults. Staining of the ventral cord and the pre-anal ganglion was more variable in young juveniles, but when staining did occur in these regions, it was usually intense and was visible as very distinct nuclei. In adults, the ventral cord stained more frequently than in young juveniles, but in contrast to the juvenile pattern, the stain was continuous along the length of the cord and was not localized to nuclei. Similarly, the dorsal nerve cord was not observed to stain at all in young juveniles and was seen first as an almost continuously staining structure about midway through juvenile development. The region of the depressor ani muscle of the tail was observed to stain only in adults, and then only infrequently. Indeed, staining in the posterior portions of adults generally was erratic, presumably because of as yet poorly understood vari-

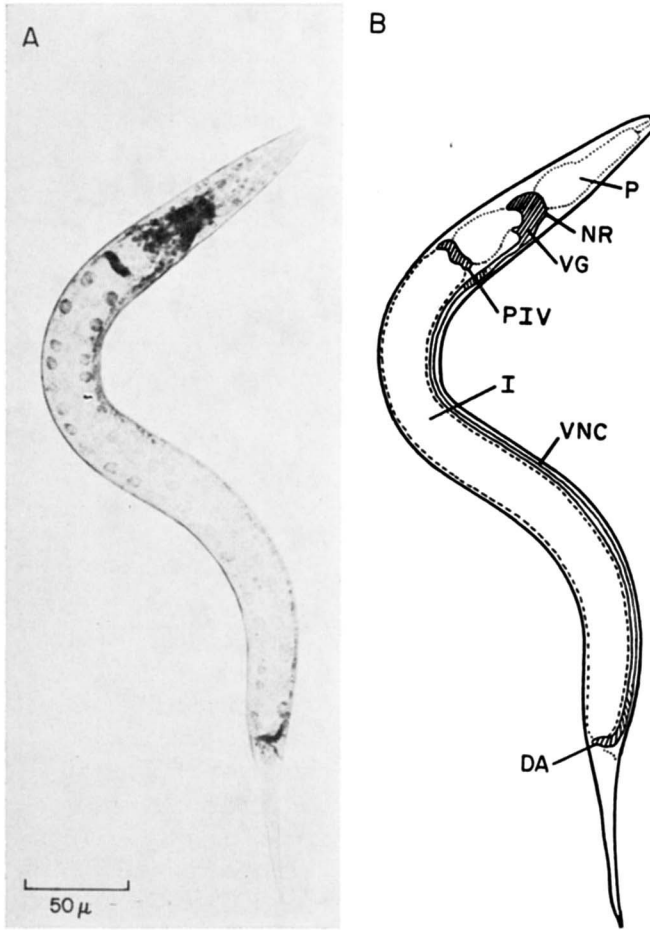


FIGURE 8.—N2 juvenile (early L3 stage) stained for acetylcholinesterase. (A) Photomicrograph (Zeiss Universal microscope, tungsten light source, green filter, Tri-X pan film). (B) Schematic diagram. P, pharynx; NR, nerve ring; VG, ventral ganglion; PIV, pharyngo-intestinal valve; I, intestine; VNC, ventral nerve cord; DA, depressor ani muscle.

ations in the acetone-based permeabilization step. Some of the age-dependent aspects of staining can be seen in Figure 9 (left panels).

Figure 9 also illustrates the effects of the *ace-1* and *ace-2* mutations on the staining pattern. In the *ace-1* mutant strain PR1000, staining resembled quite closely that seen in wild type (N2). At all ages, the nerve ring and the pharyngo-intestinal valve stained intensely and quite reproducibly, and clear examples of adult ventral and dorsal nerve cord staining could easily be found (not shown). In addition, staining in PR1000 young juveniles was primarily nuclear, as in N2. In the *ace-2* mutant strain GG202, the nerve ring also stained at all ages, although not quite so intensely as in N2. Again, examples of adult ventral and dorsal nerve cord staining could be found (not shown), and, again, staining in young

