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The Effect of Two Genes on Anesthetic Response in the Nematode *Caenorhabditis elegans*

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The authors studied the wild type strain, N2, and three mutant strains of the nematode, *Caenorhabditis elegans*, in order to measure genetically produced changes in responses to nine volatile anesthetics. They determined the anesthetic ED₅₀s of N2 for thiomethoxyflurane, methoxyflurane, chloroform, halothane, enflurane, isoflurane, fluroxene, flurothyl, and diethylether. The log-log relationship of the oil-gas partition coefficients (O/G) and the ED₅₀s of these agents for N2 yields a straight line with a slope of -0.997 with an R² of $.98$ over a range of O/G (at 37° C) from 48 to 7230. When the O/Gs are corrected to 22° C, the slope is -0.964 with an R² of $.98$. This relationship is similar to that found in other animals. Two mutant strains, *unc-79* and *unc-80*, show altered responses to these anesthetics. These strains are two to three times more sensitive than N2 to anesthetics with an O/G greater than that of halothane (220 at 37° C), yet they differ little from N2 in response to anesthetics with lower O/Gs. *unc-79* and *unc-80* are about 30% more sensitive than N2 to diethylether. The double mutant *unc-79;unc-80* is more sensitive to halothane, isoflurane, and fluroxene than is either mutant alone. The authors believe these data indicate an alteration at the site of action of volatile anesthetics in *unc-79* and *unc-80*. They also postulate that the interaction of *unc-79* and *unc-80* indicate these genes code for enzymes in a common pathway, and that *unc-79* precedes *unc-80* in this pathway. (Key words: Anesthetics, volatile: chloroform; diethylether; enflurane; flurothyl; fluroxene; halothane; isoflurane; methoxyflurane; thiomethoxyflurane. Nematodes: *Caenorhabditis elegans*. Theories of anesthesia.)

IN THE 80 YR since Meyer¹ and Overton² originally noted the correlation between potency of volatile anesthetics and their solubility in oil, no single explanation has gained universal acceptance for the mechanism of action of volatile anesthetics. Knowledge of the molecular composition of the site of action of volatile anes-

thetics should help to clarify this mechanism. Regardless of its molecular composition, the site of action of volatile anesthetics must be specified by an organism's genome. Studies in fruit flies (10⁵ neurons)³ and mice (10⁸ neurons)⁴ have documented genetic control of anesthetic sensitivity. However, the neuronal complexity of these animals limits them as models for understanding the molecular mechanism of anesthetic action.

We have proposed the nematode, *C. elegans*, as an animal in which to study how volatile anesthetics work.^{5,6} It has been said that ". . . more is known about the genetics and development of this one millimeter roundworm than about any other multicellular creature."⁷ The hermaphrodite always consists of 959 cells of which exactly 302 are neurons.⁸⁻¹¹ Every synapse of these 302 nerves is known,⁸⁻¹¹ along with all cell lineages.¹² The nematode has four neurotransmitters, acetylcholine, GABA, serotonin, and dopamine. Genetic analysis of *C. elegans* is also very extensive, and mutants are available with a variety of abnormalities in neuromuscular function. Most importantly, *C. elegans* responds to volatile anesthetics by first undergoing a period of excitation, and then by becoming immobile and unresponsive to a tap to the head.⁵ Upon removal from anesthetics, they resume movement within 2-5 min and appear normal with respect to movement, feeding, and fertility. The potency of volatile agents in *C. elegans* correlates well with their oil/gas partition coefficient (O/G); the log-log plot of ED₅₀s versus O/G yields a slope approximating -1 , from an $48 < O/G < 980$, for *C. elegans*,⁵ and all other animals tested.¹³

It is possible to clone *C. elegans* genes, i.e., produce *in vitro* DNA segments and use them to manufacture large amounts of their RNA and protein products. To select genes to clone, we initiated a search for mutants with abnormal responses to volatile anesthetics. The first mutation, *unc-79* (*unc* for uncoordinated in phenotype) was two to three times more sensitive to the highly lipid soluble anesthetics (halothane, chloroform, and methoxyflurane) than its normal counterpart N2, slightly resistant to two agents (enflurane and flurothyl), and unchanged in its sensitivity to fluroxene and isoflurane.⁵ We postulated that it represented an animal with an altered site of action of volatile anesthetics.⁵ A second mutation, *unc-80*, was also found to be hypersensi-

