# A BIOCHEMICAL AND GENETIC ANALYSIS OF THE CYCLIC AMP PHOSPHODIESTERASE DEFECT IN *dunce*, A MEMORY MUTANT OF DROSOPHILA

Thesis by

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This thesis is dedicated to my parents,

Joan Bannister Shotwell and Thomas Cooper Shotwell

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## Abstract

Drosophila can learn in several associative conditioning paradigms. Flies carrying the mutation dunce were selected for their poor performance in one such task, a negative reinforcement olfactory conditioning paradigm (Dudai et al., 1976). dunce flies express two other mutant phenotypes, female sterility, and reduced activity for one of the two cyclic AMP phosphodiesterases present in normal flies, PDE II (Byers et al., 1981). The experiments described below indicate that the normal dunce gene ($dunce^+$) probably codes for PDE II itself, rather than for a regulator that affects PDE II and possibly other activities.

A micro-assay technique is described that allows the separate measurement of PDE I and PDE II when both are present in mixture. PDE II is shown to occur at high specific activity in the nervous system, which is consistent with a role for this enzyme in neuronal function. The phenotype of female sterility associated with dunce mutants can be suppressed by any of three suppressor mutations. These do not suppress the other two phenotypes of reduced PDE II activity and poor learning, indicating that these phenotypes are closer to the primary defect associated with dunce mutants. Reduced PDE II activity correlates with poor learning in dunce flies in all three developmental stages that were tested (first and third instar larvae, and adults), as well as in response to genetic modifications of dunce gene activity. The results of several biochemical and genetic experiments fail to reveal any abnormal regulation of PDE II activity in dunce flies. In Drosophila, as a rule, the activity level of an enzyme correlates linearly with the activity of the enzyme's structural gene. The specific activity of PDE II is shown to correlate in a one to one fashion with the level of normal dunce gene activity at five different doses of $dunce^+$.

Taken as a whole, these experiments provide strong support for the hypothesis that PDE II represents the primary product of the dunce gene, indicating a role for this enzyme in the learning of Drosophila.

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## Chapter 1 - The Neurogenetics of Learning

Until a decade ago, there existed no convincing demonstration of associative learning in dipterans, that large order of insects that claims the fruitfly among its members. In fact, Vincent Dethier (1966) hypothesized that flies had lost all capacity for learning during their evolution, presumably because it was a luxury they could not afford. His implication that learning is subject to the genetic control involved in evolution later proved valid (see below). His claim that flies cannot learn, however, was disproven by one of his own graduate students, Margaret Nelson. Nelson (1971) demonstrated convincingly that the blowfly, Phormia regina, was capable of classical conditioning, a form of associative learning made famous by Pavlov's dog (Pavlov, 1927). Since then, several other workers have demonstrated classical or operant conditioning in other dipteran species, including the housefly Musca domestica (Fukushi, 1976) and the fruitfly Drosophila melanogaster (Quinn et al., 1974; Medioni and Vaysse, 1975; Menne and Spatz, 1977).

There has long been an interest in the role played by the genes in learning. One approach to studying this role has been to produce smart or stupid strains of flies by selective inbreeding, testing potential mates at each successive generation for their performance on a learning task, and breeding them accordingly. This procedure has been used by McGuire and Hirsh (1977) to produce bright and stupid strains of flies as evidenced by their performance in a classical conditioning paradigm similar to that of Nelson (1971). Such a procedure probably selects for several genetic variants in a strain of flies. Another approach to studying the role genes play in learning has been to mutate a single gene per animal in an otherwise similar genetic background, and select for mutations that affect learning. This approach has been quite successful in Drosophila, and to date it has allowed the isolation of at least five learning mutants (see below).

A broad interest among Drosophila behavioralists in learning, and the ability

to generate single gene mutants with ease, have combined to produce a plethora of learning paradigms for Drosophila. These include negative reinforcement olfactory conditioning (Quinn et al., 1974), negative reinforcement visual conditioning (Quinn et al., 1974; Menne and Spatz, 1977; Bicker and Reichert, 1978) and positive reinforcement olfactory conditioning (Tempel and Quinn, 1980). In addition, several paradigms have been devised to study the learning of individual flies, including negative reinforcement of proboscis extension (Medioni and Vaysee, 1975), conditioning of leg position (Booker and Quinn, 1981) and negative reinforcement of male courtship behavior (Siegel and Hall, 1979). Studies have shown evidence of learning as early as the first larval instar (Aceves-Piña and Quinn, 1979), as well as evidence for separate short- and long-term memory phases in adults (Quinn and Dudai, 1976).