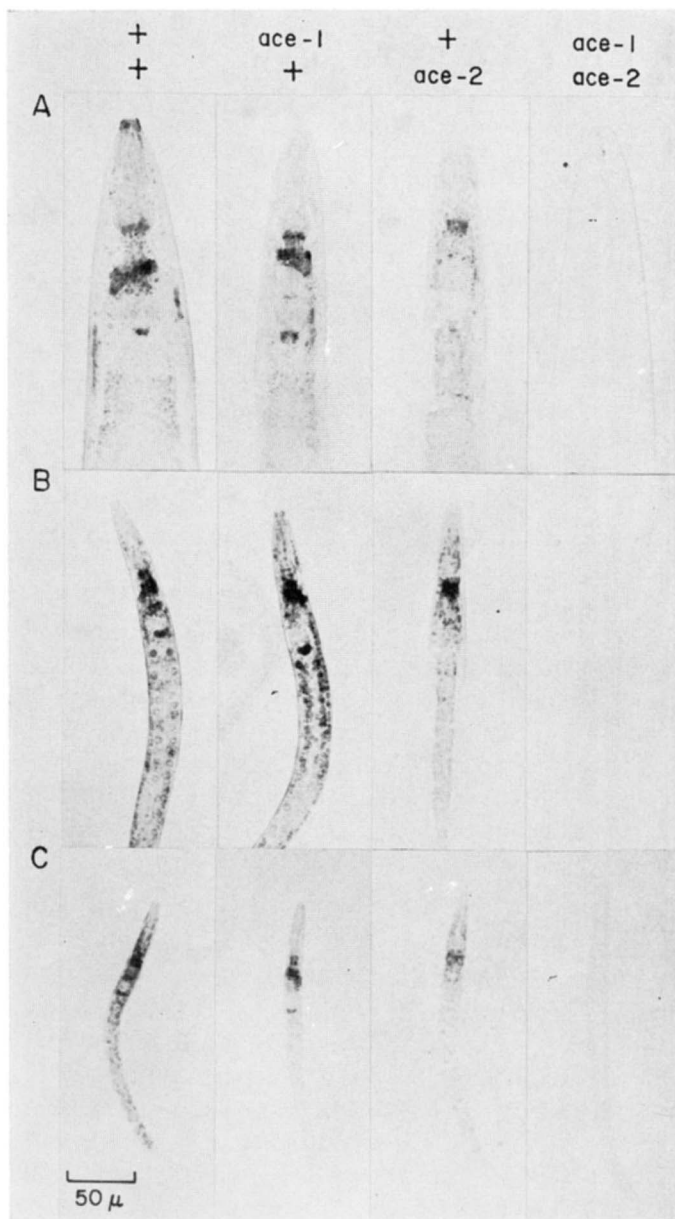


FIGURE 9.—Individuals of several genotypes stained for acetylcholinesterase. (A) Adults. (B) Mid-stage juveniles (L2 stage). (C) Young juveniles (L1 stage); left to right; N2, PR1000, GG202, GG201. For each strain, animals from an unsynchronized population were stained for 8 hr and then examined in a Zeiss Universal microscope; representative animals were chosen for photomicrography. To permit direct comparison, all animals were photographed at the same magnification, with identical illumination (tungsten lamp, green filter, unchanged intensity setting) and on the same film (Tri-X pan) for the same exposure time (1/15 sec). The resulting negatives were then printed identically (on Kodak F5 paper), with the exception that print exposure times were 30–40% longer for GG201 to permit outlines of the animals to be seen.

juveniles was primarily nuclear. However, at no age was there observable staining in the pharyngeointestinal valve of GG202 animals, in marked contrast to N2 and PR1000. Finally, in the *ace-1 ace-2* double-mutant strain GG201, all staining disappeared.

Taken together, these results suggest (1) that the observed staining is due entirely to class A and class B acetylcholinesterases, controlled respectively by *ace-1* and *ace-2*, (2) that both class A and class B acetylcholinesterases show a similar developmental shift from primarily nuclear staining in young juveniles to primarily nonnuclear staining in adults, and (3) that both classes have a similar anatomical distribution, except for the presence of only class B activity in the pharyngeointestinal valve.

We sought to confirm the differential distribution of acetylcholinesterase forms implied by these results by taking advantage of the known thermal sensitivity differences of the wild-type *C. elegans* forms (JOHNSON and RUSSELL, in press). The same two class A forms that are resistant to DOC inactivation *in vitro*; (forms III and IV) are also resistant to inactivation at 45° *in vitro*; furthermore, preliminary experiments (J. LACE, J. CULOTTI and R. L. RUSSELL, unpublished) have established that this same differential resistance occurs for 45° inactivation *in vivo*. When wild-type animals were treated at 45° for a time sufficient to inactivate all but the resistant class A forms III and IV, subsequent staining revealed a pattern essentially identical to that of untreated GG202 animals, which should also possess only class A forms. As expected, similar treatment of PR1000, which possesses only class B forms, completely eliminated staining, and similar treatment of GG202 produced little staining change. These results strongly suggest that the nerve ring and the longitudinal nerve cords contain both class A and class B acetylcholinesterases; whereas, the pharyngeointestinal valve contains only class B activity.