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tive to halothane.⁶ A nematode constructed by us to bear both mutations *unc-79* and *unc-80* was more sensitive to halothane than either parent.⁶

Before undertaking a molecular analysis of *unc-79* and *unc-80*, we chose to expose the mutants and N2 to nine volatile anesthetics with O/Gs ranging from 48 to 7230. This extends our data for *unc-79* and N2 by a factor of 7, and determines ED₅₀s for *unc-80* and the double mutant, *unc-79;unc-80*. We expected a deviation from the usual relationship between the log of the O/Gs and the log of the ED₅₀s, if these mutations do represent a change in the site of action of volatile anesthetics. Thus, any mutants which show such changes will be candidates for DNA cloning and subsequent analysis of the gene products controlling anesthetic response. In addition, if *unc-79* and *unc-80* use different pathways to affect the site of anesthetic action, we expected the double mutant, *unc-79;unc-80*, to be more sensitive to volatile agents than either *unc-79* or *unc-80*. We report here the results for all four strains of worms in nine anesthetic agents.

Materials and Methods

NEMATODES

C. elegans var. Bristol (wild type strain = N2) and the mutant *unc-80* (el272) were obtained from the Caenorhabditis Genetics Center. We isolated *unc-79* (ecl) after exposing N2 to the chemical mutagen EMS.⁵ We constructed *unc-79;unc-80* by mating the *unc-79* males (chromosome III) to *unc-80* hermaphrodites (chromosome V), bearing an easily scored homozygous marker on chromosome III.⁶ Nematode cultures were kept as previously described.⁵

ANESTHETICS

Flurothyl (FLR) was supplied by Anaquest, Inc. Thiomethoxyflurane (TMOF) was given to us by Dr. E. I. Eger II. Chloroform (CH), methoxyflurane (MOF), halothane (H), enflurane (E), isoflurane (ISO), fluroxene (FLX), and diethylether (DE) were commercial products.

DOSE-RESPONSE CURVES

Anesthetic response was assayed as described previously in detail.⁵ Briefly, synchronized cultures of worms on agar plates were placed in a glass air-tight chamber. A liquid volume of anesthetic, calculated to give an appropriate gas concentration based on the chamber's volume, was injected with a glass syringe into the sealed chamber *via* a stopcock. The worms were observed through the chamber's lid with a dissecting microscope, and judged to be anesthetized when they

assumed a straight posture and became immobile as previously described.⁵ (Normal worms move in a constant sinuous motion across the plate.) They were scored after 2 h, except those exposed to thiomethoxyflurane, which required 5 h for equilibration. Anesthetic concentrations were measured with a gas chromatograph. Dose-response curves for each anesthetic were based on a minimum of 20 different concentrations, with 50 animals per concentration. ED₅₀s were defined as that concentration at which 50% of the nematodes were immobile. Observers were unaware of the strain being scored, but were aware of the anesthetic being used.

OIL-GAS PARTITION COEFFICIENTS

We obtained O/Gs at 37° C from values published by Eger *et al.*¹³⁻¹⁵ We used O/Gs corrected to 22° C by the method of Allott *et al.*¹⁶ to determine a regression line for the log-log plot of O/Gs and ED₅₀s of the nine anesthetics.

STATISTICAL METHODS

Regression analysis, ED₅₀s, slope constants, and SEs were calculated using the methods described by Waud.¹⁷ Regression curves for the log ED₅₀s and log O/G were constructed using the least-squares method. For each anesthetic, all the ED₅₀s and the slope constants of all four strains were compared using an analysis of variance to see if they satisfied the null hypothesis (*e.g.*, all means are equal).¹⁸ If they did not satisfy the null hypothesis ($P < .05$), we compared the individual mean values of each strain. Comparison of ED₅₀s and of slope constants for the different strains was performed by Tukey's method for multiple comparisons.¹⁸ Significance was defined as $P < .01$. The increased stringency was used to avoid Type I errors. Variances for the differences between ED₅₀s used in figure 5 were calculated by adding the variances of each ED₅₀ involved.

