GENETICS OF ACETYLCHOLINESTERASE IN *DROSOPHILA MELANOGASTER*[1]

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ABSTRACT

Genes in *Drosophila melanogaster* that control acetylcholinesterase (AChE) were searched for by segmental aneuploidy techniques. Homogenates of flies containing duplications or deletions for different segments were assayed for enzyme activity. A region on the third chromosome was found for which flies having one dose consistently gave lower AChE activity than euploid flies, which in turn had lower activity than flies with three doses. The activity differences were in the approximate ratio 1:2:3. Fine structure deletion mapping within this region revealed a very small segment for which one-dose flies have approximately half-normal activity. To obtain putative AchE-null mutations, lethal mutations within this region were assayed. Four allelic lethals have approximately half-normal activity in heterozygous condition. These lethals probably define the structural locus (symbol: *Ace*) for AChE.

NEUROLOGICAL mutations in Drosophila hold promise as tools for studying the development and function of the nervous system (reviewed by BENZER 1973; GROSSFIELD 1975; PAK 1975). Most of the existing ones have been isolated on the basis of defective behavior. Another approach is to choose a gene that controls a specific protein involved in nervous system function, isolate mutations in the gene, and analyze the defective gene product's effects on the development, neurophysiology, and behavior of the organism. Potentially, such mutations can also be used as markers for nervous system tissues in genetic mosaic experiments (e.g. HOTTA and BENZER 1973; KANKEL and HALL 1976).

Acetylcholinesterase (AChE) is a favorable enzyme in Drosophila for this type of analysis. The enzyme hydrolyzes acetylcholine, which is an important excitatory central nervous system neurotransmitter in insects (reviewed by PITMAN 1971). The enzyme is a good histochemical marker for nerve cells.

Using segmental aneuploidy, the genome was screened for loci controlling the enzyme. This technique is based on the principle that three doses of the structural gene for a given enzyme could result in increased enzyme activity, compared to that in flies having the normal two doses; and one dose could lead to decreased

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activity. This has been confirmed for several Drosophila enzymes: Xanthine dehydrogenase (GRELL 1962), glucose-6-phosphate dehydrogenase (SEECOF, KAPLAN and FUTCH 1969; MARONI and PLAUT 1973; BOWMAN and SIMMONS 1973; STEWART and MERRIAM 1974), 6-phosphogluconate dehydrogenase (SEECOF, KAPLAN and FUTCH 1969; BOWMAN and SIMMONS 1973; STEWART and MERRIAM 1974), tryptophan oxygenase (TOBLER, BOWMAN and SIMMONS 1971; BAILLIE and CHOVNICK 1971), isocitrate dehydrogenase (STEWART and MERRIAM 1974; RAWLS and LUCCHESI 1974) and the soluble form of α-glycerophosphate dehydrogenase (RAWLS and LUCCHESI 1974).

Several investigators have constructed duplications (three doses) for series of second and third chromosome regions in searches for previously unlocalized enzyme-controlling segments (O'BRIEN and GETHMANN 1973; RAWLS and LUCCHESI 1974; HODGETTS 1975). Most of the duplications were generated using the *Y*-autosome translocations of LINDSLEY *et al.* (1972). In the present study, a similar screen has been carried out with respect to AChE. Three chromosome regions have been found that give lower activity for one-dose flies than two-dose flies, which in turn have lower activity than three-dose flies. Lethal mutations within one of the regions were found to cause reduced AChE activity in flies heterozygous for the mutations. These lethals are candidates for mutations that eliminate the activity of this enzyme, and are likely to be mutations in the structural gene (symbol: *Ace*) for AChE.

MATERIALS AND METHODS

A. Construction of aneuploid genotypes

The principal means of generating flies aneuploid for various portions of the genome was through the use of translocations involving the *Y* chromosome, and either the *X*, second, or third chromosome (Figure 1). For second or third chromosome segmental aneuploids (which comprise the bulk of the screening), deletions are recovered in one sex, and duplications in the other (Figure 1). AChE assays of male homogenates (see below) gave more reproducible kinetics; so in re-tests of promising segments, and in all further work on segments that gave reproducible dosage effects, activity in deleted *vs.* duplicated males was compared. For *Y-2* and *Y-3* translocations (called *T(Y;2)*'s and *T(Y;3)*'s, respectively) reciprocal crosses were carried out for a given chromosome segment, so that deleted males and duplicated males could be obtained (e.g., deleted males from the cross in Figure 1, and duplicated males from the reciprocal cross).

When using two different *T(Y;2)*'s or *T(Y;3)*'s, crosses between two translocations with *Y* chromosome breakpoints in different arms (as in Figure 1) allows all euploid and aneuploid genotypes unambiguously to be recognized among the progeny (LINDSLEY *et al.* 1972). Crosses were made between translocations with different *Y*-arm breakpoints whenever availability of stocks with appropriate autosomal breakpoints made this possible. For some regions (totalling 16% of the autosomal complement), crosses had to be made between two given translocations with the same *Y*-arm breakpoints (see LINDSLEY *et al.* 1972). Here, one can be reasonably sure of the deleted *vs.* duplicated genotype of a given progeny since meiotic nondisjunction of homologous centromeres is rare in the translocation-bearing parents (O'BRIEN and GETHMANN 1973).

Using these crosses to generate deletions, about 80% of the second-third chromosome complement could be screened for effects on AChE activity. However, virtually the entire autosomal complement was screened with respect to duplicated segments (Figure 4A). The only region that cannot be tested is 83D-E on chromosome *3*, which causes lethality in one-dose and three-dose flies (LINDSLEY *et al.* 1972).