DISCUSSION

The results presented above establish the existence of a second gene, *ace-2*, affecting acetylcholinesterase activity in *C. elegans*. This gene maps to chromosome I and is not linked to the related gene *ace-1* (JOHNSON *et al.* 1981); its two known mutant alleles, *g72* and *g73*, produce a selective deficiency of the previously identified, kinetically similar, class B acetylcholinesterase forms (JOHNSON and RUSSELL, in press). Gene dosage experiments suggest that *ace-2* is a structural gene for these forms. By itself, the enzymatic deficiency produced by the *ace-2* mutant alleles leads to no obvious behavioral or developmental alteration, under ordinary conditions. However, it does produce increased sensitivity to the acetylcholinesterase inhibitor Aldicarb, and when present in combination with the class A deficiency produced by the *ace-1* mutant allele *p1000*, it produces a specific uncoordinated behavior.

The two independent mutations that identify *ace-2* were obtained by screening behaviorally and morphologically altered mutant derivatives of the *ace-1* mutant strain PR1000. As isolated, both *ace-2*; *ace-1* double mutants showed the same

characteristic uncoordinated behavior, *viz.*, both moved forward in a slow and poorly coordinated manner, and, when mechanically induced to undergo reversal, both showed a temporary hypercontracted paralysis. In addition, both had markedly reduced levels of class B acetylcholinesterase activity. In both, the behavioral phenotype was shown to be dependent on homozygosity, not only for the new *ace-2* mutant allele (*g72* or *g73*), but also for the *ace-1* mutant allele *p1000*. Coupled with the fact that the behavioral and enzymatic phenotypes were never observed to separate from one another in several crosses, these observations strongly suggest that in each case both phenotypes resulted from a single new (*ace-2*) mutation.

The effects of the *ace-2* mutations appear to be selectively on the class B acetylcholinesterase forms; the activity present in strain PR1000, which is exclusively of class B, is markedly reduced by both *ace-2* mutant alleles. When these alleles are separated from their original *ace-1* background, both produce strains with essentially normal class A activity, but very little class B activity. In this respect, the *ace-2* mutant alleles are complementary to the *ace-1* mutant allele *p1000*, which reduces class A activity with equal selectivity (JOHNSON *et al.* 1981). The selectivity of both kinds of mutations implies that the class A and class B acetylcholinesterases can occur independently, and are therefore probably not obligatorily linked in their expression. Furthermore, quantitative data indicate that the amount of either class is not shifted markedly upward in the absence of the other class; this argues against the occurrence of control mechanisms in which the final level of acetylcholinesterase activity is somehow monitored to regulate the expression of *ace-1*, *ace-2*, or both.

Given the complementary nature of *ace-1* and *ace-2*, the fact that both must be homozygous mutant to produce a behavioral phenotype strongly suggests that the class A and class B acetylcholinesterase forms must in some sense be able to substitute for one another functionally. *A priori*, it is easiest to imagine how functional substitution might occur if both class A and class B forms are ordinarily present at the same functionally relevant sites; in this case elimination of one or the other class from these sites would be expected to have little or no consequence because the other class would remain active.

In fact, the two single mutant strains PR1000 (possessing only class B forms) and GG202 (possessing only class A forms) exhibit two primary areas of common staining for acetylcholinesterase: the nerve ring and the longitudinal nerve cords. At face value, this result would suggest that both class A and class B forms are normally present in these areas, and indeed these areas seem on anatomical grounds to be functionally relevant. The nerve ring contains many of the morphologically recognizable synapses of the whole animal, including synapses between motor neurons and the muscles that move the head (WARD *et al.* 1975; WARE *et al.* 1975). The longitudinal nerve cords also contain synapses, among which are those between motor neurons and muscles that move the body (WHITE *et al.* 1976). Furthermore, from experiments on the extensively homologous nervous system of the related nematode *Ascaris lumbricoides*, it is likely that some of the motor neurons are cholinergic; despite a considerable difference of scale, the motor

neurons of *Ascaris* longitudinal nerve codes are almost completely homologous to those of *C. elegans* (STRETTON *et al.* 1978; WHITE *et al.* 1976). Single neuron enzymatic assays in *Ascaris* show that the excitatory motor neurons contain the acetylcholine biosynthetic enzyme, choline acetyltransferase (JOHNSON and STRETTON 1977). Thus, in the simplest interpretation, the requirement that both *ace-1* and *ace-2* be homozygous mutant to generate an uncoordinated phenotype may be due to the presence of both class A and class B acetylcholinesterase forms at all functionally relevant cholinergic synapses in the nerve ring and longitudinal cords.