Results

Table 1 lists the ED₅₀s ± SEs for all four strains of worms. As previously noted with N2 and *unc-79*, the ED₅₀s for *unc-80* and *unc-79;unc-80* tended to increase as the O/G of the anesthetic decreased. Unlike N2, the mutant strains went through an "excitation phase" only in the presence of diethylether and its fluorinated derivative, flurothyl. Flurothyl in low concentrations also restored the uncoordinated movement of *unc-80* to normal, as previously reported for *unc-79*.⁵ We found a significant change in the slope constants between the mutant strains and N2 for halothane, methoxyflurane, and thiomethoxyflurane (table 2).

For all nine anesthetics, we noted four patterns of

TABLE 1. ED₅₀ ± SEs (v/v% at 1 atm, 22°C) for Four Strains of *C. elegans* in Nine Anesthetics

	N2	<i>unc-79</i>	<i>unc-80</i>	<i>unc-79; unc-80</i>
TMOF*	0.11 ± 0.01	0.09 ± 0.03†	0.07 ± 0.03†	0.04 ± 0.02†
MOF*	0.58 ± 0.02	0.28 ± 0.05†	0.46 ± 0.03†‡	0.25 ± 0.10†§
CH*	1.47 ± 0.02	0.50 ± 0.03†	0.80 ± 0.02†‡	0.54 ± 0.03†§
H*	3.18 ± 0.04	0.98 ± 0.02†	1.20 ± 0.02†‡	0.72 ± 0.02†‡§
E*	5.89 ± 0.08	6.24 ± 0.07†	6.06 ± 0.07†	5.82 ± 0.07†§
ISO*	7.18 ± 0.07	6.67 ± 0.08†	6.14 ± 0.07†‡	5.84 ± 0.07†‡§
DE*	7.53 ± 0.07	5.70 ± 0.06†	5.84 ± 0.06†	5.60 ± 0.06†
FLX*	10.8 ± 0.07	10.1 ± 0.07†	10.4 ± 0.07†	9.9 ± 0.07†§
FLR*	14.3 ± 0.10	15.9 ± 0.11†	14.9 ± 0.10†‡	14.5 ± 0.08†

TMOF = thiomethoxyflurane; MOF = methoxyflurane; CH = chloroform; H = halothane; E = enflurane; ISO = isoflurane; DE = diethylether; FLX = fluroxene; FLR = flurothyl.

* Four strains fail null hypothesis (analyzed by ANOVA) at $P < .05$

level.

† Different from N2, $P < .01$.

‡ Different from *unc-79*, $P < .01$.

§ Different from *unc-80*, $P < .01$.

TABLE 2. Slope Constants + SE for Four Strains of *C. elegans* in Nine Anesthetics

	N2	<i>unc-79</i>	<i>unc-80</i>	<i>unc-79; unc-80</i>
TMOF*	14.1 ± 5.6	3.0 ± 1.3†	4.2 ± 1.7†	3.3 ± 1.7†
MOF*	8.4 ± 3.0	2.1 ± 1.0†	1.8 ± 0.9†	0.5 ± 0.4†
CH	6.8 ± 3.8	3.2 ± 1.5	3.3 ± 1.4	4.1 ± 1.9
H*	11.6 ± 4.6	3.7 ± 1.4†	4.0 ± 1.6†	2.8 ± 1.1†
E	17.8 ± 9.6	22.4 ± 10.4	32.6 ± 14.0	30.6 ± 13.3
ISO	25.2 ± 11.5	10.8 ± 5.4	11.4 ± 5.7	16.1 ± 8.5
DE	14.3 ± 6.1	13.2 ± 6.0	9.3 ± 4.2	14.8 ± 6.1
FLX	10.7 ± 4.1	10.9 ± 4.0	12.2 ± 4.4	11.6 ± 4.2
FLR	11.7 ± 5.3	14.0 ± 6.9	8.9 ± 4.1	14.9 ± 5.6

See table 1 for abbreviations.

* Four strains fail null hypothesis (ANOVA) at $P < .05$ level.