Analysis of the role of the genes in Drosophila learning began with the isolation of the first mutant showing poor learning, dunce (Dudai et al., 1976). This mutant was discovered by screening ethylmethanesulfonate-mutagenized stocks using the olfactory conditioning paradiam devised by Quinn and his coworkers (1974). In their general behavior (including geotaxis, phototaxis, locomotion, and flight) dunce flies are similar to normal flies. In addition, the specific sensory and motor capabilities required for the learning task all seem normal in dunce. This suggests that the poor performance of dunce flies in the learning task is due to a defect in some mechanism underlying neuronal plasticity.

More detailed behavioral testing by Dudai (1976, 1981) and Tempel and Quinn (1980) indicates that dunce flies are capable of learning under certain conditions. In the learning paradigm used for the mutant screening in which dunce was isolated, a group of 20 to 50 flies is presented with a control odor (A) in a tube, into which they walk in response to phototaxis. Next they are presented with a tube containing odor (B) on an electrified grid. When the flies walk onto the grid, they receive a shock. The negative stimulus associated with the shock is sufficiently strong to

overcome their phototactic behavior, and the flies avoid the training tube. After three rounds of training [(Rest-A-Rest-B) x 3] the flies are presented with fresh tubes containing the same concentration of odorants A or B. Normal flies remember the negative stimulus that was previously associated with odor B, and selectively avoid it. Some memory can be demonstrated 24 hours later, after a large fraction of the varied experiences of fruitfly life has passed by (Quinn et al. , 1974). dunce flies, on the other hand, seem to remember odor B only if it is presented immediately after the training tube in which odor B was paired with shock. If odor A or a new odor C is interposed, dunce flies no longer specifically avoid the testing tube that contains odor B. Tempel and Quinn (1980) found a similar result on a learning paradigm that uses negative geotaxis to induce the flies to enter the tube bearing an odor, and positive reinforcement (sugar water) to affect their preference for an odor. In this paradigm, learning in normal flies persists much longer, and dunce flies show some learning that fades within one minute, when the memory of normal flies has barely begun to decline. Thus, several experiments indicate that dunce flies have all the apparatus and skills required to form associations, to learn, but that they forget very quickly. The behavioral deficit in dunce flies seems to be a defect in short-term memory.

dunce flies perform poorly in several other learning paradigms, including larval learning (Aceves-Piña and Quinn, 1979), conditioning of leg position (Booker and Quinn, 1981), and one sexual courtship paradigm (Hall, 1982). In a second sexual courtship paradigm (Hall, 1982) and a negative reinforcement visual learning paradigm (Dudai and Bicker, 1978), the behavior of dunce flies is not significantly different from that of control flies. It is possible that these last two tasks differ from the others, so that they do not require nervous system functions altered by the dunce mutation, or these tasks could be less sensitive tests of altered memory. In any case, it is clear that dunce affects learning performance in several different paradigms.

Several other learning mutants have been isolated on the basis of poor performance in the olfactory conditioning paradigm, including cabbage, turnip, rutabaga and amnesiac (see Aceves-Pina and Quinn, 1979). Two of these, cabbage and turnip, also do poorly in the leg position conditioning test (Booker and Quinn, 1981). Two mutants seem to have memory defects. They learn, but forget more rapidly than normal flies. One is the mutant amnesiac, and the other is turnip heterozygous females (turnip/normal) (Quinn, Sziber and Booker, 1979). The ranking among mutants that affect memory, beginning with the most severely affected, is as follows: dunce < turnip heterozygotes < amnesiac < normal. Mutagenesis and selection based on learning performance thus has allowed the identification of several genes affecting learning or memory in Drosophila.

Interest in the biochemical genetics of learning has led several workers to test mutants with known biochemical defects for their learning performance. The dopa-decarboxylase gene (Ddc), identified by Wright (1977) as the structural gene for the enzyme dopa decarboxylase, reduces the levels of the neurotransmitters dopamine and serotonin (Wright, 1977; Livingstone, 1981). Tempel and Livingstone (1981) found that learning ability in flies mutant or deficient for Ddc closely parallels the activity levels of the enzyme dopa decarboxylase found in these flies. This result suggests a role for dopamine and/or serotonin in the learning of Drosophila. Another approach has been to test learning mutants for defects in biochemical processes thought to be involved in learning. This approach led to the finding that rutabaga flies have altered adenyl cyclase activity (Uzzan, 1981 and M. Livingstone, personal communication).

Yet a third approach to uncovering the biochemical defect in a learning mutant utilizes a combination of classical genetics and intuition. This is the approach taken by Duncan Byers with the dunce mutant. Byers (1980) found that the dunce gene mapped close to a region of the X chromosome known to affect the activity of cyclic AMP phosphodiesterase (Kiger and Golanty, 1977). Given the postulated roles for cyclic AMP in the function of the nervous system, Byers was suspicious that

these two genes might be one and the same, particularly in view of the fact that mutants for each gene had a shared phenotype, female sterility. He, Ron Davis and John Kiger demonstrated that the dunce locus affected the activity levels of one of the two soluble cyclic AMP phosphodiesterase activities found in Drosophila, which led to elevated levels of cyclic AMP in dunce mutants (Byers et al., 1981). It is this observation that served as the starting point for the studies presented in this thesis.