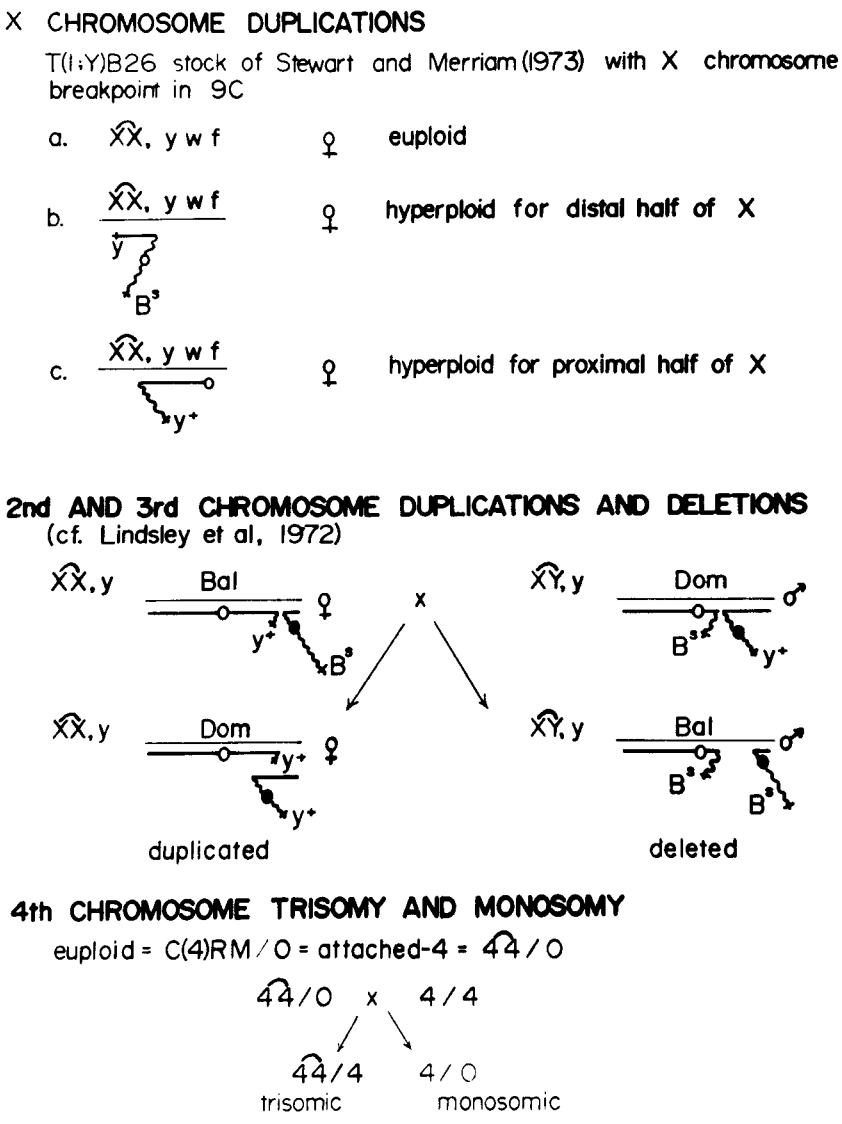


FIGURE 1.—Generation of flies aneuploid for various portions of the genome. $\hat{XX}$ = attached-X chromosome; $\hat{XY}$ = attached-*XY* chromosome; *Bal* = dominantly-marked inversion balancer; *Dom* = autosomal dominant mutation. Wavy lines designate heterochromatin; circles designate centromeres.

To screen the *X* chromosome for aneuploidy effects on AChE activity, the *T(1;Y)*'s of NICOLETTI and LINDSLEY (1960) and STEWART and MERRIAM (1973), plus additional strains listed in Table 1, were used. The initial chromosome screen (Figure 1) involved large duplications. This was followed by more detailed analysis (Figure 4B). Fourth chromosome aneuploidy was tested for effects on AChE activity using a stock of *C(4)RM, ci ey^R/gvl sv^n* (attached-*4*, see Figure 1).

TABLE 1

List of chromosome aberrations used in production of aneuploids assayed for AChE

Stock	Description
A. Sources of *X* chromosome deletions and duplications (cf. Figure 4B). All but the first three stocks were provided by G. LEFEVRE.	
T(1;3)sn^{13a1}	*X* chromosome region 6C; 7C9 inserted into region 79 of chromosome *3*; segregates flies duplicated or deleted for 6C; 7C9
Df(1)B263-70	*X* chromosome deletion of region 15F9–16A1; 16A6–B1
Dp(1;1)B	Bar duplication, of *X* chromosome region 15F9–16A1; 16A7–B1
T(1;2)FN107	*X* chromosome region 7A8; 8A5 inserted into chromosome *2* between pericentric inversion breakpoints in 2L + 2R; segregates flies duplicated for 7A8; 8A5
T(1;2)63i	*X* chromosome region 9E1; 10A11 inserted into 2R of chromosome *2*; segregates flies duplicated for 9E1; 10A11
T(1;2)65b	*X* chromosome region 9F13; 11A7 inserted into heterochromatin of chromosome *2*; segregates flies duplicated for 9F13; 11A7
y+Y mal^{106}	*Y* chromosome containing most of *X* chromosome region 1A, and also 18F to the centromere, allowing flies duplicated for these regions to be generated
y^{2}Y67g$^{19.1}$	*Y* chromosome containing *X* chromosome regions 1A1–2; 2B18 and also 20A3 to the centromere, allowing flies duplicated for these regions to be generated
B. Sources of duplications which include third chromosome region 87. (cf. Table 4). The first two stocks are described in LINDSLEY and GRELL (1968); the second two are from W. M. GELBART (unpublished).	
Stock	Description
T(3;1)ry^{35}	87C–E; 91B–C inserted into region 20 of the *X* chromosome
Dp(3;4)ry+	86D3; 88B attached to centromere and region 101 of fourth chromosome
T(3;1)kar^{5l}	87C8–D1; 88D12–E1 inserted into region *20* of the *X* chromosome
T(3;2)ry+^{w70hR}	87C2–3; 88C2–3 inserted into chromosome *2*, in *2L* euchromatin
C. Sources of small deletions in and near third chromosome region 87 (cf. Figure 5). The most recent cytological information on these stocks is from W. M. GELBART (unpublished).	

TABLE 1—Continued

Stock	Description—deletion of:
Df(3R)ry^{85}	87B15–C1; 87F15–88A1
Df(3R)ry^{615}	87B12–15; 87E8–11
Df(3R)ry^{619}	87D7–9; 87E12–F1
Df(3R)l26c	87E1–2; 87F11–12
Df(3R)ry^{27}	87D1–2; 87F1–2
Df(3R)l26d	87D11–13; 87E3–5
Df(3R)lC4a	87E5–7; 87E11–F1
Df(3R)ry^{614}	87D2–4; 87D11–14
Df(3R)kar^{3l}	87B15–C2; 87C9–D2
Df(3R)ry^{1402}	87D2–4; 87D14–E2
Df(3R)ry^{75}	87D1–2; 87D14–E1
Df(3R)ry^{81}	87C1–3; 87D14–E2
Df(3R)kar^{1G27}	87B3–5; 87D6–12
Df(3R)red^{3l}	87F12–14; 88C1–3

The translocation, deletion, or duplication stocks used in the current segmental aneuploid screen—that are not included in the initial reports of the segmental aneuploidy principles and techniques (LINDSLEY *et al.* 1972; STEWART and MERRIAM 1973)—are listed above. The tables or figures containing the results of assaying these aneuploids are noted.

