AN ACETYLCHOLINESTERASE-DEFICIENT MUTANT OF THE NEMATODE *CAENORHABDITIS ELEGANS*

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ABSTRACT

Within a set of five separable molecular forms of acetylcholinesterase found in the nematode *Caenorhabditis elegans*, previously reported differences in kinetic properties identify two classes, A and B, likely to be under separate genetic control. Using differences between these classes in sensitivity to inactivation by sodium deoxycholate, a screening procedure was devised to search for mutants affected only in class A forms. Among 171 previously isolated behavioral and morphological mutant strains examined by this procedure, one (PR946) proved to be of the expected type, exhibiting a selective deficiency of class A acetylcholinesterase forms. Although originally isolated because of its uncoordinated behavior, this strain was subsequently shown to harbor mutations in two genes; one in the previously identified gene *unc-3*, accounting for its behavior, and one in a newly identified gene, *ace-1*, accounting for its selective acetylcholinesterase deficiency. Derivatives homozygous only for the *ace-1* mutation also lacked class A acetylcholinesterase forms, but were behaviorally and developmentally indistinguishable from wild type. The gene *ace-1* has been mapped near the right end of the *X* chromosome. Gene dosage experiments suggest that it may be a structural gene for a component of class A acetylcholinesterase forms.

THE small soil nematode *Caenorhabditis elegans*, now being used to investigate a number of problems in metazoan organization (for review, see RIDDLE 1978), is particularly well suited for genetic studies of neural function and development, in part, because *C. elegans* possesses a remarkably simple 350-cell nervous system, much of which has been analyzed in synaptic detail by serial-section electron microscopy (WARD *et al.* 1975; WARE *et al.* 1975; WHITE *et al.* 1976; ALBERTSON and THOMSON 1976; HALL and RUSSELL submitted for publication), and in part because *C. elegans* is a self-fertilizing hermaphrodite, a feature which has greatly facilitated the isolation of a large number of behaviorally defective mutants (BRENNER 1974; see RIDDLE 1978).

Among the problems for which these advantages should be useful, an important one is the question of the genetic control mechanisms that lead to the

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selective synthesis and degradation of different neurotransmitters in different neurons. To approach this problem in *C. elegans*, it seems essential to us to identify mutations affecting enzymes of neurotransmitter metabolism, and among several possibilities we have focused attention first on acetylcholinesterase, which hydrolyzes the apparent neurotransmitter acetylcholine.

This choice is based on several considerations. First, physiological and pharmacological observations on the related nematode *Ascaris lumbricoides* already suggest that acetylcholine is a likely neurotransmitter in Ascaris and, by homology, in other nematodes as well (DEL CASTILLO, DE MELLO and MORALES 1963). Second, a variety of acetylcholinesterase inhibitors have been shown to produce a hypercontracted paralysis in *C. elegans* (BRENNER 1974; J. A. LEWIS, personal communication; R. L. RUSSELL, C. D. JOHNSON, J. B. RAND and J. G. CULOTTI, unpublished observations), indicating that acetylcholinesterase plays an important behavioral role in *C. elegans*. Third, histochemical staining of *C. elegans* has shown selective localization of acetylcholinesterase in areas of high synaptic density, suggesting a synaptic role for the enzyme (R. L. RUSSELL, unpublished results; CULOTTI *et al.* 1981). Finally, an especially convenient assay for acetylcholinesterase is available (JOHNSON and RUSSELL 1975).

Before attempting to isolate mutants with acetylcholinestrase defects, we thought it wise to characterize the wild-type *C. elegans* enzyme. In a previous report (JOHNSON and RUSSELL, in press), we described the results of this characterization. Briefly, as in other systems, multiple molecular forms of acetylcholinesterase are found; in *C. elegans* there are five forms that can be separated by a combination of selective solubilization, velocity sedimentation and ion exchange chromatography. Comparison of their properties allows these forms to be divided into two classes, three forms in Class A and two in class B (see Table 1). Within each class, the kinetic properties are sufficiently similar to suggest a common active site, presumably carried on a common active subunit. Between classes, however, the differences are sufficient to suggest different active sites, presumably carried on different active subunits. Thus, the situation in *C. elegans* is different from the well-studied one in electric organs, where all the multiple forms of acetylcholinesterase apparently possess a common active subunit, differently assembled into synaptic structures (MASSOULIE and RIEGER 1969; for review, see ROSENBERRY 1976). Instead, it somewhat resembles the case of the vertebrate "true" and "pseudo" cholinesterases, in the sense that two kinetically distinct enzyme species can be recognized (although the two *C. elegans* classes A and B clearly do not correspond directly to the two vertebrate enzyme types and do not differ as markedly). Genetically, these observations suggest the possibility of two (or possibly more) structural genes encoding active subunits of acetylcholinesterase in *C. elegans*.

Because each of the two *C. elegans* enzyme classes A and B constitutes about half of the total acetylcholinesterase activity seen in extracts, and because we expected the most common kind of acetylcholinesterase mutation to affect either class A or class B, but not both, it seemed impractical to search for such mutations by screening for an expected 50% reduction in activity. Instead, we decided to

use one of the differences between the classes as a way of eliminating class B activity in extracts, thereby allowing us to search much more sensitively for mutations affecting class A activity. We describe below how this selective screening procedure was devised and how it was used to identify a mutant with a selective deficiency of class A acetylcholinesterase forms. The mutant gene involved, now called *ace-1*, is apparently a structural gene for class A acetylcholinesterase forms. A preliminary report of some of this work as appeared (RUSSELL *et al.* 1977). CULOTTI *et al.* (1981) describe how the original mutant has been used to isolate additional mutants that identify a second gene, *ace-2*, apparently a structural gene for the class B acetylcholinesterase forms.

MATERIALS AND METHODS

Chemicals: Sodium deoxycholate (DOC), bovine serum albumin (BSA), Tween 80, and Tris (Tris(hydroxymethyl)amino methane) were from Sigma Chemical Co. (St. Louis, MO). Renografin (Squibb) was obtained from a local apothecary. [^{3}H]-acetylcholine was TRA277 from Amersham/Searle Corporation (Arlington Heights, IL). All other chemicals were of standard reagent grade.

Media: NGM solid culture medium has been described by (BRENNER (1974).

Nematodes: All nematodes were cultured at 20° on NGM agar petri dishes with an *E. coli strain* OP50 as food source, as described by BRENNER (1974). The strains used were:

N2: wild type

CB xxx: mutant strains isolated from N2 by BRENNER (1974), most showing aberrant behavior (originally called E xxx by BRENNER 1974).

CB678: *lon-2 (e678) X* (long; originally E678)

CB1324: *dpy-7 (e1324) X* (dumpy; originally E1324)

CB 151: *unc-3(e151) X* (uncoordinated; originally E151)

CB54: *unc-3(e54) X* (uncoordinated; originally E54)

CB95: *unc3(e95) X* (uncoordinated; originally E95)

CB121: *unc-3(e121) X* (uncoordinated; originally E121)

PR xxx: mutant strains isolated from N2 in our laboratory (COLEMAN *et al.* 1972), most showing aberrant behavior.

PR808: *osm-1(p808) X* (osmotic avoidance defective, originally called P808 by CULOTTI and RUSSELL 1978).