Thus, in the last few years, several biochemical correlates of learning in Drosophila have emerged. Not surprisingly, they all focus on pre- or post-synaptic mechanisms related to neuronal transmission, including transmitter synthesis, transmitter-sensitive adenyl cyclase activity, and control of cyclic AMP levels by phosphodiesterase. One value of the neurogenetic approach is to make a conclusive demonstration of the participation of a biochemical activity in learning, where it may have been implicated before. This is an aim of several of the studies that have been cited, as well as of this thesis. Another goal is to uncover previously unsuspected functional correlates of learning. In addition, anatomical substrates and physiological correlates of learning are being sought through the use of genetic mosaics, as well as with traditional histological and physiological techniques.

## Chapter II

## Analysis of the cyclic AMP Phosphodiesterase Defect Associated With dunce

### Introduction

Benzer, Quinn, and their coworkers have isolated several mutants of Drosophila melanogaster that affect learning or memory. The first such mutant allele isolated, dunce, is also the best characterized both behaviorally and biochemically. Dudai et al. (1976) initially studied the learning behavior of these mutant flies using the olfactory, negative reinforcement conditioning paradigm of Quinn et al. (1974). In this test, dunce flies showed greatly reduced learning compared with that of controls. Further study by Dudai (1979, 1981) and Tempel and Quinn (1980) using different olfactory paradigms showed that dunce flies were capable of almost normal learning, but that memory decayed very rapidly. These results led them to suggest that defective short-term memory storage represents the primary behavioral lesion in dunce.

Byers et al. (1981) provided the first information about the biochemical defect that potentially underlies the short-term memory deficit when they found that dunce flies exhibit abnormal regulation of cyclic adenosine 3',-5'-monophosphate (cyclic AMP). Increased levels of cyclic AMP in the flies were associated with reduced levels of one of the two soluble cyclic AMP phosphodiesterases (PDEs) characterized by Davis and Kiger (1980), PDE II. Cyclic AMP functions as a second messenger mediating the effects of many hormones and neurotransmitters (Robison et al., 1971; Bloom, 1975; Greengard, 1976; Nathanson, 1977). It is thought to participate in synaptic facilitation (Brunelli et al., 1976; Shimahara and Tauc, 1977) a simple form of non-associative learning at the cellular level. In addition, it has been implicated in the learning of mammals (Sattin, 1981). Thus it was of great interest to find a defect in cyclic AMP metabolism associated with a known memory mutant in Drosophila.

One major value of these studies lies in the potential ability of this genetic

mutation to provide a direct link between PDE activity and learning. If the normal dunce gene (dunce$^+$) encodes the cyclic AMP phosphodiesterase molecule itself, then other phenotypes (such as poor learning) associated with mutations at the dunce locus must result directly from abnormal PDE II activity (see Fig. 1). If, on the other hand, the dunce gene regulates PDE II activity indirectly, the possibility would remain that the dunce gene affects learning behavior via some other activity also regulated by this gene. Distinguishing between these two logical possibilities represents an important first step toward understanding the role of cyclic AMP in learning in Drosophila.

The experiments presented below demonstrate that PDE II activity is present in the nervous system and that its reduced activity correlates with poor learning at three different developmental stages as well as in response to genetic modifications of the activity of the dunce gene. Further studies indicate that dunce mutants appear to have normal regulation of PDE II, and that PDE II activity is quantitatively altered by alterations in the dose of the dunce$^+$ gene, in a manner suggesting that dunce$^+$ is the structural gene for this enzyme. Additional support for this hypothesis comes from the recent studies of Davis and Kiger (1981) and Kauvar (1982) on abnormalities in the PDE II activity associated with two different dunce alleles.

## Materials and Methods

Mutations and chromosome rearrangements. Normal Drosophila melanogaster were from the wild-type stock Canton Special (CS). The mutant alleles dunce$^1$ and dunce$^2$ were induced in this stock by ethylmethanesulfonate mutagenesis (Dudai et al., 1976; Byers, 1980). The alleles dunce$^{M11}$ and dunce$^{M14}$ were induced by the same method in a stock carrying the X-linked visible markers yellow, crossveinless, vermilion and forked (Mohler, 1977). Two deficiency chromosomes [Df(1)N$^{71h24-5}$, Df(1)dm$^{75e19}$] and two duplication chromosomes [Dp(1;Y)w$^+$, Dp(1,2)w$^{+51b7}$] previously described by Kiger and Golanty (1977) were used to alter the number of copies of the region of the X chromosome near to or including dunce$^+$. The regions

either duplicated or deleted by these chromosomes are shown in Figure 2. An explanation of the genetic symbols is included in the legend to this figure.