B. Chemistry of Drosophila AChE

1. *Histochemistry*: Ten micron serial frozen sections of adults were fixed in glutardaldehyde plus formaldehyde, as described in KANKEL and HALL (1976). Following fixation, slides containing the sections were washed in running tap water for approximately 3 minutes followed by a 30-second rinse in distilled water. They were then ready for either of the two histochemical procedures described below:

General esterase: The procedure was essentially that of PEARSE (1972), where α-naphthyl acetate is the substratee and Fast Blue B salt is the capture agent. The cholinesterase inhibitor eserine sulfate (Sigma Chemical Co.) was sometimes added to the incubation mixture to a final concentration of 10^{-6} to 10^{-4} M. Counter staining in Mayer's haemalum was eliminated. Sections were incubated for 2 minutes at room temperature.

Cholinesterase: The direct coloring method of KARNOVSKY and ROOTS (1964) was used, with acetylthiocholine (ATCh) as the substrate. The following cholinesterase inhibitors were sometimes added to the incubation medium in the indicated final concentrations: BW284 C51 dibromide (Burroughs Wellcome & Co.), 10^{-6} to 10^{-5} M; iso-OMPA, tetraisopropylpyrophosphoramide (ICN Life Sciences—K & K Rare and Fine Chemicals), 10^{-5} to 10^{-4} M; eserine sulfate, 10^{-6} to 10^{-4} M. Sections were incubated for 25 minutes at room temperature.

2. *Quantitative assays for acetylcholinesterase*: The measurement of enzyme activity was the spectrophotometric assay of ELLMAN *et al.* (1961), which uses ATCh as substrate. HEPES, N-2-hydroethylpiperazine-N'-2-ethanesulfonic acid (final concentration 0.05 M) was substituted for phosphate as a buffer, and the pH of the reaction was 7.8 instead of 8.0. This change decreased the spontaneous color formation in the blank without homogenate. Optical density measurements were carried out either on a Beckman DU spectrophotometer with recorder and Gilford kinetic attachments, on the Beckman Model 25 Kinetic system, or on a Zeiss Model M4QIII spectrophotometer with recorder.

Single flies were briefly anesthetized by cold and placed in 0.05 ml of 0.05 M HEPES buffer, pH 7.8, in a 10–50 μl ultramicrohomogenizer (Radnoti Glass Technology, Inc., Arcadia, Ca.) which was standing on ice. The fly was homogenized thoroughly by approximately 6 turns of the pestle and the reaction mixture minus substrate (which had been kept at the temperature of the assay, 25°) was added in a volume of 0.90 ml. The pestle was used to stir this mixture and the mixture was then transferred to a 1.2 ml semi-micro cuvette, which was then placed in the spectrophotometer for the next 60 seconds. The substrate was then added in a volume of 0.05 ml and

TABLE 2

Acetylcholinesterase activity in euploid flies

Genotype		AChE activity	Number of single fly assays
Canton-S	♂	10.4 ± 1.0	15
Canton-S	♀	10.8 ± 2.2	13
$\widehat{XY},Y$; $\frac{In(2L+2R)Cy}{T(Y;2)\ y^+B^S}$	♂	10.8 ± 1.7	37
$\widehat{XX},Y$; $\frac{In(2L+2R)Cy}{T(Y;2)\ y^+B^S}$	♀	9.4 ± 1.2	12
$\widehat{XY},Y$; $\frac{In\ (3LR)\ TM6}{T(Y;3)\ y^+B^S}$	♂	11.3 ± 1.6	37
$\widehat{XX},Y$; $\frac{In\ (3LR)\ TM6}{T(Y;3)\ y^+B^S}$	♀	11.1 ± 2.7	9
Canton-S ♂ no substrate		0	2
Canton-S ♂ 10^{-5} M eserine present		<.005	2
Canton-S ♂ 10^{-5} M BW284 C51 dibromide		<.006	2

Single flies were assayed spectrophotometrically for AChE activity, as described in MATERIALS AND METHODS. The numbers represent mean $\Delta O.D._{412} \times 100$, per minute reaction, ± the standard deviation.

the contents of the cuvette mixed vigorously with a Pasteur pipet. The final substrate concentration was 10^{-3} M. The increase in O.D. at 412 nm as a function of time was monitored.

A fly of the Canton-S wild-type strain (and other euploid flies, Table 2) produced an increase in O.D. of about 0.1 units per minute. The reaction is linear for at least 15 minutes. Increase or decrease of the substrate concentration by a factor of 10 has no effect on the reaction rate. The use of two flies instead of one approximately doubles the rate, while the use of a single head or thorax cuts the rate to approximately half that of the whole fly. Addition of AChE inhibitors in the reaction mixture virtually abolishes the reaction (see Table 2).

3. *Protein determinations*: Protein content was determined by the method of LOWRY *et al.* (1951). For assays in which protein content was measured a single fly was homogenized in twice the usual volume of buffer; half was used for protein determination and half for the measurement of enzyme activity.

4. *Electrophoresis*: The high pH disc acrylamide gel system of DAVIS (1964) was used, with the modification that all components of the system were made 1% (w/v) with the non-ionic detergent Tween 80. This procedure proved effective in allowing most of the enzyme activity to enter the stacking gel and to minimize trailing in the observed bands. When the detergent was omitted, more activity remained at the origin, yet the pattern of bands in the running gel was unchanged. Electrophoretic runs were made either with a Buchler apparatus accepting gels cast in cylindical glass tubes, or in a Hoeffer apparatus in which gels were cast as slabs between two glass plates. Electrophoresis was carried out in the cold (4°). Electrophoretic separations were run with or without the circulation of water as a coolant through the apparatus and at constant current, constant voltage, or constant power. These variations had little if any effect on the results. With the Buchler apparatus most runs were made using a constant current of 2 mA per tube

and with the Hoeffer apparatus at a constant voltage of approximately 250 volts for a 0.75 mm thick gel.

A single fly head, thorax, or whole fly was homogenized in 60 μl of running buffer with 40% (w/v) sucrose and 1% (w/v) Tween 80. This homogenate was centrifuged for 5 minutes in a clinical centrifuge, and 10 μl were applied to the top of the stacking gel in tube or column. When the tracking dye (1% Bromphenol Blue) had reached within a cm of the end of the gel, the electrophoretic run was terminated, and the gel was removed for the staining of cholinesterase activity. The staining solution was that of KARNOVSKY and ROOTS (1964) as described here in the *Histochemistry* section. Cylindrical gels were placed in individual test tubes containing 10 ml of the histochemical stain minus the substrate and these placed in the cold for approximately one hour. A slab gel was placed in 100 ml of the same solution in a shallow tray. This pre-incubation sharpened the staining of the bands. At the end of the hour, the old solution was replaced by fresh solution containing substrate. Staining was carried out at room temperature or 37° for 1–6 hours in the dark. Bands of cholinesterase activity, appearing as brown precipitates in the gels, could be intensified by treating with 1% ammonium sulfide. As with the histochemical experiments, various inhibitors were sometimes added to the incubation medium.