SP219: *mnDp1(X,V)/+; unc-3(e151) X*

SP5: *dpy-11(e224) V; unc-3(e151) X*

SP40: *dpy-11(e224) V; unc-7(e5) X*

SP115: *mnDp8(X;I); unc-3(e151) X*

SP116: *mnDp9(X;I); unc-3(e151) X*

SP75: *mnDp25(X;I); unc-3(e151) X*

SP76: *mnDp27(X;II); unc-3(e151) X*

SP301: *mnDp8(X;I); unc-7(e5) X*

SP265: *mnDp1(X;V)/+; mnDf4 X*

SP269: *mnDp1(X;V)/+; mnDf8 X*

Sp395: *mnDpl(X;V)/+; mnDf42 X*

SP394: *mnDp1(X;V)/+; unc-3(e151) mnDf41 X*

Strains are listed according to a recently standardized nomenclature (HORVITZ *et al.* 1979).

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 [^{3}H]-labelled acetylcholine, undiluted by unlabelled acetylcholine, at a final concentration 1×10^{-6} M and specific activity of ~250 mCi/mmole. In the latter case, because the assay was per-

formed at a substrate concentration well below the K_m of the enzyme(s) for acetylcholine, it was necessary to correct for substrate depletion during the assay. This was done by using the formula $f_{corr} = -\ln(1-f)$, where f is the observed fraction of substrate converted to product and f_{corr} is the fraction that would have been converted if substrate concentration had not changed. The correction is based on assumed exponential depletion of substrate and is observed experimentally to give values of f_{corr} that remain linearly dependent on time even when observed f values exceed 0.5.

For "individual assays," the desired number of worms (usually 1–5), grown at 20° for 72 hr from the time of egg deposit on the plate, were transferred on a sterile, slightly bent tip of a No. 25 syringe needle into 40 μl of a buffer (10 *m*M borate, pH 8.8; 2 mg/ml BSA; 1 mM NaN_3) in a 1-dram mini-scintillation vial. After observation to confirm transfer, the vial was subjected to 6–9 cycles of freeze-thawing (liquid nitrogen alternating with room-temperature water), capped, and incubated overnight (12–16 hr) at 25–26°. One hr before assay, the vial received an additional 10 μl of either H_2O (for total activity measurecents) or 2% DOC (for DOC-resistant activity measurements). Assays were begun by adding 50 μl of 50 mM potassium phosphate buffer, pH 7.0, containing 2×10^{-6}M [^{3}H]-acetylcholine (~250 mCi/mmole), and stopped at the desired time, as for the usual acetylcholinesterase assay (JOHNSON and RUSSELL 1975).

RESULTS

Design of a screening procedure: Table 1 lists some of the properties of the five separable forms of *C. elegans* acetylcholinesterase and indicates the two classes into which these properties divide the forms. An additional difference among these forms is their sensitivity to inactivation by the anionic detergent sodium deoxycholate (DOC). As Figure 1 shows, the two largest forms, III and IV, both of which belong to class A, are relatively unaffected by concentrations of DOC that produce rapid and extensive inactivation of other forms. To see whether the same difference would occur in crude extracts, where it could be conveniently used to screen potential mutants, we carried out the experiments of Figure 2. In crude extracts, the total acetylcholinesterase activity, due to both class A and class B forms, was clearly inactivated by DOC with biphasic kinetics; a rapid initial phase of inactivation was followed by a relatively stable plateau (Figure 2A). When partially inactivated extracts taken from near the beginning of the plateau

TABLE 1

Properties of separated forms of C. elegans *acetylcholinesterase*

	Form				
	Class A			Class B	
	IV	III	IA	II	IB
Sedimentation constant (in Svedbergs)	13.0 ± 0.2	11.4 ± 0.1	5.4 ± 0.1	7.3 ± 0.2	5.1 ± 0.1
Approximate molecular weight (in daltons × 10^{-3})	343	282	92	145	84
K_m for acetylcholine, μM	12 ± 1	15 ± 1	12 ± 1	67 ± 5	80 ± 5
K_i for Triton X-100, μM	18	19	33	> 3000	300
[NaCl] at which elution from DE52 occurs	~ 200	~ 200	~ 200	~ 60	~ 60

Data are taken from JOHNSON and RUSSELL (in press).

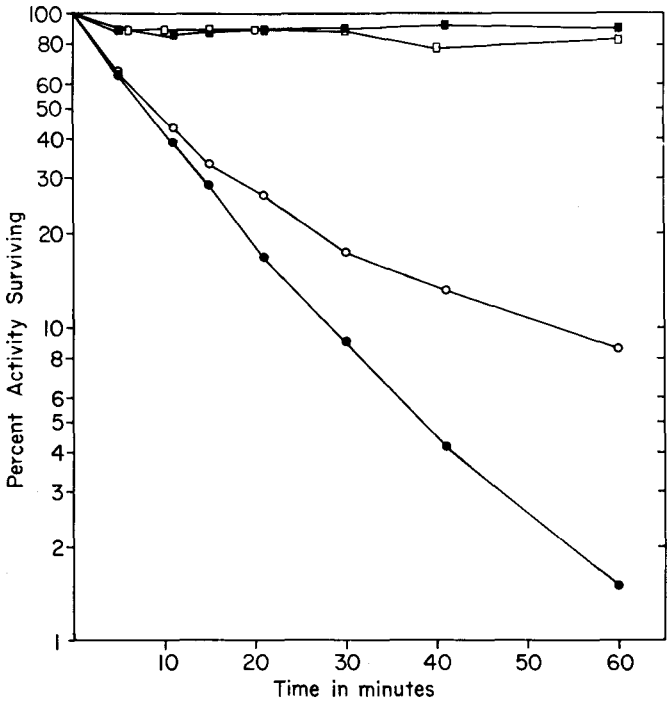

FIGURE 1.—Inactivation of separated *C. elegans* acetylcholinesterase forms by sodium deoxycholate (DOC). N2 forms were separated by a combination of selective solubilization, velocity sedimentation and ion exchange chromatography, as described by JOHNSON and RUSSELL (in press). Before inactivation, all forms were dialyzed against 50 mM Tris buffer, pH 7.5. For inactivation, DOC was added to a final concentration of 0.2%, and, at the indicated times, duplicate 5 μl aliquots were withdrawn and immediately assayed, for 30 min, in an assay volume of 100 μl (20 × dilution, final concentration of DOC in assay, 0.01%). Surviving activities are plotted as a percentage of the original activity, determined with the same 0.01% DOC in the assay. Inactivation was at 25°. —●—, form IA; —○—, form IB; —□—, form III; —■—, form IV.

phase were compared with the starting material by velocity sedimentation (Figure 2B), it was clear that the rapid inactivation had indeed affected the low molecular weight forms selectively, leaving the large molecular weight forms III and IV virtually unchanged.

These observations suggested a relatively simple screening procedure by which it might be possible to identify potential mutants with a deficiency of class A acetylcholinesterase forms; in short, such mutants should exhibit very little resistant acetylcholinesterase activity in crude extracts after treatment with DOC.