Behavioral testing. Adult flies (4-10 days of age) were tested in the negative reinforcement olfactory conditioning paradigm of Quinn et al. (1974). In brief, groups of 20-40 flies were alternately presented with two odorants, one of which was paired with shock. After three training trials, the flies were presented with fresh tubes bearing the same odorants. Learning is demonstrated by selective avoidance of the tube containing the odor that was previously paired with shock. The learning index ($\Lambda$) indicates the fraction of flies selectively avoiding the odorant that was previously paired with shock. These experiments were carried out at 20°C using 3-octanol (1%) and 4-methylcyclohexanol (0.5%) as odorants (for further details, see Quinn et al., 1974).

Sample preparation. Three different procedures were used to prepare either whole animals, dissected tissues or cell lines for homogenization. Larvae or etherized 3-5 day old adults were suspended at a concentration of 10 animals/ml in 0.2 -1.0 ml of buffer A (40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM 2-mercapto-ethanol). For tissue distribution studies, tissues from 5-10 animals were dissected out under cold isotonic saline solution (128 mM NaCl, 2 mM KCl, 4 mM $MgCl_2$, 1.8 mM $CaCl_2$, 35.5 mM sucrose, 5 mM HEPES, pH 7.1) and transferred to 0.2 ml buffer A. To prepare clonal cell lines for homogenization, approximately $10^7$ cells from flask cultures containing modified Schneider's medium plus 14% calf serum were pelleted at 200 x g for 5 min. After removal of the supernatant, the cells were washed in 2 ml of buffer A, pelleted and resuspended in 0.2 - 0.25 ml buffer A. All three types of samples were homogenized at 0°C using 10 complete strokes with a motorized teflon/glass homogenizer. An aliquot of each homogenate was frozen at -20°C and later used for protein assay (see below). A second aliquot of the homogenate was mixed with 1/10th volume of buffer A containing 12.5 mg/ml bovine serum albumin (BSA). This sample was used for enzyme assay (see below), yielding 1 mg/ml BSA

in the final incubation mixture. Flies for the homogenate mixture and cohomogenization experiments presented in Figure 5 were homogenized directly in buffer A containing 1 mg/ml BSA.

Protein assay. Protein concentrations were determined using the Bio-Rad dye-binding assay with BSA as the standardizing protein (Bradford, 1976). Samples were assayed in duplicate at protein concentrations in the linear range of the absorbance vs. concentration curve.

Ion exchange column chromatography. The sample for DEAE chromatography was prepared by homogenizing 2.2 g of etherized 4-6 day old CS adults in 10 ml of buffer B (40 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 100 mM NaCl, 2 mM 2-mercapto-ethanol) at 0°C using a Brinkman Polytron homogenizer. After centrifugation at 100,000 X g for 1 hr, the supernatant was dialyzed against 800 ml buffer B for 2.5 hr at 4°C. Six ml of dialyzed supernatant were applied to the top of a 30 X 1 cm column of precycled DE52 cellulose (Whatman) equilibrated with buffer B, and the column was washed with 90 ml of buffer B. Bound material was eluted with a 90 ml gradient of 100 to 300 mM NaCl in buffer B, and 2 ml fractions were collected into tubes containing 0.22 ml of buffer C (40 mM Tris-HCl , pH 7.5, 100 mM $MgCl_2$, 10 mM $CaCl_2$, 2 mM 2-mercaptoethanol, 10 mg/ml BSA). The resultant fractions were thus effectively equilibrated in buffer A containing 1 mg/ml BSA.

Cyclic nucleotide phosphodiesterase assay. PDE activities were assayed using a modification of the procedure of Rangel-Aldao et al. (1978) similar to that used by Kauvar (1982). The reaction was initiated by adding 5 µl of $^3$H-cyclic nucleotide (New England Nuclear) in buffer A to 20 µl of sample at 30°C, and terminated by the addition of 10 µl of a quench solution (0.2 M EDTA containing 5 mg/ml each of cAMP, 5'-AMP and adenosine for cAMP-PDE assay, or cGMP, 5'-GMP and guanosine for cGMP-PDE assay). Unhydrolyzed substrate was separated from products by ascending polyethyleneimine thin layer chromatography developed for 20 minutes

with 50 mM KCl. cNMP and 5'-NMP bands were visualized under UV-illumination, cut, and placed in separate scintillation vials. The radioactive material was eluted from the bands in 1 ml of 0.7 M $MgCl_2$, 20 mM Tris-HCl, pH 7.5 , for 10 min. Then 10 ml of scintillant (Aquasol-2, New England Nuclear) was added to each vial, and radioactivity was determined using a liquid scintillation counter.