C. Experimental design for screening the genome.

Flies were grown on the medium of LEWIS (1960), or on cornmeal, agar, molasses medium. The adults were 3–4 days post-eclosion when assayed. At this age, AChE activity levels reach a plateau (DEWHURST, MCCAMAN and KAPLAN 1970; DEWHURST and SEECOF 1975).

The most laborious part of the project was to screen chromosomes *2* and *3*. Thus, for the initial assays, spectrophotometric measurements were done on only two flies (assayed singly) which were heterozygous for a given deletion; the enzyme activities were compared to values from two single-fly assays of individuals duplicated for the same interval. For chromosome regions where viable, deletion heterozygotes could not be generated, activity levels in two translocation-bearing euploid flies were compared to activity levels from two duplicated flies.

For any region that gave a suggestion of increased activity in duplication-bearing flies compared to deleted or euploid flies, the initial test was repeated. For the three third-chromosome regions that persistently gave positive results (see below), the following procedures were adopted: (1) The segments that yielded the dosage effects were sub-divided, using additional translocations of LINDSLEY *et al.* (1972); single-fly assays were performed on aneuploid male progeny to localize further the putative AChE-controlling loci. (2) For the smallest segments which showed dosage effects, specific activity determinations were carried out to see if activity differences could be due to the possibility that deleted flies were simply smaller and duplicated flies larger. (3) Aneuploids which gave positive results were examined histochemically and electrophoretically, to see if there would be a general reduction in staining intensity in tissues or bands, a change in activity in only part of the nervous system, or a change in activity in only some of the bands.

In the initial screen of the *X* and fourth chromosomes the number of different aneuploid types which had to be tested was smaller, and so several single-fly specific activity assays were performed for each of the genotypes in Figure 1.

For one chromosome segment (87B–E on chromosome *3*) which yielded the most dramatic dosage effects, additional independently derived deletions and duplications (Table 1) were assayed, to confirm the true dosage effect.

D. Search for AChE-null mutations

Assuming that segment 87B–E contains one or more AChE structural loci, and that an AChE-null mutation would be a recessive lethal, such a mutation in heterozygous condition might have reduced AChE activity. Therefore, lethal mutations located in region 87 were assayed. Many such mutations have been found and have had mapping and complementation tests carried out on them (SCHALET, KERNAGHAN and CHOVNICK 1964; DELAND 1971; W. GELBART, in preparation).

The following tests were carried out on the lethals which effect a reduction in AChE activity: (1) Histochemical and electrophoretic assays were carried out on *l(3)26/+* flies (one of four allelic lethals affecting AChE activity—Figure 6). (2) The lethal phases of *l(3)26*, *l(3)m38*

and *l(3)m115* were determined, by crossing lethal/*In(3LR)Ubx*^{A} females to Canton-S males, then crossing non-*Ubx*^{A} progeny to each other. Eggs from this mating were collected on agar and counted. The proportions of survivors at subsequent development stages were determined, relative to developing progeny from a cross of wild-type parents.

RESULTS

A. AChE activity in euploid flies.

The results of several single-fly assays of wild-type or euploid *T(Y;2)* and *T(Y;3)* males showed that consistent values could be obtained for a variety of genotypes (Table 2).

In frozen sections of wild-type flies, histochemical procedures specific for acetylcholinesterase demonstrate activity almost exclusively in the nervous system (Figure 2). This activity is abolished by 10^{-5} M eserine and by 10^{-5} M BW 254 C51 dibromide. Sections stained for general esterases show activity in both the nervous system and a variety of other tissues. 10^{-5} M eserine, when used in this procedure, seems to abolish staining only in the nervous system (Figure 2).

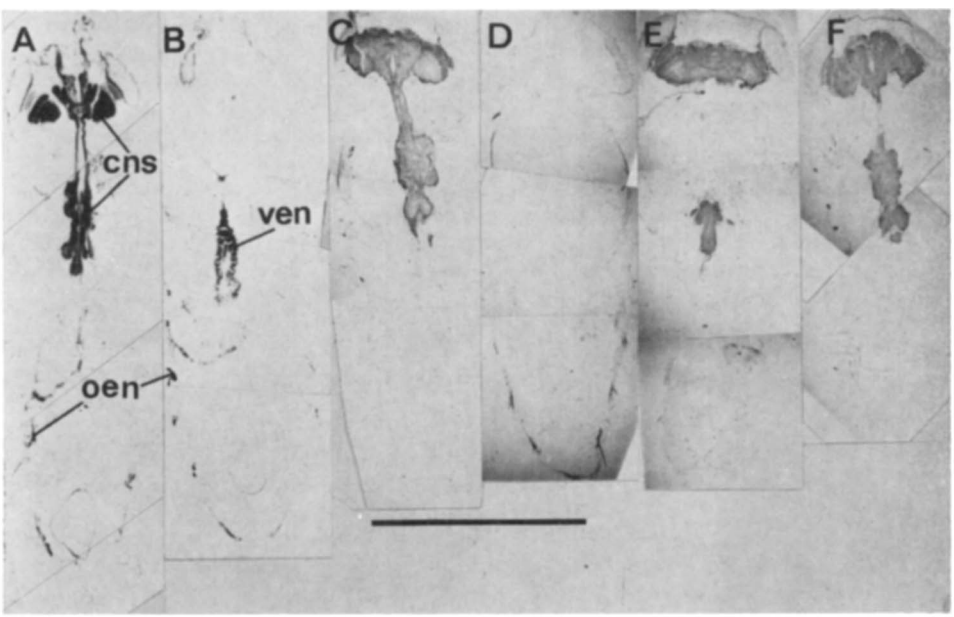


FIGURE 2.—Esterase histochemistry in *Drosophila melanogaster*. Histochemical procedures were carried out as described in MATERIALS AND METHODS. A. General esterase staining (substrate = α-naphthyl acetate). Central nervous system (CNS) and one cluster of oenocytes (oen) are stained. B. General esterase staining, with 10^{-5} M eserine in reaction mixture. Note the absence of staining in CNS, while the oenocytes and ventriculus (ven) retain activity. This is a more dorsal 10 μ section than the one shown in A., which is from the same fly. C. AChE staining. D. AChE staining, with 10^{-5} M eserine in reaction mixture (section from same fly represented in C.) Sections A. through D. are from Canton-S males. E. AChE staining in *Df(3R)l26d*/+ male. F. AChE staining in *Ace*^{lm15}/+ male. Black bar represents approximately one mm.