† Different from N2, $P < .01$.

dose-response curves, which are represented in figures 1–4. Figure 1, the response of N2, *unc-79*, and *unc-80* to thiomethoxyflurane, is representative of their behavior in the four anesthetics with the highest O/G (i.e., also methoxyflurane, chloroform, and halothane). The

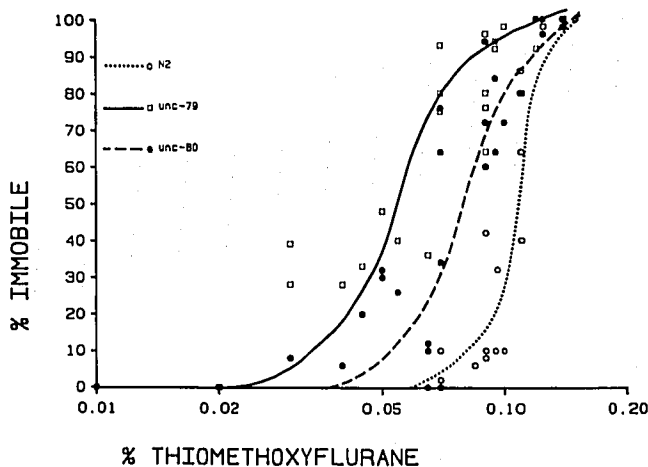


FIG. 1. Percent *C. elegans* immobile versus percent (v/v%) thiomethoxyflurane in air. Each point represents 50 organisms observed for 10 s each. All experiments were performed at 20–22°C and 1 atmosphere for this figure, as well as figures 2–4.

ED₅₀s of *unc-79* and *unc-80* are much less than those of N2, with the ED₅₀s of *unc-79* less than those of *unc-80*. The double mutant, *unc-79; unc-80*, has ED₅₀s not statistically different from *unc-79*. An exception exists with halothane, to which *unc-79; unc-80* is more sensitive than *unc-79*, as noted before.⁶

In contrast to the above, *unc-79* and *unc-80* are resistant to flurothyl compared to N2 ($P < .01$ for *unc-79*, $P < .01$ for *unc-80*) (fig. 2). This pattern is characteristic for both flurothyl and enflurane, two agents with convulsant activity in mammals. The ED₅₀ of the double mutant is indistinguishable from those of N2 in these two agents.

In fluroxene, the mutant strains have ED₅₀s about 5–10% less than that of N2 ($P < .01$) (fig. 3). This was also true for isoflurane. The double mutant, *unc-79; unc-80*, has an ED₅₀ indistinguishable from *unc-79* in fluroxene, but its ED₅₀ is less than that of *unc-79* in isoflurane ($P < .01$).

With diethylether, both mutants have an ED₅₀ approximately 30% less than that of N2 ($P < .01$); the double mutant resembles *unc-79* (fig. 4).

Figure 5 summarizes the data as percent change of ED₅₀ for all mutant strains compared to N2. The differences in ED₅₀s for all anesthetics with an O/G

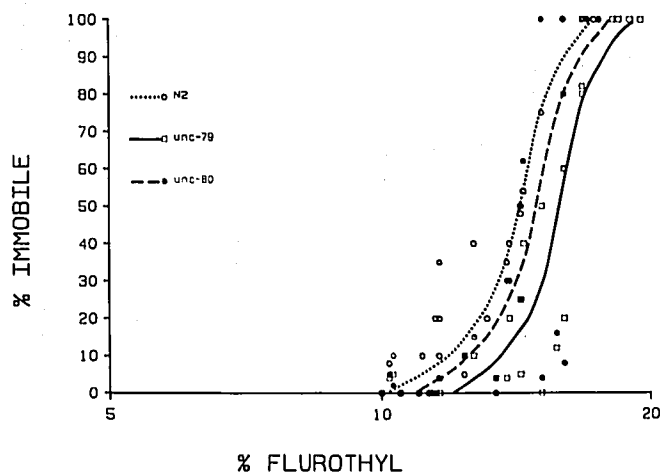


FIG. 2. Percent *C. elegans* immobile versus percent (v/v%) flurothyl in air.