The concentration of cyclic nucleotide in the assay was 20 μM, at least five times the $K_m$'s for PDE I and II reported by Davis and Kiger (1980). Thus the amount of enzyme was rate-limiting for hydrolysis. In addition, 2 mM 5'-AMP was present in the reaction mixture. This greatly reduced further degradation of the hydrolytic products $^3$H-5'-AMP and $^3$H-5'-GMP, without inhibiting PDE activity. Percent hydrolysis was defined as 100 X (cpm in 5'-NMP band) ÷ (cpm in 5'-NMP band + cpm in cNMP band). Blank values were determined using buffer A in place of the enzyme sample. This background hydrolysis (usually less than 1%) was subtracted from each sample. Under these conditions, the hydrolysis of $^3$H-cAMP and $^3$H-cGMP was linear up to at least 40% and 30% hydrolysis, respectively. Hydrolytic activity was also linear to dilution in the presence of 1 mg/ml BSA, and with time of assay up to at least 30 min at 30°C.

## Results

Separate assay of two enzyme activities in mixture. Davis and Kiger (1980) studied the kinetic properties of PDE I and II separated by gel filtration, and found that both enzymes have similar affinities for cAMP ($K_M$ = 4 μM for PDE I, $K_M$ = 2 μM for PDE II). In addition, they showed that PDE I hydrolyzes both cAMP and cGMP with high affinity ($K_m$ about 4 μM for each substrate). Each substrate serves as a competitive inhibitor for hydrolysis of the other with similar $K_I$ values (about 3 and 4 μM, respectively). For the studies presented here, this competitive inhibition has been exploited to develop a technique which allows independent assay of the two cAMP-PDEs without requiring their physical separation. Unlabeled cGMP is

used to inhibit $^3$H-cAMP hydrolysis by PDE I without affecting PDE II activity. In theory, total cAMP hydrolysis is represented by the summed activities of PDEs I and II:

$$v_o = \frac{V_{max(I)cAMP} \times [cAMP]}{[cAMP] + K_{M(I)cAMP}(1 + \frac{[cGMP]}{K_{I(I)cGMP}})} + \frac{V_{max(II)cAMP} \times [cAMP]}{[cAMP] + K_{M(II)cAMP}}$$

As the concentration of cGMP increases, the contribution of PDE I to this equation approaches zero, and cAMP hydrolysis represents the activity of PDE II alone:

$$v_o = \frac{V_{max(II)cAMP} \times [cAMP]}{[cAMP] + K_{M(II)cAMP}}$$

In practice this technique was tested by applying it to the assay of separated forms of Drosophila PDE activities. Figure 3 represents a DEAE fractionation of the soluble PDE activities of normal flies. Assaying fractions with $^3$H-cAMP as substrate revealed two separate peaks of hydrolytic activity. When $^3$H-cGMP served as substrate, only the first peak contained hydrolytic activity, identifying it as PDE I. Hydrolysis of $^3$H-cAMP by this form was inhibited by 3 mM cGMP, while the hydrolytic activity of the second peak, PDE II, was unaffected. Thus hydrolysis of $^3$H-cAMP in the presence of cGMP represents PDE II activity, while cAMP-hydrolysis that is inhibited by cGMP represents PDE I activity. In practice, cAMP hydrolysis by both enzymes in a sample can be measured separately by assaying with (a) $^3$H-cAMP, and (b) $^3$H-cAMP plus 3 mM cGMP. PDE II activity is measured directly by (b), PDE I activity is calculated from (a-b). This procedure allows rapid measurement of the separate PDEs from a sample as small as one tenth of a fly, and is useful over at least a ten-fold range of PDE I/PDE II ratios (Table 1).

Tissue and cellular distribution of PDE activities. For PDE II to participate in neuronal events underlying memory, it must be present in the nervous system. Analysis of tissues dissected from normal flies revealed the presence of PDE II in

the brain at high specific activity (expressed in picomoles of substrate hydrolyzed per minute per mg of protein). It is not restricted to the brain, however, nor is it the only PDE activity found there. In fact, both PDE I and PDE II activities occur in readily measurable amounts in each of the tissues or body regions tested (see Table 2). Between body regions, enzyme specific activities vary considerably, as do the ratios of the two activities. Brain and gut contained the highest concentration of both enzymes, ovaries the lowest. PDE II specific activity was consistently higher than that of PDE I for a given tissue. The ratio of PDE II to PDE I ranged from a low of about 1.2 in the head, brain and ovaries to a high of over 10 in the thorax.