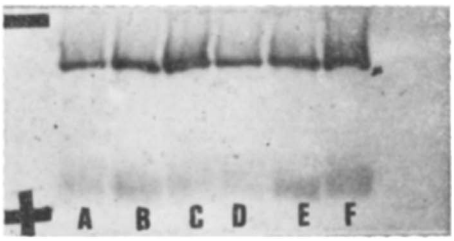


FIGURE 3.—Electrophoretogram of AChE activity. Conditions of sample preparation and electrophoresis are described in MATERIALS AND METHODS. There are two bands here for each of the isolated head homogenates run on a slab gel: A. and D. are patterns from *Df(3R)l26d*/+ males; B. and E. are from wild-type (Canton-S) males; and C. and F. are from Ace^{l26}/+ males. The — and + show the relative positions of the cathode and anode, respectively. All bands have migrated toward the anode. Neither the origin nor the tracking dye front are shown. Both bands for given assay of a deletion heterozygote or a lethal heterozygote stain more faintly than the corresponding bands from wild type—but this is not discernible here, because the gels had to be over-stained (6 hours) in order that the faint (more anodal) bands could be photographed. For gels stained for only 2–3 hours, the decreased staining in the assays of the mutant or deletion heterozygotes is observable.

The results of electrophoresis show two AChE bands (Figure 3). In other gels third and fourth minor bands were seen. All bands are eliminated by eserine or BW 254 C51 dibromide, in the concentrations stated above. The same banding patterns appear after electrophoresis of a head, a thorax, or whole fly.

B. AChE-controlling loci detected by segmental aneuploidy.

The initial screen of nearly the entire genome showed that most chromosome segments have no influence on AChE activity, when present in one or three doses (Figure 4, Table 3). However, three segments in the right arm of chromosome 3 each caused reductions in AChE activity when present in one dose, and increases when present in three doses: 86F–87E, 90E–91B, and 96B–97A (Figure 4A; for the latter region, the dosage effect was initially detected only in duplicated *vs.* euploid flies).

The dosage effect of aneuploidy for the 86F–87E interval resides in 87B–E: From the progeny of crosses made with additional *T(Y;3)*'s, it was found that 87B–E deletions and duplications affect AChE activity levels, but aneuploids for 86F–87B do not (Table 3). 87B–E dosage effects are such that normalized absolute activity values of 1.2: 2.0: 2.7 are obtained for one-dose, two-dose, and three-dose flies, respectively (euploid activity is set equal to 2.0). For specific activity determinations on these three genotypes (Table 4), the normalized ratios are 1.2 : 2.0 : 3.1. All these activity (or specific activity) differences are highly significant, by a non-parametric statistical test (Table 5).

The dosage effects on absolute activity for the 90D–91B and 96B–97A regions (which are less dramatic that those for 87B–E) seem to be false positives for the following reasons: (1) The differences in specific activity for different 90E–91B dosages are all non-significant (Table 5)—the smaller average protein content of the deletion-bearing males caused the deletion *vs.* euploid difference in abso-

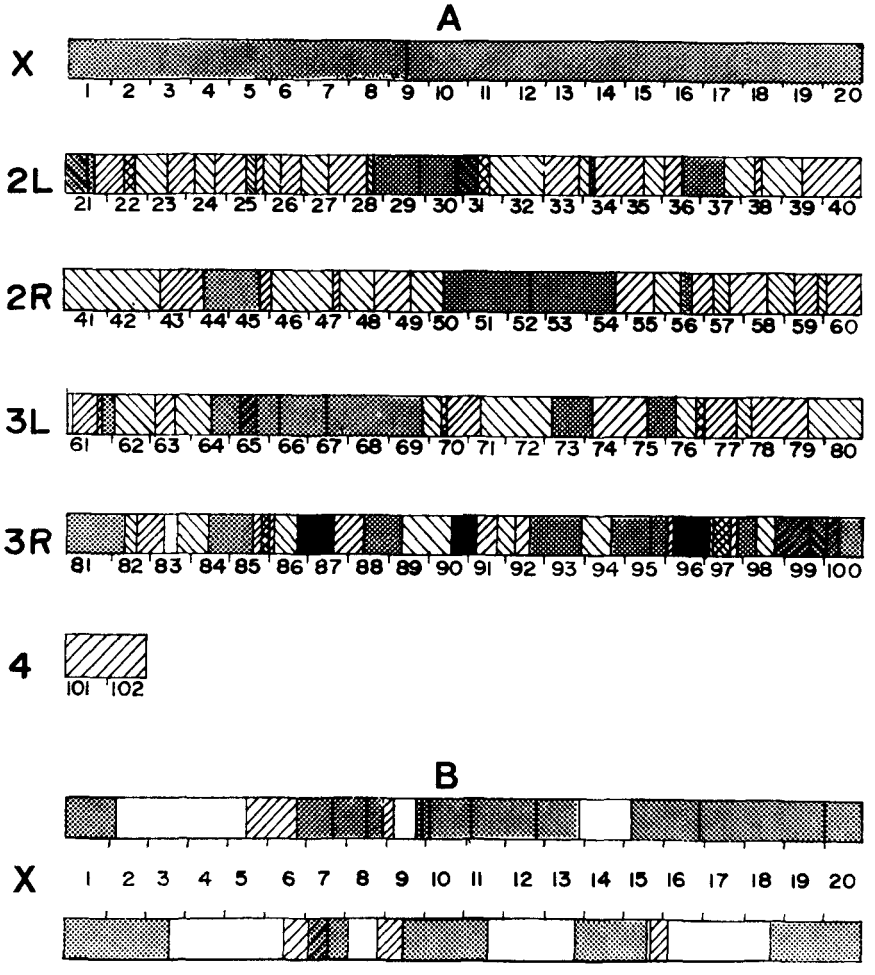


FIGURE 4.—Segmental aneuploidy and acetylcholinesterase activity. A. Schematic map of the four chromosomes of *D. melanogaster*. Each major chromosome arm is divided into twenty numbered segments, and the fourth chromosome into two segments, as shown in LINDSLEY and GRELL (1968). Flies aneuploid for parts of a given segment, for more than one adjacent segment, etc., were constructed as described in Figure 1, and then assayed spectrophotometrically for AChE activity. A region with diagonal lines indicates that there was no difference in activity for deleted (*one-dose*), *vs.* euploid (two-dose), *vs.* duplicated (three-dose) flies. A shaded region indicates that a deletion heterozygote could not be generated (because of lethality), and that there was no difference in activity for euploid *vs.* duplicated flies. A solid region indicates that there was lower activity for deleted flies than euploid flies, which in turn had lower activity than duplicated flies (see Tables 3 and 4). Blank regions were not tested. Some of the segments tested overlapped adjacent tested segments, and this is indicated by, for example, contiguous shading and cross-hatching. B. Finer subdivision of *X* chromosome. Flies aneuploid for different regions of the *X* chromosome were constructed, using the *X-Y* translocations of NICOLETTI and LINDSLEY (1960) and STEWART and MERRIAM (1973), and in addition, the strains listed in Table 1. The results of the AChE assays of aneuploids are indicated by shading and diagonal lines as in part A (two *X* chromosomes are used to display the results, merely for the sake of clarity, because of the several overlaps among aneuploids). All *X* chromosome regions were tested, except for 3E;5C, for which one of the relevant *T(1;Y)*'s proved too inviable for crosses.