Identification of an altered mutant strain: In a first attempt to use the newly devised screening procedure, we examined crude extracts of a set of 171 mutant strains, most of which had been selected for their "uncoordinated" behavior in a large previous mutant isolation (COLEMAN *et al.* 1972). Our reasoning was that a class A acetylcholinesterase defect might generate a behavioral deficit. A sample of the results is presented in Figure 3. In almost all cases, incubation with DOC

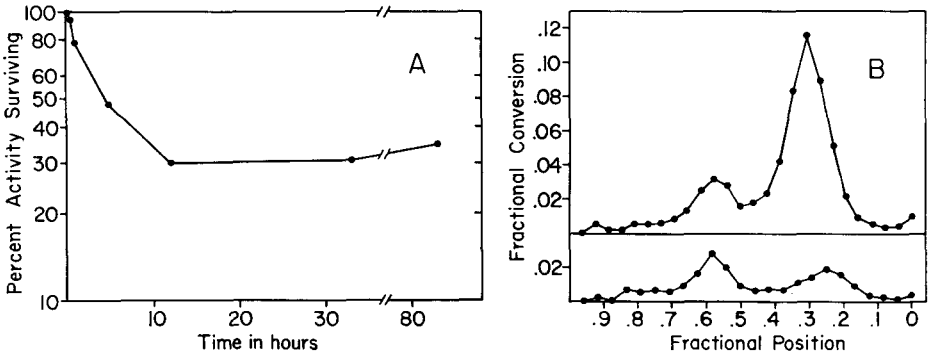

FIGURE 2.—Inactivation of *C. elegans* acetylcholinesterase activity in crude extracts by DOC. (A) A 100 μl aliquot of freeze-powdered N2 *C. elegans* homogenate was added to an equal volume of 0.1 M borate buffer, pH 8.8, and 10 μl of 10% DOC was added. At the indicated times, aliquots were withdrawn for acetylcholinesterase assay, as in Figure 1. Inactivation was at 4°. Activity is plotted as a percentage of the original activity, as in Figure 1. (B) Homogenate was diluted with borate and DOC was added as in (A). After 30 min (top gradient) or 8 hr (bottom gradient) at 4°, 100 μl aliquots were loaded onto 4.5 ml 5–20% sucrose gradients (0.05 M borate, pH 8.8; 0.1% Tween-80) with 0.5 ml Renografin cushions. After centrifugation for 4 hr at 65,000 rpm in a Beckman L265B ultracentrifuge (SW65 rotor), fractions were collected and 10 μl of each was assayed for 60 min for acetylcholinesterase activity. Activity is plotted as the corrected fractional conversion of substrate. Sedimentation from right to left in both panels.

produced the same biphasic pattern of inactivation as in wild type, with the stable plateau representing 30–50% of the total activity. In one case, however, the pattern was markedly different. The mutant strain PR946 (originally called BC46 by RUSSELL *et al.* 1977) appeared to be of the type anticipated in the design of the screening procedure, since it showed a very low level of activity resistant to DOC.

Retesting of a second PR946 crude extract confirmed the original inactivation pattern, and we next determined whether PR946 exhibited the expected deficiency of high molecular weight acetylcholinesterase forms III and IV. Figure 4A and 4B depict the sucrose gradient sedimentation patterns of easily solubilized acetylcholinesterase forms from wild-type N2 (A) and PR946 (B). A large peak of activity sedimenting at approximately 13s in the wild-type extract, corresponding to form IV, was completely missing from the PR946 extract, but the other peak, sedimenting at approximately 5s, was relatively little affected. That this difference was not due simply to a failure to solubilize 13s activity in PR946 is indicated by Figures 4C and 4D. These depict the sucrose-gradient sedimentation patterns of acetylcholineserase forms extracted from wild type (C) and PR946 (D) by a rapid, detergent-based procedure that solubilized most of the measurable activity (see legend to Figure 4). The sedimentation patterns were more dispersed than those of Figures 4A and 4B, primarily because the rapid procedure solubilized significant additional amounts of forms II and III, sedimenting at approximately 7s and 11s respectively. However, the difference between wild type and PR946 persisted, there being essentially no activity peak in the region of the PR946 gradient

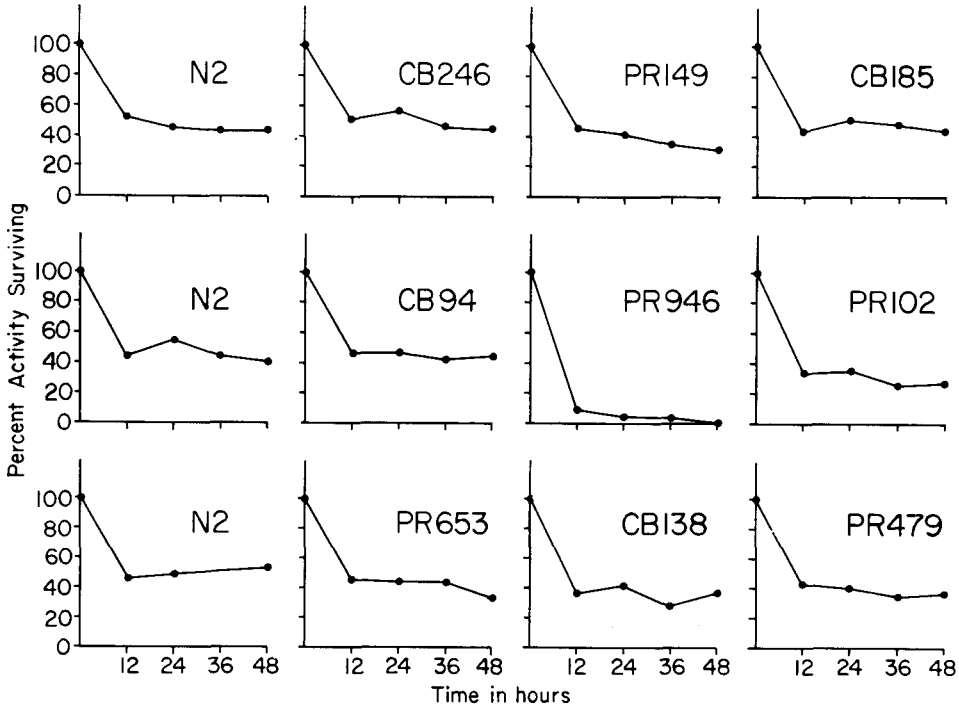

FIGURE 3.—Detergent inactivation of acetylcholinesterase activity in mutant crude extracts. For each mutant strain, 10 plates of animals were eluted, washed with distilled water, and freeze-powdered in 1 ml. To 100 μl aliquots of this homogenate were added 100 μl of 0.1 M borate buffer, pH 8.8 and 10 μl of 10% DOC, and the aliquots were incubated at 4°. At the indicated times, 10 μl aliquots were withdrawn and assayed for acetylcholinesterase activity. Activity is plotted as a fraction of the 0-time control. Only a sample of the 171 strains tested is given here. N2 is the wild type, and PR946 is the one strain showing a virtual absence of activity after about 24 hr treatment with DOC.

where forms III and IV should have appeared. In short, PR946 appeared to be the anticipated type of mutant strain, with a selective deficiency of acetylcholinesterase forms III and IV.