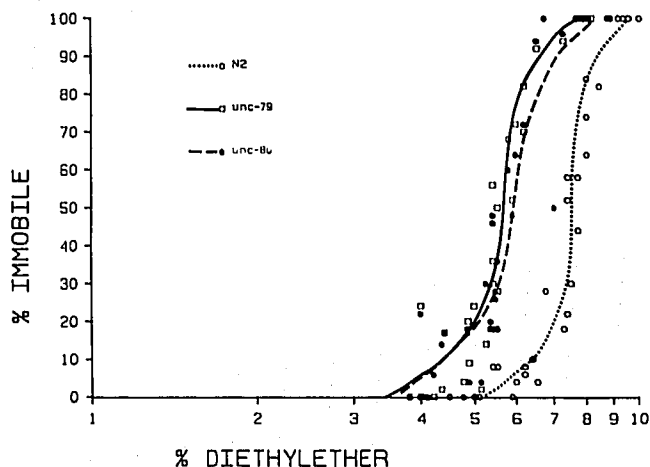


FIG. 4. Percent *C. elegans* immobile versus percent (v/v%) diethylether in air.

greater than or equal to that of halothane are seen to the left of the histogram; also shown are the similarities of ED_{50} s for the double mutant and *unc-79* in these agents.

The log-log relationship of O/G versus ED_{50} s for N2 is a straight line, with a slope of -0.997 and an R^2 of $.98$ (fig. 6). The values for these O/G were determined at $37^\circ C$ [O/G (37°)]. However, our experiments were performed at $22^\circ C$. We used a standard temperature correction described by Allott *et al.*¹⁶ for O/Gs (37°) from 0.1 to 980 to determine O/Gs at $22^\circ C$ [O/G (22°)]. Since this method has only been shown to be applicable to an O/G ($37^\circ C$) as high as 980 (MOF), we did not use TMOF [O/G ($37^\circ C$) = 7230] to calculate the slope of the regression line. When we applied this temperature correction, we obtained a slope of -0.964 with an R^2 of

.98. Plotting the same data for *unc-79* or *unc-80* does not yield a straight line at either temperature. Figure 6 presents these relationships at $22^\circ C$. Each mutant generates a set of points that deviates from those of N2 for O/Gs greater than or equal to that of halothane (H, CH, MOF, TMOF).

Discussion

Our experiments show genetic control of anesthetic response in *C. elegans*, an animal unfamiliar to most anesthesiologists. Introduction of this unfamiliar model warrants discussion of limitations of our methods in scoring animals for immobility. The accuracy of scoring a plate was approximately $\pm 5\%$ between different scorers, which is about the same as the reproducibility of counting for any individual scorer. The animals' characteristic phenotypes were usually obvious to anyone scoring plates, making true "blinding" as to strain

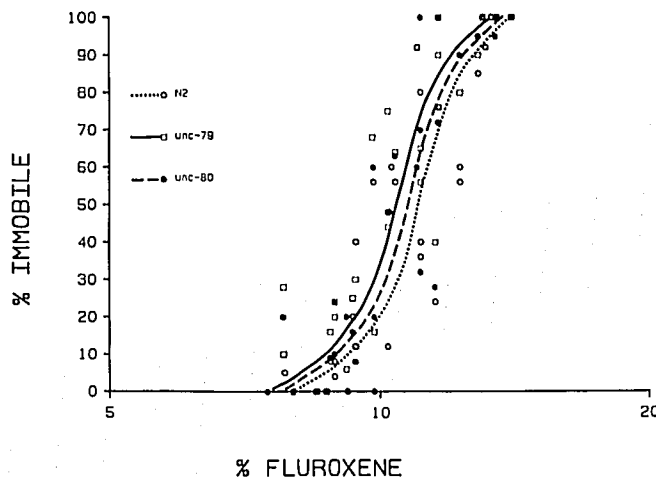


FIG. 3. Percent *C. elegans* immobile versus percent (v/v%) fluroxene in air.