TABLE 3

Tests of gene dosage effects on AChE activity

Schemes for generating these aneuploid flies are in Figure 1. AChE activity in single-fly assays is expressed as in Table 2; in parentheses are the number of assays for each genotype.

A. Original screen (see Figure 4)

	Source of aneuploids	Deletion	AChE activity Euploid	Duplication
3rd chromosome segment which is aneuploid				
86F;87E	*T(Y;3)J141* × *T(Y;3)D226*	6.2 (8)	—	12.9 (4)
90E;91B	*T(Y;3)B116* × *T(Y;3)R135*	8.0 (2)	—	13.9 (3)
96B;97A	*T(Y;3)B197* × *T(Y;3)R87*	—	9.8 (4)	15.5 (4)
X chromosome segment which is duplicated				
1A;9C	*T(1;Y)B26*	—	10.9±2.9 (16)	11.4±2.1 (22)
9C→ centromere	*T(1;Y)B26*	—	same	11.1±2.2 (26)
4th chromosome segment which is aneuploid				
101A;102F	*C(4)RM*	9.6±1.8 (16)	9.0±1.8 (22)	8.5±1.6 (18)

B. Subdivisions of 3rd chromosome segments (and retests of 90E–91B): assays of males only

Autosomal segment which is aneuploid				
86F;87B	*T(Y;3)J141* × *T(Y;3)A78*	13.2 (2)	10.8 (2)	12.4 (2)
87B;87E	*T(Y;3)A78* × *T(Y;3)D226*	6.7±0.8 (10)	11.1±1.3 (10)	15.1±1.4 (11)
96A;96C	*T(Y;3)B217* × *T(Y;3)H135*	8.8 (3)	—	9.9 (4)
96B;96C	*T(Y;3)B197* × *T(Y;3)H135*	10.1±0.7 (10)	12.6±1.8 (14)	14.9±2.0 (11)
96C;97A	*T(Y;3)H135* × *T(Y;3)R87*	11.7 (2)	11.6 (2)	11.4 (2)
90E;91B	*T(Y;3)B116* × *T(Y;3)R135*	9.9±2.1 (13)	12.8±1.9 (16)	14.1±2.9 (11)

lute activity to vanish. (2) Within region 96B–97A, 96B–C aneuploids gave slight dosage effects, and 96C–97A aneuploids did not (Table 3); *but* 96A–C aneuploids also gave no dosage effects.

Of four strains duplicated for region 87, other than the two constructed with *T(Y;3)*'s, only two gave greater-than-euploid levels of AChE activity (Table 4). The other two duplications—involving insertion of region 87 into chromosome *2* or *4*—may for unknown reasons have position effects exerted on the translocated segments, which leads to suppression of action of the putative AChE gene(s) in the segment. The insertions of region 87 into chromosome *2* or *4* do not involve any known heterochromatic breakpoints; so this hypothesis of position effect suppression of the translocated AChE gene apparently does not refer to the standard "V-type" position effect (as reviewed by BAKER 1968).

TABLE 4

Specific activities of aneuploids

	Source of aneuploids	AChE activity Deletion	AChE activity Euploid	AChE activity Duplication
A. 3rd chromosome segment which is aneuploid				
87B–E	*T(Y;3)*'s	42±5 (5)	72±4 (8)	112±8 (11)
90E;91B	*T(Y;3)*'s	73±4 (14)	75±8 (16)	85±4 (19)
B. 3rd chromosome segment which is duplicated				
87C–E;91B–C	*T(3;1)ry*^{35}	—	52±3 (23)	87±4 (20)
86D3–88B	*Dp(3;4)ry+*	—	same	47±2 (21)
87C8–D1;88D12–E1	*T(3;1)kar*^{51}	—	same	90±4 (5)
87C2–3;88C2–3	*T(3;2)ry+*^{w70hR}	—	same	56±5 (21)
C. *X* chromosome segment which is duplicated				
1A;9C	*T(1;Y)B26*	—	78±7 (9)	88±9 (9)
9C → centromere	*T(1;Y)B26*	—	same	80±10 (6)
D. 4th chromosome segment which is aneuploid				
101A–102F	*C(4)RM*	71±3 (15)	63±5 (15)	63±3 (14)

AChE-specific activities were obtained by normalizing enzyme activity with respect to protein content of the single fly homogenates (see MATERIALS AND METHODS). The values are mean ΔO.D. min^{-1} μg $protein^{-1} \times 10^5$, ± standard deviation; the numbers of assays for each genotype are in parentheses. For part A., the euploid flies are from *T(Y;3)* stocks. For B., euploids are Canton-S males, and the stocks used to produce the duplications are described more fully in Table 1. The sources of euploids for C. and D. are described in Figure 1.

TABLE 5

Statistical significance of activity differences found for flies aneuploid for segments on chromosome 3

	Third chromosome segments 87B–E	90E–91B	96B–C
i. Absolute activities			
deletion *vs.* euploid	<.01	<.01	<.01
euploid *vs.* duplication	<.01	>.05	>.05
ii. Specific activities			
deletion *vs.* euploid	<.01	>.05	—
euploid *vs.* duplication	<.01	>.05	—

Numbers in the table are P values from the non-parametric Mann-Whitney test (two-tailed), performed on data listed in Tables 3 and 4.

The histochemical and electrophoretic procedures applied to 87B–E aneuploids showed: (1) no noticeable change in histochemically detectable activity for deletion heterozygotes or duplications (Figure 2); and (2) the same number and electrophoretic mobility of all bands, for assays of aneuploids; but the bands in the deletion heterozygote *Df(3R)l26d/+* seem to have less intense staining (Figure 3).