Most of the samples tested comprise multiple cell types. Does the presence of both PDE activities indicate that both enzymes exist in a single cell type, or are their activities being contributed separately by distinct classes of cells? Clonal cell lines were assayed to determine the PDE activities present in a single cell type. The lines studied had been established by R. Konopka and S. Wells from late Drosophila embryos, and cloned by limiting dilution in soft agar. Three such clonal lines each exhibited both PDE activities (see Table 3). The specific activities of the enzymes were lower in the cell lines than in most adult tissues sampled, with the exception of ovary. This may reflect the cell type (unknown) from which the cell lines initially were derived, or a generally lower specific activity of PDEs early in development. The fact that the specific activities found were within the range seen in adult material, and that each clonal cell line clearly expresses both PDE I and PDE II, is consistent with the idea that both enzymes can coexist in a single cell type.

Developmental relationship between learning behavior and enzyme activity levels. As mentioned above, dunce adults fail to learn in the negative reinforcement olfactory conditioning paradigm of Quinn et al. (1974). More recently, Aceves-Piña and Quinn (1979) developed a similar learning paradigm for larvae and found that

both first and third instar larvae bearing the dunce$^1$ allele failed to demonstrate learning. (Data from their learning experiments have been graphed in Figure 4.) If PDE II activity is responsible for poor learning in these developmental stages, then the enzyme defect should occur in dunce$^1$ larvae as well. The experiments presented in Figure 4 show that, as in adult dunce$^1$ flies, PDE II activity in dunce$^1$ first and third instar larvae is reduced to about one third of the levels in normal animals. In contrast, PDE I activity remains at normal levels in each case. Aceves-Piña and Quinn's observation of poor larval learning in animals bearing a mutation that affects adult learning demonstrates that this behavior is under similar genetic control throughout development. The present finding, that the cAMP-PDE abnormality of dunce$^1$ first and third instar larvae parallels that of dunce$^1$ adults, suggests that learning at all of these developmental stages requires the regulation of levels of cyclic AMP. Normal function of the dunce gene apparently is critical for both PDE II activity and learning from the first instar larva to the adult.

Regulation of dunce PDE II activity. While reduced PDE II activity in dunce flies may be due to a mutation affecting the structure of the enzyme itself, it also could result from abnormal regulation of enzyme activity. It is important to distinguish between these possibilities in order to understand the relationship between the known biochemical defect and the poor learning behavior. The fact that flies mutant at the dunce locus fail to learn tells us that this behavior is critically dependent upon the normal product of the dunce gene. If that product is PDE II, then a role for this enzymatic activity in learning is demonstrated. If, on the other hand, the dunce gene regulates PDE II activity indirectly, then it may also affect the activities of other substances which could be responsible for the effect on learning behavior associated with this gene. For this reason, several experiments were carried out to test for abnormal regulation of PDE II in dunce flies.

One possibility is that dunce flies might produce an inhibitor, not present in normal flies, that reduces the activity of an otherwise functional PDE II. If so,

such an inhibitor should be capable of reducing the activity of PDE II extracted from normal flies. To test this possibility, mixing experiments were performed using material from normal and dunce flies. Flies carrying a small deficiency for the dunce region of the X chromosome ($\underline{Df(1)N^{71h24-5}}/\underline{Dp(1;Y)w^{+}}$) (see Fig. 2) and containing no PDE II activity (see below) were homogenized, and varying amounts of this material were added to homogenates of normal (CS) flies. Mixtures containing dunce: normal homogenate volumes in the ratios 1:3, 1:1 and 3:1 were assayed for PDE II activity (Fig. 5a). In a second set of experiments, normal and dunce flies were cohomogenized in varying ratios (1:4 to 4:1), and the PDE II activity of the cohomogenate was measured (Fig. 5b). In both cases, using either homogenate mixture or cohomogenization, the level of PDE II activity in the combined samples was exactly that expected from the contribution of the normal fly material present. Thus, the dunce fly material failed to reduce the PDE II activity extracted from normal flies, even when present in a four-fold excess over normal fly material. There does not appear to be an inhibitory substance present in excess in dunce flies that can act either during or after homogenization to reduce the activity of the normal PDE II enzyme.

These experiments also argue strongly against the possibility that the PDE II defect in dunce flies is due to the lack of an activator that is required for normal PDE II activity. Calmodulin, the calcium-dependent regulator protein of mammalian PDE is an example of such an activator (Cheung, 1971; Teo et al., 1973). Yamanaka and Kelly (1981) have found a calmodulin-like substance in Drosophila, and have shown that it increases the activity of an enzyme that corresponds to PDE I (Kauvar, 1982). Although this regulatory molecule does not appear to affect PDE II, another similar activator could exist in normal Drosophila to regulate PDE II. If the dunce defect resulted from its absence, such an activator, when extracted from normal tissue, would be expected to increase the activity of PDE II in the mutant. Such an increase was not observed in the experiments presented in Figure 5, however,

indicating that the dunce defect cannot be explained by the absence of a required PDE II activator. These experiments would not detect an abnormal regulatory molecule that was labile to homogenization, nor would they detect abnormal regulation at the level of transcription or translation. These possibilities are examined in the next set of experiments.