Genetic analysis of the mutant strain PR946: The strain PR946 was originally isolated by its uncoordinated phenotype, which consisted of a selective deficiency in the propagation of contractile waves from the head through the body (RUSSELL *et al.* 1977). To determine whether the same mutation produced both the acetylcholinesterase deficiency and the uncoordinated behavior, we crossed PR946 hermaphrodites with wild-type (N2) males. The cross progeny contained normally coordinated hermaphrodites in large numbers, indicating that the mutation producing the uncoordinated phenotype was recessive. In the same cross, all male progeny were uncoordinated, indicating that the same mutation was sex linked. When the normally coordinated hermaphrodite cross progeny were allowed to self-fertilize, very nearly one-fourth of the resulting progeny were uncoordinated (Table 2A), indicating that the uncoordinated phenotype was most probably due

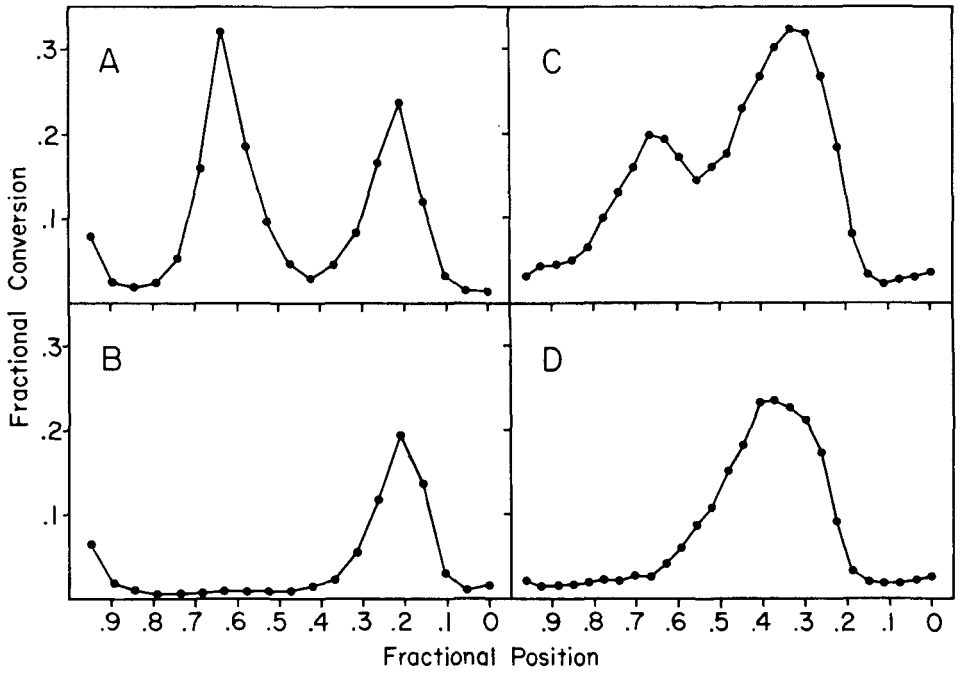

FIGURE 4.—Velocity sedimentation of wild-type N2 and mutant PR946 extracts. (A) and (B), extracts made without detergent. Fresh freeze-powdered homogenates of N2 (A) and PR946 (B) were diluted three-fold with 0.1 M borate buffer, pH 8.8. After stirring 24 hr at 4°, 1 ml aliquots of a 1500 g, 1 min supernatant were loaded onto 38 ml 5–20% sucrose gradients containing 0.05 M borate buffer, pH 8.8 and centrifuged for 29 hr at 27,000 rpm (BECKMAN *L265B ultracentrifuge, SW27 rotor*). Fractions were collected and 10 μl of each was assayed for acetylcholinesterase activity. (C) and (D), extracts made with detergent. Fresh freeze-powdered homogenates of N2 (C) and PR946 (D) were diluted 1.8-fold with 0.1 M borate buffer, pH 8.8. At 4°, DOC was added to a final concentration of 0.2% and, after 2 min, a 1500 g 1 min supernatant was prepared and 100 μl aliquots were loaded onto 5 ml, 5–20% sucrose gradients in 0.05 M borate buffer, pH 8.8, plus 0.1% Tween-80. Gradients were centrifuged for 4.5 hr at 65,000 rpm (SW 65 rotor), fractions were collected and 10 μl of each was assayed for acetylcholinesterase activity. Activity is plotted as the fractional conversion of substrate.

to a single (*unc*) mutation, which we named *p1001*. Twenty of the uncoordinated F_2 progeny were cloned, and all twenty of the clones, when tested for DOC-resistant acetylcholinesterase, had the same deficiency as the original PR946 strain. In addition, five other uncoordinated clones were assayed by velocity sedimentation, and all lacked acetylcholinesterase forms III and IV. In these twenty-five clones, then, the uncoordinated behavior and enzymatic deficiency of PR946 were not separated. One such clone was again backcrossed to N2 males with the same results, and one of the resulting uncoordinated clones, now the result of two backcross cycles to wild type, was selected for further work. Like its predecessors, this clone lacked acetylcholinesterase forms III and IV, as shown both by DOC resistance and velocity sedimentation. That this clone had been significantly freed

TABLE 2

Phenotypic ratios of progeny segregating from certain hermaphrodites

Parents of hermaphrodite	Classes among progeny of hermaphrodite				Map distance
A. N2 ♂ × *unc(p1001)* ♀	non-Unc	Unc			
	110	40			
B. N2 ♂ × *lon-2 unc(p1001)* ♀	non-Unc	Unc	Unc	non-Unc	
	non-Lon	Lon	non-Lon	Lon	
	509	69	136	123	38.3
C. N2 ♂ × *dpy-7 unc(p1001)* ♀	non-Unc	Unc	Unc	non-Unc	
	non-Dpy	Dpy	non-Dpy	Dpy	
	612	161	91	96	21.9

The origins of the hermaphrodites used are given in the text. Phenotype designations are: Unc, uncoordinated; Lon, long; Dpy, dumpy. Map distances are the percentages of recombination, calculated as described by BRENNER (1974), between *unc(p1001)* and the marker mutation, *lon-2* or *dpy-7*.

of additional genetic damage present in PR 946 was indicated by the fact that it no longer showed PR946's inability to grow at 25°.

To obtain a rough map location of *unc(p1001)*, crosses were performed between hermaphrodites of the doubly backcrossed clone and males of two sex-linked marker strains, CB678:*lon-2* × and CB1324:*dpy-7* × (BRENNER 1974). The normally coordinated hermaphrodite progeny were allowed to self-fertilize in each case, and from their progeny the relatively rare double-mutant segregants, exhibiting both the marker phenotype (Lon or Dpy) and the uncoordination of PR946, were cloned. Hermaphrodites of each double-mutant type were crossed to N2 males, and the resulting *cis* double heterozygotes, recognizable as normally coordinated unmarked hermaphrodites, were allowed to self-fertilize. The distribution of phenotypes among their progeny is given in Table 2B and 2C, which also gives the derived map distances between each marker and *unc(p1001)*. While the map distances are relatively large, they indicate that *unc(p1001)* is located on the right arm of the *X* chromosome (see Figure 5).

The region to which *unc(p1001)* mapped, as Figure 5 shows, was already known to contain four genes capable of generating *unc* mutations, *unc-3*, *unc-7*, *unc-9* and *unc-84*. Fortuitously, it also had been selected by one of us (R.K.H.) for intensive genetic mapping, and as a result several useful strains harboring duplications or deficiencies in this region were available (HERMAN, MADL and KARI 1979; MENEELY and HERMAN 1979). We first made use of one of these strains, harboring the duplication *mnDp1*, to test for possible allelism between *unc(p1001)* and *unc-3*, whose map position and mutant phenotype were closest to those of *unc(p1001)*. Figure 6 depicts the method used. The results unambiguously established *p1001* as a new allele of *unc-3*.