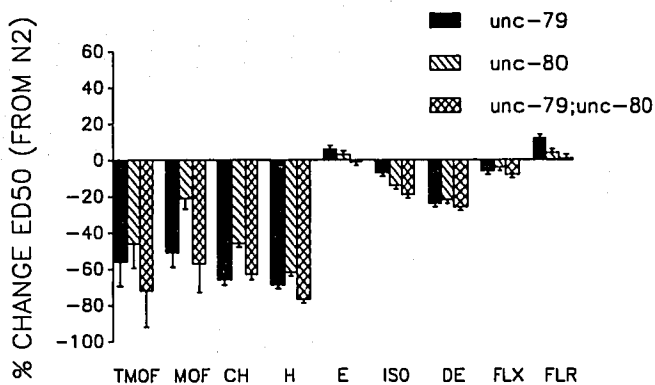


FIG. 5. Percent change in ED_{50} s for three mutant strains compared to the wild type strain (N2). Calculated as $ED_{50}(\text{strain}) - ED_{50}(N_2) / ED_{50}(N_2)$.

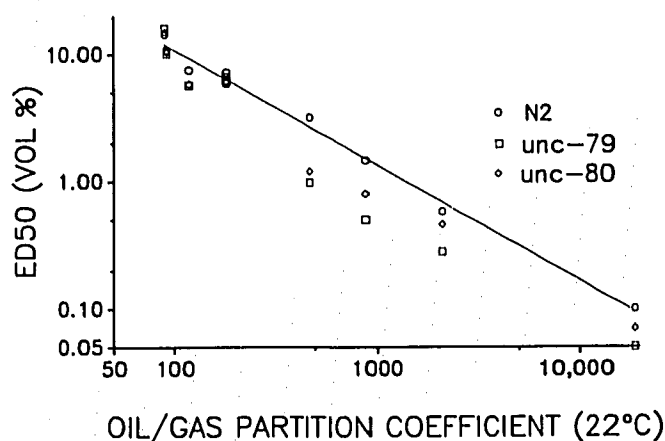


FIG. 6. Log dose required to immobilize 50% of nematodes (ED_{50}) versus log oil/gas partition coefficient (O/G) for nine anesthetics. O/G values determined at 37° C¹³⁻¹⁵ are corrected to 22° C, as described by Allott *et al.*¹⁶

of worm intrinsically impossible. Although anesthetic agent and approximate concentration were known to the scorer, the exact concentration of gas was not known until after all worms were counted. However, we think that true double blinding of these dose-response curves is probably unnecessary. To score worms for immobility, one records an objective behavioral endpoint that is a quantal response; there is no gradation of behavior that requires evaluation by an observer. A major advantage of dose-response curves in *C. elegans* are the large numbers (relative to mammals) of animals in each curve. We have scored at least 1500–2000 animals for each dose-response curve; this, in turn, leads to small standard errors of each ED_{50} .

We do not know why 5 h of exposure were necessary for maximum effect of thiomethoxyflurane. *C. elegans* is always enveloped in a thin film of water as it moves across an agar plate; we thought that thiomethoxyflurane may be less water soluble than the other anesthetics tested. However, the water/gas partition coefficient of thiomethoxyflurane is no lower than anesthetics that required only 2 h for maximum effect.¹³

To use *C. elegans* as a model, it was necessary to establish that the non-mutated animal, N2, responds to anesthetics like other animals. We found that the log/log plot of ED_{50} s versus O/Gs for N2 gives a straight line with a slope very close to -1 for nine volatile anesthetics. Like all other animals tested to date, the response of N2 adheres closely to the Meyer-Overton correlation; the responses of the two mutant strains, *unc-79* and *unc-80*, do not. These two strains represent the first animals with a documented deviation from this correlation; this may have profound implications. As stated by Koblin and Eger, "The amazing closeness of this correlation (the Meyer-Overton rule) implies a *unitary molecular site of action* (the italics are ours) and sug-

gests that anesthesia results when a specific number of anesthetic molecules occupy a crucial hydrophobic region within the CNS."¹⁹ We agree with their conclusion and postulate that our mutants represent a genetic manipulation of the otherwise "unitary molecular site of action." Whatever the precise nature of the change in these two mutants, their deviation from the Meyer-Overton rule is consistent with a change in the "crucial hydrophobic region" or the "specific number of anesthetic molecules" necessary to achieve the anesthetic state.