Deficiency chromosomes, such as those used in the preceding experiments, generally produce flies with a simple absence of the functions encoded by the missing genes. Point mutations, on the other hand, can have one of several effects. Hypomorphic mutations produce lowered levels of activity while amorphic alleles produce none. A third class of mutations, neomorphs, produce new activities that may actively interfere with the function of a normal allele of the gene. Three of the alleles of dunce ($dunce^1$, $dunce^2$, and $dunce^{CK}$) are hypomorphic alleles; they have reduced levels of PDE II activity. I have examined the stronger mutant alleles, $dunce^{M11}$ and $dunce^{M14}$, which completely remove PDE II activity, to see if they demonstrate any evidence of abnormal regulation. Are these zero-activity mutant alleles, or do they produce a potent inhibitor of PDE II activity? To address this question, flies were produced that were homozygous or heterozygous for either $dunce^{M11}$ or $dunce^{M14}$. Some of the flies also carried a duplication for the normal dunce gene, in an attempt to restore genetically the PDE II activity lost as a result of the mutations at the dunce locus. If $dunce^{M11}$ and $dunce^{M14}$ are zero-activity mutants, then the addition of a copy of the normal gene should compensate quantitatively for the reduced enzyme activity. If, however, these mutations produce a potent inhibitor of PDE II activity, the addition of a normal copy of the gene may yield a fly with less than the normal level of PDE II activity. The results of these experiments are presented in Figs. 6 and 7. In flies hemizygous (males) or heterozygous (females) for either $dunce^{M11}$ or $dunce^{M14}$, the addition of a normal dose of the dunce gene via a genetic duplication quantitatively restores PDE II activity to the

level seen in control flies. These results do not support the hypothesis of production of a potent inhibitor, but rather indicate that these two alleles represent zero-activity mutations. When a mutant and normal copy of the dunce gene are present in the same cell, PDE II activity is the linear sum of the activities produced by each gene alone. This indicates that regulation is normal in dunce from the level of transcription to that of enzyme activity.

Just as a duplication of the $dunce^{+}$ gene compensates quantitatively for the PDE II activity lost as a result of the mutant alleles $dunce^{M11}$ or $dunce^{M14}$, behavioral experiments show that the activity of the $dunce^{+}$ gene also compensates for the poor learning associated with these mutant alleles. The olfactory paradigm of Quinn et al. (1974) was used to test the learning of $dunce^{M11}$ or $dunce^{M14}$ males with or without an added copy of $dunce^{+}$. These alleles originally were induced in an X chromosome with the visible markers yellow, crossveinless, vermilion and forked. In these experiments (Fig. 8), flies bearing the control chromosome obtained a learning index of 0.25 [similar to the value of 0.23 reported by Byers (1980)]. Flies bearing the mutant alleles $dunce^{M11}$ and $dunce^{M14}$ have considerably reducing learning. The mean learning indices reported here of 0.03 and 0.00 for $dunce^{M11}$ and $dunce^{M14}$, respectively, are similar to the values of 0.01 and -0.01 obtained by Byers. In contrast, male flies bearing an added copy of $dunce^{+}$ in addition to one of the mutant alleles demonstrate learning similar to that of control flies. Males with the mutant allele $dunce^{M11}$ and a duplication for the $dunce^{+}$ region [$Dp(1;2)w^{+51b7}$] achieved a mean learning index of 0.28, while flies bearing the duplication in addition to the mutant allele $dunce^{M14}$ scored 0.22.

Thus, the results of several biochemical, genetic and behavioral experiments fail to demonstrate any evidence of abnormal regulation of PDE II activity in the dunce mutant, increasing the probability that this locus encodes the enzyme molecule itself.

Gene dosage studies. In Drosophila, the genes that code for enzyme molecules and possibly other proteins exhibit a strict dosage sensitivity that has come to serve as a diagnostic indicator of a structural gene (O'Brien and MacIntyre, 1978). Enzyme levels correspond to the number of copies per cell (the dosage) of the normal structural gene for the enzyme. By choosing the appropriate duplication chromosome(s) from the set available in Drosophila, the experimenter can produce flies with added copies of part of a chromosome to search for regions which, when duplicated, increase levels of an enzyme's activity. In addition, deficiency chromosomes allow the production of flies with fewer than normal copies of a region. Such flies will have lowered activity levels for an enzyme encoded there. This screening procedure, termed segmental aneuploidy, has been used successfully to locate the structural gene for several enzymes, including acetylcholinesterase (Hall and Kankel, 1976).