Anticipating that strains with previously isolated *unc-3* alleles might resemble PR946, we examined the strain CB151 *unc-3 X* and observed that its uncoordi-

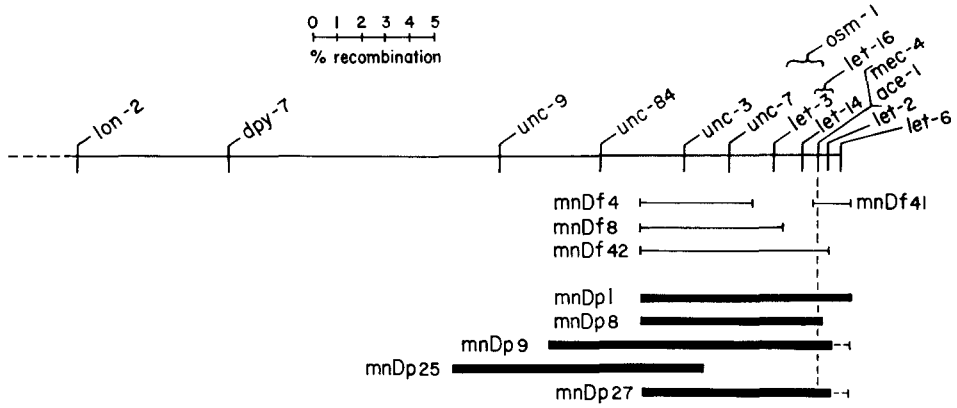

FIGURE 5.—Partial map of the *C. elegans X* chromosome. Only the right hand portion of the *X* and only the relevant genes are included. The map is redrawn slightly from that of MENEELY and HERMAN 1979. *let-8* on the previous map has since been shown to be an allele of *let-2*. In addition, two new deficiencies (*mnDf41*, *mnDf42*) are included. Dotted lines indicate uncertainty of extent for duplications in which practical constraints currently preclude testing against the gene *let-6*.

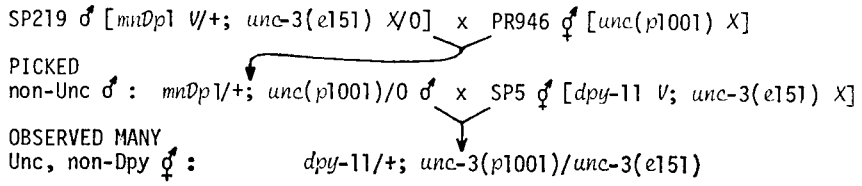

FIGURE 6.—Test for allelism between *unc(p1001)* and *unc-3*. *mnDp1* is a translocation of a portion of the *X*, including the *unc-3(+)* allele, to chromosome *V* (see Figure 5); it is used to permit mating by the otherwise uncoordinated *unc-3/O* ♂. The presence of non-Unc ♂ ♂ (and ♀ ♀) in the progeny of the first cross shows that *unc(p1001)* is covered by *mnDp1*. In the second cross, *dpy-11* is used as a recessive marker for chromosome *V*; the presence of many unc, non-Dpy ♀ ♀ in the progeny of the second cross shows that *unc(p1001)* is indeed an allele of *unc-3*. Among the progeny of the second cross, there were also the expected other classes: non-Unc, non-Dpy ♀ ♀; non-Unc, non-Dpy ♂ ♂; Unc, non-Dpy ♀ ♀; Unc, Dpy ♀ ♀.

nated phenotype resembled that of PR946. However, when an extract of CB151 was assayed for DOC-resistant acetylcholinesterase, we found to our surprise that it showed wild-type levels, or about 30–40% of the total activity, resistant to DOC. In confirmation, velocity sedimenetation of extracts from CB151 and three other *unc-3* mutant strains, (CB54, CB95 and CB121) all showed essentially normal levels of acetylcholinesterase forms III and IV.

Although other interpretations were certainly possible, these results suggested to us that the acetylcholinesterase deficiency of PR946 might be due, not to its *unc-3* mutant allele *p1001*, but instead to a second mutation. Realizing that such a second mutation, by its failure to segregate out in the original backcrosses to wild type, would probably be quite closely linked to *unc-3*, we set up crosses in which we could selectively identify recombinants derived from recombination

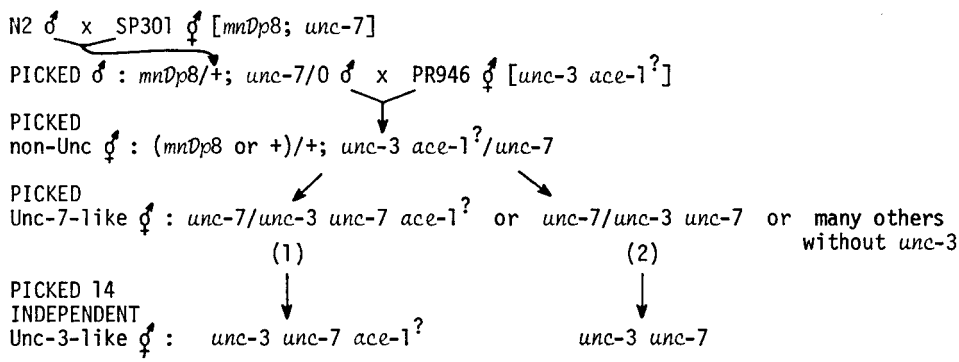

FIGURE 7.—Selection of recombinants from events between *unc-3* and *unc-7*. The potential second mutation of PR946 is indicated as *ace-1*?. The duplication *mnDp8*, which covers *unc-7*, is used to allow mating by *unc-7/O* males in the second cross; it may still be present in the selected progeny of this cross, but must have been lost in the selected progeny of the next generation. The *unc-7* allele used was *e5*, whose uncoordination is much less severe than that of all *unc-3* alleles; thus Unc-3-like segregants can be phenotypically picked in an Unc-7 background. The genotype labelled (1) is the most common one expected to segregate Unc-3-like progeny if *ace-1*? exists and maps near *unc-3* or to the left (see Figure 5). The genotype labelled (2) is the most common one expected to segregate Unc-3-like progeny if *ace-1*? exists and maps near *unc-7* or to the right. The Unc-3-like progeny from (1) should be enzymatically defective; whereas, those from (2) should not. The procedure involving *unc-3* and *unc-9* recombinants was operationally identical except that the *unc-9* allele *e101* was used instead of *e5*, *mnDp9* (which covers *unc-9*) was used instead of *mnDp8* and only 6 recombinants were analyzed. In this case, the genotype analogous to (1) is expected if *ace-1*? maps near *unc-3* or to the right; whereas, the (2) analog is expected if it maps near *unc-9* or to the left.

events between *unc-3(p1001)* and closely linked markers on either side. Figure 7 shows the method used. When recombinants between *unc-3(p1001)* and the leftward marker *unc-9* were examined, 6 of 6 recombinants retaining *p1001* also retained the enzymatic deficiency of PR946. However, when recombinants between *unc-3(p1001)* and the rightward marker *unc-7* were examined, only 2 out of 14 recombinants retaining *unc-3(p1001)* also retained the enzymatic deficiency; the remaining 12 were uncoordinated but enzymatically normal. These results clearly established that the enzymatic deficiency of PR946 was indeed due to a second mutation and, in addition, located this mutation somewhere to the right of *unc-3*. (While the occurrence of two recombinants that retained the enzymatic deficiency might additionally suggest that the new mutation lies slightly to the left of *unc-7*, subsequent mapping against duplicated and deficiency strains indicated an alternative explanation, *viz.*, that these two recombinants arose by double exchanges. The newly identified gene harboring this mutation was given the name *ace-1*, and the mutation itself was named *p1000*.