Before this interpretation can be adopted, however, it will clearly be necessary to carry out acetylcholinesterase histochemical staining at the electron microscope level to determine whether (a) the observed staining is indeed localized to synapses and if so, whether only neuromuscular synapses stain, (b) the localized distribution is the same in N2, PR1000 and GG202, and (c) the nerve ring and the nerve cords are indeed the only areas of staining overlap. It should also be emphasized that the functional overlaps between class A and class B acetylcholinesterases is indicated only for the major behavioral function of coordinated movement. It could well be that there are more subtle aspects of behavior, involving only one or the other class, and that defects in these more subtle aspects have been inadvertently missed in examining the single mutant strains.

The selective staining of the pharyngeointestinal valve in N2 and PR1000, but not in GG202 or heat-treated N2, suggests strongly that only class B acetylcholinesterase is present in this structure. The functional significance of this acetylcholinesterase is uncertain; the valve itself, although known to be composed of 6 cells (SULSTON and HORVITZ 1977), is of unknown function and has not been reported to be connected to the nervous system proper. Furthermore, the absence of an observable phenotype for GG202 suggests either that this enzyme has no major functional role or that the small amount of residual class B activity in this strain is adequate for any function it may have. In any case, selective localization of class B activity to the pharyngeointestinal valve suggests that *ace-1* and *ace-2* are not necessarily coordinately expressed.

The reported gene dosage experiments are an indication that *ace-1* and *ace-2* may be structural genes for the class A and class B acetylcholinesterase forms, respectively. More definitive testing of this idea could be achieved by identifying *ace-1* and/or *ace-2* alleles that spare sufficient amounts of the relevant activity to permit qualitative characterization. The existing *ace-1* allele (*p1000*) is probably inadequate in this respect; kinetic studies of the inactivation of PR1000 crude extract acetylcholinesterase activity by DOC indicate that there is less than 0.1% having the DOC resistance characteristics of class A forms III and IV (K. MURPHY and R. L. RUSSELL, unpublished). The existing *ace-2* alleles, *g72* and *g73*, however, may be more promising; these two mutations spare an amount of activity that is 2–5% of the normal level of class B forms. Qualitative characterization of this residual activity should establish whether it represents mutationally modified class B forms.

If *ace-2* is indeed a structural gene for the class B acetylcholinesterase forms,

there remains the question of the relationship between the two forms, IB and II, comprising this class. Presumably, like the three forms of class A for which *ace-1* is most probably a structural gene, the two forms of class B must share at least one component in common, and their kinetic similarity (JOHNSON and RUSSELL, in press) would again suggest that the common subunit may carry the active site. The larger form II, again in parallel with the class A situation, is quite difficult to solubilize in the absence of detergents, implying a membrane localization, and again it may be the case that it differs from the smaller form IB in possessing additional component(s) that confer its localization.

The availability of *ace-1* and *ace-2* mutations makes possible some preliminary statements about the relationship between acetylcholinesterase level and behavioral function. Animals homozygous mutant at one of these loci and heterozygous at the other (e.g., *ace-2/ace-2;ace-1/+*) are, by the tests so far carried out, behaviorally and developmentally normal, despite the fact that they have only about one fourth the normal level of total acetylcholinesterase activity. On the other hand, animals homozygous for the currently available mutant alleles at both loci (*ace-2/ace-2;ace-1/ace-1*) have about 2% of the normal activity level and are uncoordinated, but viable and not markedly affected in development. It would seem that the levels of acetylcholinesterase activity required for viability and coordination are considerably less than those ordinarily present in wild-type animals.

Finally, mutations affecting acetylcholinesterase activity have also been identified in *Drosophila melanogaster*, following an analysis by segmental aneuploidy of the chromosomal regions likely to contain a structural gene (HALL and KANKEL 1976). Interestingly, the existence of multiple genes with related gene products, as seen in *C. elegans*, does not appear to hold for *D. melanogaster*; although multiple molecular species of acetylcholinesterase are indeed observed in *D. melanogaster* (DUDAI 1977), all appear to be controlled by a common chromosomal region, 87E1-E5, and apparent point mutations in this region appear to affect all activity by the criterion that activity levels are effectively halved in mutant heterozygotes (HALL and KANKEL 1976). Further evidence that these mutations affect all acetylcholinesterase is provided by the observation that most are homozygous lethal, in contrast to the *ace-1* or *ace-2* mutations in *C. elegans*. In view of these differences, it will be of interest to determine whether the anatomical abnormalities seen in *Drosophila* mosaic patches homozygous for such mutations (HALL, GREENSPAN and KANKEL 1979; GREENSPAN, FINN and HALL 1979) are also observed in the much simpler *C. elegans* nervous system, with its readily identifiable neurons (WHITE *et al.* 1976; WARD *et al.* 1975; WARE *et al.* 1975) and its highly lineage-dependent pattern of development (SULSTON 1976; SULSTON and HORVITZ 1977; DEPPE *et al.* 1978).

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