C. Localization of an 87B–E subsegment influencing AChE.

The above results suggest that there is only one structural locus for AChE in Drosophila, and that it is contained in 87B–E, which gives the most obvious and reproducible dosage effects, near to what one might expect for one-, two-, and three-dose flies. It was found that some of the additional deletions partitioning 87B–E lead to only 50%–60% of the normal level of enzyme activity (Figure 5). The deletion mapping unambiguously placed the putative AChE-controlling site in sub-segment 87E1 to 87E5, which includes only about four chromomeres. Thus, all deletions of the appropriate region affect the level of AChE activity, unlike the case of two of six tested duplications (cf. Table 4).

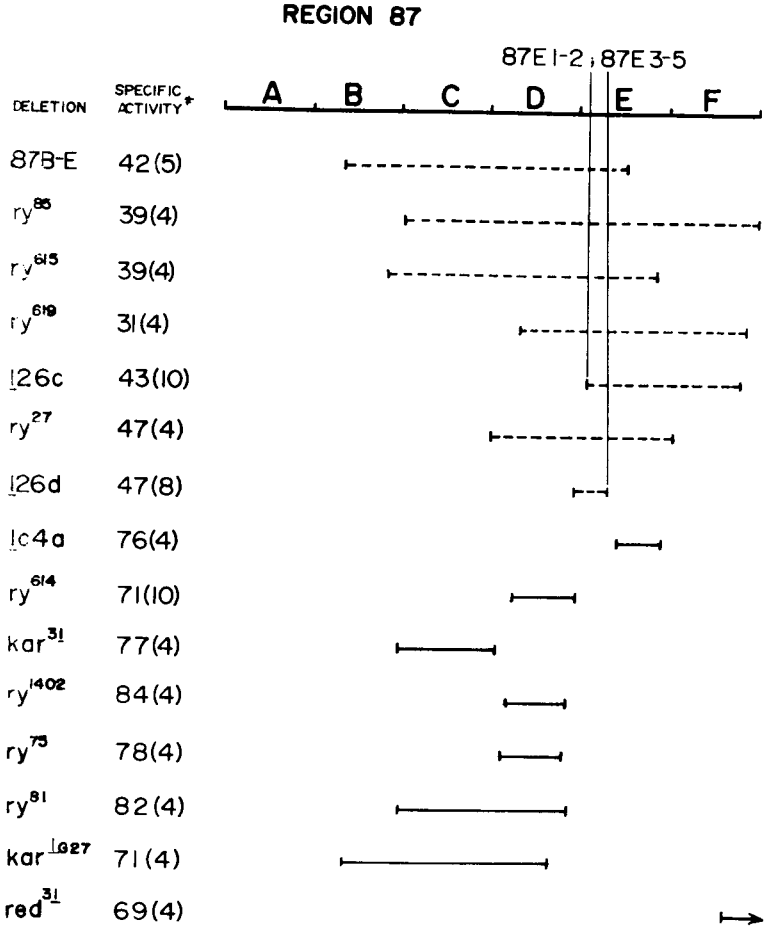
D. Lethal mutations influencing AChE.

Assays were performed on heterozygous lethal mutations localized within 87E1; 87E5. The assumptions were that a mutation that eliminates (or nearly so) the activity of this nervous system enzyme would be a homozygous lethal; but a decrease in AChE severe enough to kill homozygotes would show up as a substantial reduction in enzyme activity for heterozygotes. There are four allelic lethals within 87E1; 87E5 that have nearly the same effects on AChE as does a relevant deletion (e.g. *Df(3R)l26d*—Figure 5): *l(13)26*, *l(3)m15*, *l(3)m38*, *l(3)m115* (Figure 6). The first lethal listed was discovered by SCHALET, KERNAGHAN and CHOVNICK (1964), and the last three by DELAND (1971). Other lethals, mapping to the left and right of this group, have essentially wild-type as heterozygotes. In Figure 6 the lethal mutations are placed within the subsequent to which they map, on the basis of complementation tests with the deletions shown in Figure 5. Lethals that fail to complement are placed at the same position within their subsegment (based on complementation and deletion mapping results of W. GELBART's manuscript in preparation). The left-right positioning of the five lethals within sub-segment E5;F1 has not been determined; *l(3)m102* may be an allele of one of the other three lethals in its sub-segment, none of which are alleles of each other.

It is proposed that the locus defined by these lethals be named *Ace*, so that the lethal alleles are symbolized Ace^{l26}, etc.

E. Characterization of putative AChE-null mutants.

The lethal phase for the *Ace* alleles in homozygous condition is late embryonic/early larval: the development of progeny from crosses of lethal heterozygotes to each other was compared to the development of progeny from Canton-S parents. An excess of unhatched embryos was seen among the progeny of the



* Δ OD min^{-1} μg^{-1} protein $\times 10^5$

FIGURE 5.—Fine-structure localization of AChE-controlling segment within region 87 of chromosome 3. The deletion heterozygotes, on which the single-fly assays (males only), plus protein determinations were made are listed in Table 1, except for "*Df(3R)87B–E*", which was constructed with translocations (see Tables 3 and 4). Dotted lines are deleted segments, for which deletion heterozygotes gave significantly reduced activity compared to assays of euploid flies (Mann-Whitney tests). Solid lines are deleted segments for which deletion heterozygotes gave activities which were nearly the same as for euploids. The numbers corresponding to each deletion are the mean AChE-specific activities (expressed as in Table 4) for the deletion heterozygotes (which had been outcrossed to a Canton-S strain); numbers in parentheses are the numbers of single fly assays which were performed. The standard euploid values for these tests are 76 ± 13 (N=24) for Canton-S males, and 72 ± 4 (N=8) for euploid *T(Y;3)* males.

crosses with three of the lethals, relative to the control. At no other point in development was there appreciable death relative to the control (Table 6). The fourth lethal that decreases AChE activity when heterozygous—*l(3)m15*—has been preliminary tested for lethal phase, with results qualitatively similar to those for the other three lethals; however, *l(3)m15* may be a semi-dominant, in that substantially less than three-quarters of the eggs develop into viable first