To obtain the newly identified *ace-1* mutation *p1000* by itself, we carried out two independent sets of crosses, both with the tentative assumption that an *ace-1 (p1000)* homozygote might not have a visible phenotype accompanying its enzymatic deficiency. First, as indicated in Figure 8A, we used the marker *osm-1*, previously mapped to the right end of the *X* chromosome (CULOTTI and RUSSELL

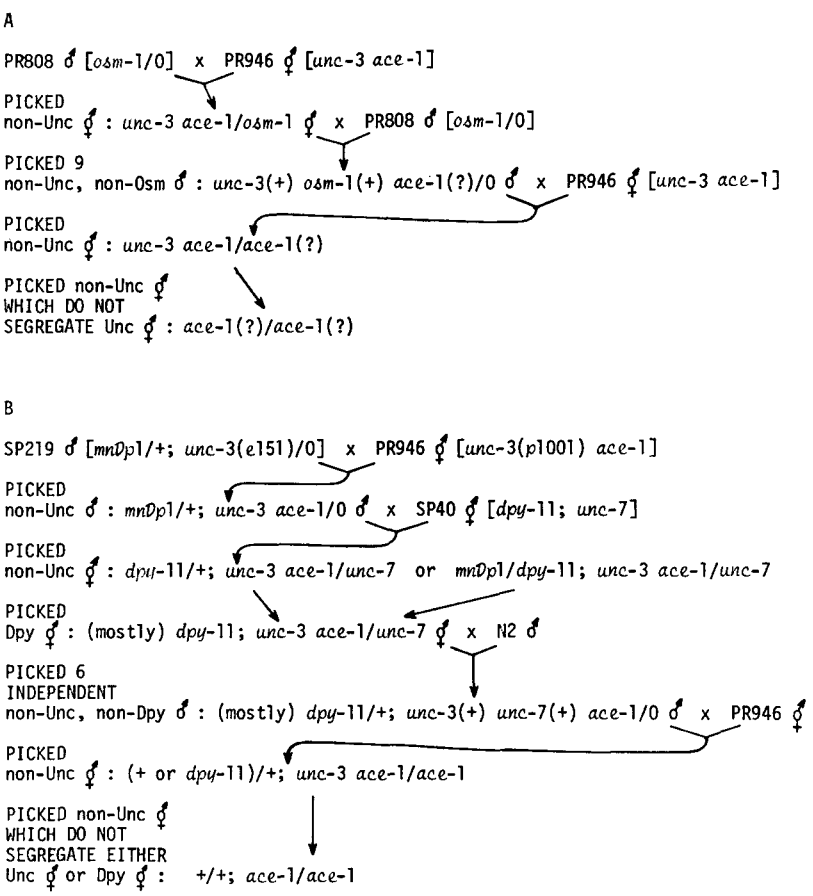

FIGURE 8.—Isolation of recombinants homozygous for *ace-1(p1000)*. (A) First approach. Mutations in the gene *osm-1* eliminate osmotic avoidance behavior, but leave mating ability unimpaired. The symbol *ace-1*(?) indicates the expected, but not ensured presence of *ace-1(p1000)*. (B) Second approach. The duplication *mnDp1* is used to permit mating by the otherwise uncoordinated *unc-3(e151)/O* males. In the third generation, the *mnDp1* may or may not be present in the selected animals; selection of Dpy segregants in the next generation ensures that it has been lost. In the final generation, progeny testing reveals those animals that have lost the *dpy-11* marker. The dpy-11 allele used was *e224*, the *unc-7* allele used was *e5* and, unless otherwise indicated, the *unc-3* and *ace-1* alleles are those of PR946.

1978; see Figure 5), to create heterozygotes of genotype *unc-3 ace-1/ osm-1*. These were mated to *osm-1/0* males, and 300 non-Unc male progeny were screened for their osmotic avoidance behavior, as described by CULOTTI and RUSSELL (1978). Of these 300, 9 were normal in avoidance, and therefore carried on their single *X* chromosome the recombinant genotype *unc-3* (+) *osm-1*(+); because *ace-1* should be closely linked to *osm-1*, most or all of these 9 were also expected to carry *ace-1(p1000)* on their single *X*. To obtain the recombinant *X* chromosome in homozygous form, these 9 males were individually mated to PR946 hermaphrodites, the non-Unc hermaphrodite progeny were allowed to

self-fertilize, and their non-Unc progeny were cloned to identify those that did not segregate Uncs. In all 9 cases, these final progeny were the expected *ace-1(p1000)* homozygotes, as judged by their lack of DOC-resistant acetylcholinesterase activity.

In a second approach, shown in Figure 8B, duplication *mnDp1* was used to cover the usual mating deficiency of *unc-3 ace-1* males, allowing heterozygotes of genotype *unc-3 ace-1/unc-7* to be created. Derivatives of these (which were similarly heterozygous, but carried an unlinked *dpy* mutation in homozygous form) were mated to wild-type males, and among the progeny were 6 non-unc males whose single *X* chromosome was therefore of the recombinant genotype *unc-3*(+) *unc-7*(+); as before, most or all of these were expected to contain *ace-1(p1000)*. Again, when the recombinant *X* chromosomes were recovered in homozygous form, in all 6 cases the resulting animals were *ace-1* homozygotes, as judged both by DOC resistance and by velocity sedimentation.

In both approaches, all animals either hemizygous or homozygous for *ace-1 (p1000)* were normally coordinated, developed at apparently normal rates, and in simple tests exhibited normal sensitivity to mechanical, thermal and chemical stimuli. These results confirmed the tentative assumption that *ace-1* mutants might not have a visible phenotype, and also suggested that the uncoordination of PR946 might be due entirely to its *unc-3* allele *p1001* and not to its *ace-1* allele *p1000*. In confirmation, the behavioral phenotype of other progeny, homozygous for the *unc-3* allele *p1001*, but normal at the *ace-1* locus, was indistinguishable from that of PR946 itself. For further work, one *ace-1(p1000)* homozygote from the first approach was chosen and called PR1000.

Mapping the gene ace-1: The absence of a visible phenotype for *p1000* considerably complicated attempts to map *ace-1* further. Specifically, because cross progeny could not be scored visually, it was necessary, instead, to score by growing clones of sufficient size to permit enzymatic assay. To circumvent this problem, we devised an "individual assay" capable of measuring accurately the acetylcholinesterase activity of single, adult *C. elegans* (wet weight about 4 μg). The details of this method are given in MATERIALS AND METHODS, and Figure 9 demonstrates that, for wild-type animals, the observed activity is directly proportional both to worm number and to time. For scoring purposes, the assay was modified by the inclusion of a period of pretreatment with DOC, and under these conditions, as Table 3A shows, individuals homozygous for *ace-1(p1000)* could be readily distinguished.