The significant differences in slope constants for the mutant strains (table 2) may indicate a change in the type of molecular interaction at the site of anesthetic action for thiomethoxyflurane, methoxyflurane, and halothane. If the change in ED_{50} s for these agents were merely due to a decreased dissociation coefficient at the site of action, the slope constants should remain unaffected. Thus, something other than mere increased affinity for these anesthetics seems to be responsible for the change in ED_{50} s.

Nothing is known of the neuroanatomy or molecular defects of *unc-79* or *unc-80*. However, based on the behavior of the double mutant, *unc-79;unc-80*, we can attempt to explain the role of these two genes. *unc-79* and *unc-80* are recessive mutations with respect to halothane sensitivity.⁶ This implies that both genes code for enzymatic products, as opposed to structural proteins required in stoichiometric amounts. If *unc-79* and *unc-80* affect the same enzymatic pathway, then the ED_{50} s of *unc-79;unc-80* should be the same as the ED_{50} s of either *unc-79* or *unc-80*. If the two genes affect separate pathways, the ED_{50} s of the double mutant should be less than those of either single mutant. Thus, we can speculate that *unc-79* and *unc-80* may act *via* a common enzymatic pathway in causing increased sensitivity to the more lipid-soluble anesthetics. In addition, the ED_{50} s of the double mutant are closest to those of *unc-79* in all of the more lipid soluble anesthetics, leading us to conclude that the product of the normal *unc-79* gene (*unc-79*⁺) precedes that of *unc-80* in this pathway (fig. 7). Figure 7 shows compound A converted into B by the product of the *unc-79*⁺ gene, and B into C by the product of the normal *unc-80* gene (*unc-80*⁺). Accumulation of C leads to normal anesthetic sensitivity.

This model is particularly interesting in light of recent data presented by Evers *et al.*²⁰ These investigators showed that, by altering fatty acid composition of rat brains, they were able to change sensitivity to certain anesthetics. The fatty acid compositions were altered by controlling the diet of the animals. Genetically produced enzymatic changes may affect the same or similar systems.

We have previously described a mutation in another gene, *unc-9*, which causes *unc-79* and *unc-80* to respond

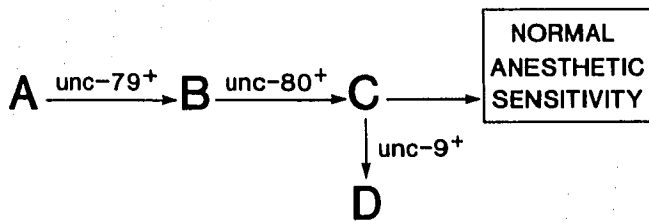


FIG. 7. A postulated metabolic pathway leading to normal anesthetic sensitivity in *C. elegans*. Compound A is converted into B by the product of the normal gene *unc-79*⁺. B is converted, in turn, to C by the product of the normal gene *unc-80*⁺. Adequate levels of C cause normal anesthetic sensitivity. The product of *unc-9*⁺ normally converts C into D and, thus, levels of C may rise if the *unc-9*⁺ gene is inactivated.

like N₂ to halothane.⁶ In figure 7, the *unc-9*⁺ product degrades C to D; a mutated *unc-9* allows C to accumulate, leading to normal response in halothane by the mutant. If our model is correct, *unc-9* will be a suppressor in other volatile anesthetics.

In addition to suppressor studies, we are now screening for mutants with increased sensitivity to flurothyl and enflurane, and mutants resistant to halothane. To date, we have screened 90 of the 110 known uncoordinated mutants in *C. elegans* for alterations in anesthetic sensitivity, and have found none other than the two reported here. However, we cannot say that *unc-79* and *unc-80* are the only genes affecting anesthetic response in *C. elegans*. We are also beginning to clone both the *unc-79* and *unc-80* genes, in order to identify their gene products.

In summary, we have identified specific genes that alter anesthetic sensitivity in *C. elegans*, and proposed a model for their interaction. These genetic studies may lead to understanding the molecular determinants of anesthetic response in *C. elegans*.

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