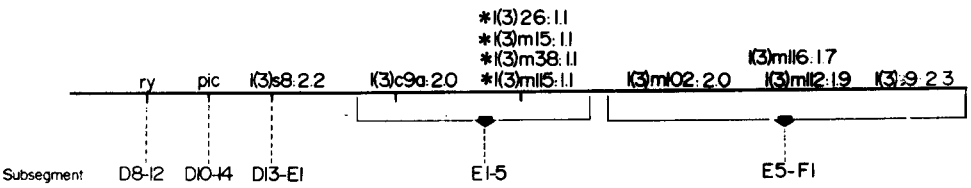


FIGURE 6.—Third-chromosome recessive lethal mutations which affect AChE activity. The drawing is a map of part of region 87 of chromosome 3, showing the locations of lethals that were assayed in heterozygous condition. The locations of the *ry* and *pic* genes are included (third chromosome map positions 52.0 and 52.1, respectively). The lethals with an asterisk (*) are those for which males heterozygous for the mutations give significantly reduced AChE activity (Mann-Whitney tests). The mean activity values (relative to the euploid value, set equal to 2.0) are just to the right of the symbol for each lethal mutation (e.g. *l(3)s8* has an activity level of 2.2, relative to wild type =2.0). The actual activities (expressed as in Table 2, with number of single-fly assays in parentheses) are: Canton-S control: 8.7 ± 1.7 (63); *l(3)s8*/+: 9.6 ± 2.5 (10); *l(3)c9a*/+: 8.7 ± 1.1 (7); *l(3)26*/+: 4.9 ± 1.2 (13); *l(3)m15*/+: 4.7 ± 1.2 (15); *l(3)m38*/+: 4.9 ± 0.8 (10); *l(3)m115*/+: 4.7 ± 1.2 (11); *l(3)m102*/+: 8.8 ± 1.0 (6); *l(3)m116*/+: 7.5 ± 0.8 (6); *l(3)m112*/+: 8.3 ± 0.9 (6); *l(3)s9*/+: 9.9 ± 1.1 (5).

instar larvae; yet, no death at subsequent developmental stages was seen.

In histochemical assays, there were virtually no differences between Ace^{lm15} heterozygotes and Canton-S adults. As for the deletion heterozygotes for this region, Ace^{lm15} heterozygotes show a slight reduction in activity for all detectable cholinesterase bands. Figure 3 does not show a reduction, because staining time of the gel was increased (to 6 hours) beyond the time (2–3 hours) at which there is a noticeable difference between wild-type and mutant heterozygotes. (For gels stained for the shorter times, the fainter bands for any genotype are nearly impossible to reproduce photographically.)

TABLE 6

Lethal phase for lethal mutations affecting AChE activity

Allele	N	No. eggs laid	Number (and %) of animals surviving to 1st instar larvae	Pupae	Adults
l(3)26	3	830	632 (73.5±6.8)	544 (88.1±9.2)	488 (92.8±4.2)
l(3)m38	4	708	494 (68.9±9.0)	484 (97.9±1.0)	476 (98.8±1.0)
l(3)m115	3	1,376	993 (70.3±4.1)	897 (94.4±7.5)	794 (87.8±5.8)
+	5	998	936 (92.0±5.4)	833 (93.5±5.5)	756 (91.6±12.0)

l(3)26, *l(3)m38* and *l(3)m115* are different lethal alleles at the acetylcholinesterase locus. + is a wild-type allele at the same locus from the Canton-S strain. The data are from crosses of *a*/+ males × *a*/+ females (where *a* is any of the alleles in the table). *N* = number of independent egg layings. Numbers in parentheses are unweighted averages for all egg layings of the percentage of individuals that have developed from the previous stage of the life cycle ± standard deviation.

DISCUSSION

Genetic changes that influence Drosophila acetylcholinesterase activity have been identified. The purpose of the search was to isolate mutations that lead to severely reduced enzyme activity. It would then be possible to eliminate AChE activity in different parts of the nervous system, and determine the effects of such elimination on the physiology of the nervous system and on the behavior of the fly. Flies with AChE missing from only part of the nervous system can, if viable, be generated by genetic mosaic techniques completely analogous to those used by KANKEL and HALL (1976—to study acid phosphatase activity). That is, an *X* chromosome containing the normal allele of *Ace* (Table 1 of the present paper) could be somatically lost from an individual homozygous for one of the lethal alleles.

It is also desirable to turn off AChE activity at different stages of the life cycle to determine effects of altered acetylcholine metabolism on development. For this purpose, conditional alleles of the AChE-controlling gene are necessary. These can be found through attempts to induce temperature-sensitive (ts) lethal alleles of *Ace*, by making mutagenized third chromosomes heterozygous with the current lethals (none of which is temperature-sensitive). Such ts *Ace* alleles would also allow flies missing AChE in all or part of the nervous system to be grown up at a permissive temperature, then tested for behavioral abnormalities at the restrictive temperature.

Thus, it is hoped that the *Ace* lethals are mutations in the gene that codes for AChE. A hypothetical ts AChE-null mutant would presumably have a thermolabile form of the protein. Several lines of evidence suggest that *Ace* lethals are structural gene mutations: (1) The effects of different gene dosages on AChE activity are on the same order as found for the structural genes of other Drosophila enzymes (noted at the beginning of this paper). (2) The multiple bands of AChE activity on gels are by no means necessarily due to products of different structural genes, since (a) changes at the *Ace* locus effect decreases in all the electrophoretic bands, and (b) DUDAI (1976) has shown that the *relative* amounts of different molecular forms of *Drosophila melanogaster* AChE (detected on sucrose gradients) are the same when comparing detergent homogenates made from wild-type flies to homogenates of flies heterozygous for a deletion of the *Ace* locus.

It is very important that AChE electrophoretic variants that are allelic to the lethals be isolated. This would give perhaps the strongest suggestion that the lethals in region 87 are AChE structural gene mutations. Temperature-sensitive alleles of the AChE-nulls—a search for which is in progress—might have altered electrophoretic mobility of AChE bands, for gels run at a permissive temperature. One might have suggested from our results that, with respect to electrophoretic variants, Esterase-C (*Est-C*—BECKMAN and JOHNSON 1964) is an AChE gene, since it is near region 87 (based on limited genetic mapping data of the *Est-C* variant). However, it seems unlikely that this gene is concerned with AChE, because the *Est-C* bands (in starch gels stained for non-specific esterase) are not

affected by eserine, though another band in these same gels ("E") is eliminated (BECKMAN and JOHNSON 1964).

Further studies of embryos homozygous for *Ace*^{126} and its other alleles are in progress. Preliminary histochemical assays of embryos from a cross of lethal heterozygotes to each other show that some of the animals—examined approximately 80% of the way through embryogenesis—have no AChE in any tissues. The late embryo/early larval phase of these homozygotes is also consistent with the notion that such homozygotes contain extremely low levels of AChE. That is, the developing animal may first be required to carry out nervous system function controlling movement at this stage, during hatching. However, in normal individuals, movements begin earlier, at about 60% of the way through the embryonic period (POULSON 1950). Also, cholinesterase activity is first detectable at about 45% of the way through embryogenesis, and the level of the enzyme increases rapidly through the period when there is increasing movement (POULSON 1950; DEWHURST, MCCAMAN and KAPLAN 1970; DEWHURST and SEECOF 1975).

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