We used the individual assay first to determine whether *ace-1* fell within the intervals covered by the partial *X*-chromosome duplications *mnDp8*, *mnDp9*, *mnDp25* and *mnDp27* (see Figure 5). In each case, animals homozygous for *p1000* and for the translocated duplication segment were produced according to the general scheme outlined in Figure 10. Then, individuals of this genotype were examined by the individual assay, with and without pretreatment by DOC. As Table 3B shows, *ace-1* fell by these tests within the interval covered by *mnDp8*, *mnDp9* and *mnDp27*, but not within that covered by *mnDp25*. This confirmed its location to the right of *unc-3* and also showed that it lay to the

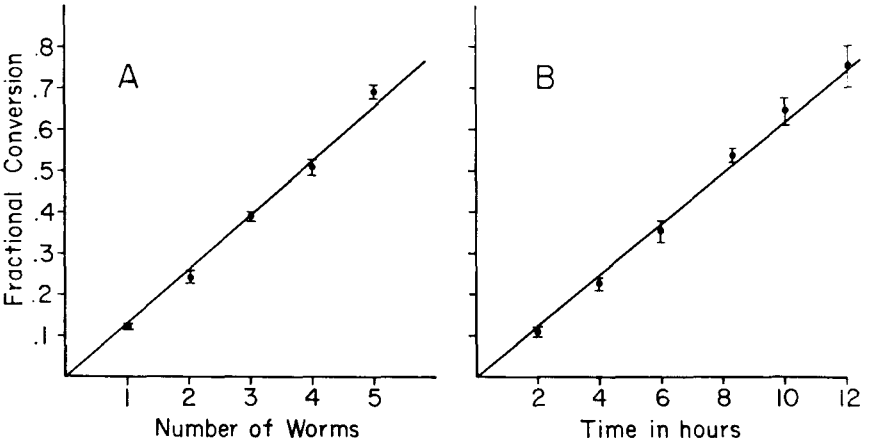

FIGURE 9.—Linearity of the "individual assay". Synchronously hatched worms were grown to adulthood (70 hr after hatching) at 20°, then assayed at 23° for acetylcholinesterase activity as described in MATERIALS AND METHODS. Fractional conversion (ordinate) is the corrected fraction of acetylcholine hydrolyzed. (A) Assays for 2 hr with the indicated number of worms per vial. (B) Assays with 1 worm per vial for the indicated times. Error bars indicate standard deviations for five assays.

TABLE 3

Acetylcholinesterase activities of several genotypes, as determined by "individual assay"

	Genotype	Acetylcholinesterase activity		
		Total	Resistant to DOC	Percent DOC-resistant
	+/+ (N2)	0.312 ± 0.013	0.159 ± 0.006	51
A.	*ace-1* (PR1000)	0.160 ± 0.033	0.000 ± 0.000	< 1
	unc-3 ace-1 (PR946)	0.130 ± 0.003	0.000 ± 0.000	< 1
	mnDp8; unc-3 ace-1	0.241 ± 0.007	0.139 ± 0.005	58
B.	*mnDp9; unc-3 ace-1*	0.331 ± 0.012	0.165 ± 0.004	50
	mnDp25; unc-3 ace-1	0.166 ± 0.003	0.000 ± 0.000	< 1
	mnDp27; unc-3 ace-1	0.333 ± 0.007	0.157 ± 0.006	47

Animals of the indicated genotype were grown for 72 hr at 20° from the time of egg laying, picked into vials (5 per vial) and assayed for 1 hr by the individual assay as described in MATERIALS AND METHODS. During the last 60 min before the assay, vials received either 10 μl of 2% DOC or 10 μl of H_2O. The values reported are the means, ± standard errors, for 6 assays each, expressed as corrected fractional conversion of substrate, f_{corr}.

right of the right end of *mnDp25* and the left of the right end of *mnDp8* (see Figure 5).

Further refinement was possible using the partial *X*-chromosome deficiencies *mnDf4*, *mnDf8*, *mnDf41* and *mnDf42*. In this case, however, because these deficiencies were homozygous lethal, it was necessary to test individuals harboring the deficiency on one *X* chromosome and *ace-1(p1000)* on the other. These were produced as outlined in Figure 11 and tested individually. As shown in Table 4A, the results established that *ace-1* fell outside the intervals covered by *mnDf4* and

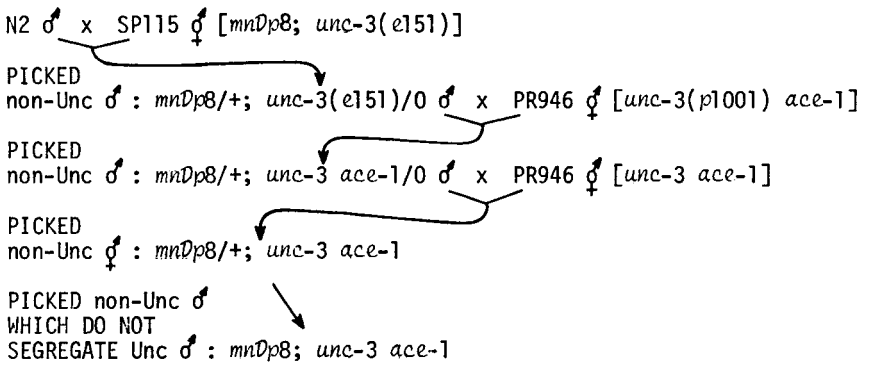

FIGURE 10.—Production of animals homozygous for *ace-1(p1000)* and for a duplicated *X* segment. The procedure is illustrated for *mnDp8*, but the procedure for *mnDp9*, *mnDp25 and mnD27* was operationally identical. All four duplicated segments include the *unc-3(+)* allele (see Figure 5).

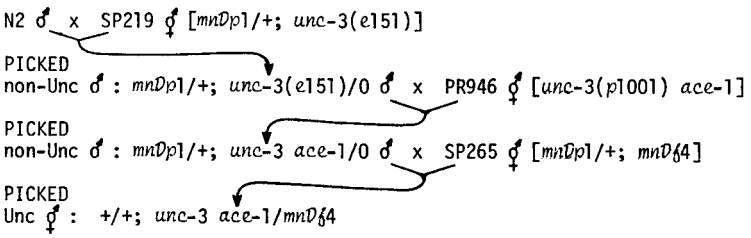

FIGURE 11.—Production of animals doubly heterozygous for *ace-1(p1000)* and a deficiency. The crosses are illustrated for *mnDf4*, but were operationally identical for all the deficiencies used, with the exception that *mnDf41*, the only deficiency that does not cover *unc-3*, was used in combination with the *unc-3* mutant allele *e151*. Details on the extents of the deficiencies are given in Figure 5.

mnDf8, but within those covered by *mnDf41* and *mnDf42*. These results localize *ace-1* to a limited region between the left end of *mnD41* and the right end of *mnDf42* (see Figure 5).

The region to which *ace-1* mapped also contains the genes *let-2* and *let-16*, whose only known alleles are lethal when homozygous (MENEELY and HERMAN 1979). It seemed possible, although not likely, that *p1000* might be a less severe allele of one of these. To test this possibility, we assayed individuals carrying *p1000* on one *X* and a lethal allele of one of these genes on the other, producing them by a method analogous to that of Figure 11. As Table 4B shows, all possessed significant levels of DOC-resistant acetylcholinesterase, establishing that *ace-1* is not allelic to either of the *let* genes. The same region also contains *mec-4*, whose mutant allele confers lack of sensitivity to mechanical stimulation (SULSTON, J. unpublished). As Table 4C shows, individuals homozygous for the *mec-4* mutant allele *e1479* had normal levels of DOC-resistant acetylcholinesterase activity. In addition, PR1000 [homozygous for *ace-1(p1000)*] showed normal sensitivity to mechanical stimulation (our direct observation). By these criteria, *ace-1* is not an

TABLE 4

Localization and allelism tests of ace-1

	Genotype	Acetylcholinesterase activity: Total	Resistant to DOC	Percent DOC-resistant
	unc-3 ace-1/mnDf4	0.158 ± 0.004	0.087 ± 0.002	55
A.	*unc-3 ace-1/mnDf8*	0.147 ± 0.020	0.086 ± 0.009	54
	unc-3 ace-1/mnDf41	0.092 ± 0.007	0.000 ± 0.000	< 1
	unc-3 ace-1/mnDf42†	0.026 ± 0.007	0.000 ± 0.000	< 4
	unc-3 ace-1/unc-3 let-2 (mn143)	0.160 ± 0.007	0.071 ± 0.004	45
B.	*unc-3 ace-1/unc-3 let-2 (mn153)*	0.185 ± 0.017	0.073 ± 0.004	40
	unc-3 ace-1/unc-3 let-16 (mn117)	0.188 ± 0.007	0.084 ± 0.005	45
	unc-3 ace-1/unc-3 let-6 (mn130)	0.165 ± 0.008	0.075 ± 0.004	45
C.	*unc-3 mec-4/unc-3 mec-4*	0.275 ± 0.015	0.140 ± 0.005	51

Assays as in Table 3 except that each vial contained a single animal and was assayed for 5 hr. The *mec-4* allele used is *e1497*. Results are means, ± standard errors, of 5–10 assays each, expressed as corrected fractional conversion of substrate, f_{corr}.

† Although of the same chronological age, the *unc-3 ace-1/mnDf42* animals were noticeably smaller; slower growth has been observed in other instances for *mnDf42* heterozygotes.

allele of *mec-4* and is therefore a new gene, but it has not been mapped further with respect to *let-2*, *let-16* and *mec-4*.

Gene dosage experiments: As a preliminary test of the idea that *ace-1* might be a structural gene for class A acetylcholinesterase forms, we carried out individual assays on animals possessing 0, 1, 2 or 4 copies of the wild-type allele *ace-1* (+), using the available *mnDp* strains as necessary. Table 5 show that the observed levels of DOC-resistant acetylcholinesterase activity are almost directly proportional to the number of *ace-1* (+) gene copies present. This gene dosage effect supports our view that *ace-1* is a structural gene.

DISCUSSION

The results presented above reveal a previously unidentified gene, *ace-1*, that affects acetylcholinesterase activity in *Caenorhabditis elegans*. This gene maps to the right end of the *X* chromosome, and its one known mutant allele, *p1000*, produces a selective deficiency of acetylcholinesterase forms of the previously (kinetically) identified class A. Gene dosage experiments suggest that *ace-1* is a structural gene for these forms. Somewhat surprisingly, the enzymatic deficiency produced by *p1000* leads to no obvious behavioral or developmental alteration.

The initial occurrence of the *ace-1* mutation *p1000* in combination with an allele of the closely linked behavioral gene *unc-3* appears to have been a chance consequence of the relatively heavy mutagenesis used (exposure to 0.1 M ethyl methanesulfonate for 4 hr at 20°). There was no indication of any functional interaction between *p1000* and the *unc-3* phenotype, and the relative normality of recombination between *ace-1* and *unc-3* argues against the occurrence of any massive chromosomal event by which both mutations might have been generated.

TABLE 5

Gene-dosage effects of ace-1(+)

	Genotype	Number of *ace-1*(+) gene copies	DOC-resistant Acetylcholinesterase activity	% of N2 control
	ace-1 (PR1000)	0	0.000 ± 0.000	< 0.5
	unc-3 ace-1 (PR946)	0	0.000 ± 0.000	< 0.5
A.	*dpy-11*/+; *ace-1*/+	1	0.113 ± 0.006	52
	unc-3 ace-1/++	1	0.130 ± 0.004	60
	+/+ (N2)	2	0.218 ± 0.009	(100)
	mnDp25; unc-3 ace-1	0	0.000 ± 0.000	< 0.5
	+/+ (N2)	2	0.159 ± 0.006	(100)
	mnDp8; unc-3 ace-1	2	0.139 ± 0.005	87
	mnDp9; unc-3 ace-1	2	0.165 ± 0.004	104
B.	*mnDp27; unc-3 ace-1*	2	0.157 ± 0.006	99
	mnDp25; unc-3	2	0.162 ± 0.003	102
	mnDp8; unc-3	4	0.311 ± 0.006	196
	mnDp9; unc-3	4	0.280 ± 0.014	176
	mnDp27; unc-3	4	0.279 ± 0.002	176

Assays were conducted exactly as in Table 3; some data from that table reappear here. A and B are two different batches, performed on different days. Batch-to-batch variability of the kind seen for N2 is common and probably due to lack of temperature control in the assays. Within-batch reproducibility, as the reported standard errors indicate, is good.

That the mutagenesis was indeed heavy is illustrated by the fact that the original *p1000*-containing strain, PR946, also failed to grow at 25° In view of the mutagenesis conditions and of the fact that only a single *ace-1* mutation was recovered, it is clearly impossible to comment on the mutagenic target size for *ace-1* or on the likelihood that additional *ace-1* alleles might be recovered by an enzymatic screen of behaviorally *un*selected F_2 clones from a mutagenized population.

The absence of a behavioral phenotype for the *ace-1* homozygous strain PR1000 was initially a surprise, and could have any of several possible explanations. It could be, for instance, that the class A acetylcholinesterase forms in PR1000 are present in the animal, but extremely labile in extracts, or that an extremely small and as yet undetected amount of class A acetylcholinesterase remains in PR1000 and is adequate for normal behavioral function, or that class A acetylcholinesterases normally have no behavioral role. In fact, as CULOTTI *et al.* (1981) shows, none of these explanations is likely, and it appears instead that the class A acetylcholinesterase deficiency of PR1000 is of no obvious behavioral consequence simply because the remaining class B acetylcholinesterases are adequate for normal behavior. Further discussion of this point is found in CULOTTI *et al.* (1981).

The enzymatic deficiency of PR1000 has been shown to include both of the normally DOC-resistant acetylcholinesterase forms III and IV, but the methods reported are not sufficient to determine whether the remaining member of class A, form IA, is also deficient. A forthcoming publication will examine this and other quantitative questions in detail, but at this point it can be said that PR1000

also lacks detectable amounts of form IA, and is therefore deficient in all class A forms. The gene-dosage effects of *p1000* on the DOC-resistant forms III and IV makes it likely that *ace-1* is a structural gene for these forms and most probably for all three class A forms (IA, III, and IV). If so, then these three forms, despite their size differences, would appear to contain at least one common subunit, and their kinetic similarities would suggest that this common subunit might carry the active site. Whether they differ simply in the number of common subunits possessed, or perhaps in the differential possession of other components as yet not identified, is a question that can be answered only by enzyme purification or identification of additional structural genes. However, it is intriguing to note that the two large forms, III and IV, require detergent for effective solubilization (JOHNSON and RUSSELL, in press) and would therefore appear to be membrane bound; whereas, the smaller form, IA, does not. Perhaps the larger size of forms III and IV could be due to the presence of components that confer membrane association.

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