Kiger and Golanty (Kiger and Golanty, 1977) used segmental aneuploidy to screen for regions affecting the activity of PDE. They found that total cyclic AMP hydrolytic activity (presumably the summed activities of PDE I and PDE II) was sensitive to the dose of the dunce region of the X chromosome, chromomere 3D4 (see Fig. 1). They demonstrated further, using physical separation techniques to allow independent analysis of the two PDE's, that flies with no copies of the normal dunce gene appear to lack PDE II activity, while retaining activity for PDE I (Kiger and Golanty, 1979; Davis and Kiger, 1981).

The assay developed here (see above) allows separate measurement of the two enzymes when both are present in mixture. Homogenates of flies can be assayed directly and rapidly without being subjected to the decays in activity and protein losses that can be a consequence of physical separation techniques. This technique has been used to analyze the levels of PDE's I and II in flies with five different doses, the equivalent of 0, 0.5, 1.0, 1.5 and 2.0 times the usual dose of the normal dunce gene. Duplication and deficiency chromosomes in the dunce region of the X chromosome (see Fig. 2) were used to produce flies with the genotypes listed in Table 4.

Single-pair backcrosses yielded flies with similar mixtures of autosomes to reduce any effect of genetic background on enzyme activities. To allow quantitative comparisons, enzyme activities for each sample were normalized to protein content, and the specific activities in flies with abnormal doses of dunce$^+$ were compared with those of normal sibling flies of the same sex that were assayed in parallel.

The results of these experiments are shown in Figure 9. Cyclic GMP hydrolysis and cyclic AMP hydrolysis by PDE I are unaffected by alterations in the dose of the dunce$^+$ gene, indicating that this region does not contain the structural gene(s) for these enzyme activities. In contrast, PDE II activity varies linearly with the dose of the dunce$^+$ gene. Male flies having zero copies of the gene display essentially no PDE II activity, as reported by Kiger and his co-workers (Davis and Kiger, 1981). In duplication-bearing male flies, where duplicating the dunce$^+$ region of the X chromosome doubles the dose of the gene, PDE II activity is 170% of that found in normal males. Female flies with half the normal dose of dunce$^+$ (one normal X chromosome and one deficient for dunce$^+$) have 63% of the PDE II activity measured in control females, while females with 1 1/2 times the normal dose (two normal X chromosmes plus a small duplication for the dunce$^+$ region) have 140% of the normal PDE II activity. Thus, at five different doses of the dunce$^+$ gene, PDE II activity corresponds one to one with the level of the gene, as would be expected if this were the structural gene for the enzyme.

Similar results occurred in a separate set of experiments that utilized a second, overlapping deficiency to produce female flies with no copies of dunce$^+$, or, as in the previous experiment, half the normal dose. The results presented in Table 4 show that these flies contained 3.7% and 50%, respectively, of the PDE II specific activity found in normal flies. Thus similar results are obtained with two different deficiencies for the dunce$^+$ region, and flies of both sexes show essentially no PDE II activity in the absence of the dunce$^+$ gene. These results, combined with the biochemical,

genetic, and behavioral data presented above, argue strongly that dunce$^{+}$ encodes PDE II, and thereby indicate a role for this enzyme in learning.

## Discussion

The enzyme assay technique described here (Fig. 3) allows separate assay of the two PDEs that hydrolyze cyclic AMP in Drosophila, even when both are present in mixture. This proved particularly advantageous for measuring the PDE activities in small samples such as tissues dissected from fruit flies. Both PDE I and PDE II have been shown to occur at high specific activity in the brain as well as in several non-neuronal tissues (Table 2). Both also occur in clonal Drosophila cell lines (Table 3), indicating that multiple enzymes for the hydrolysis of a substrate coexist in a single cell type.

Multiple forms of enzymes catalyzing the same reaction have been seen in many species, and for several enzyme systems, including PDE (Strada and Thompson, 1978; O'Brien and MacIntyre, 1978). Speculation arises as to the purpose of such apparent duplication of effort, especially in those cases where more than one isozyme is thought to exist within a single cell. While the ubiquitous presence of two cyclic AMP-PDEs in varying proportions in Drosophila tissues suggests that their regulation of cyclic AMP levels may affect different cellular functions in different tissues, their coexistence in a single cell type suggests that they may also share common functions. Perhaps the most striking observation is that flies completely lacking one of the enzyme activities, PDE II, can exist at all. This is true for two point mutations, dunce$^{M11}$ and dunce$^{M14}$, as well as for flies deficient for a small region of the X chromosome around dunce. Clearly PDE II activity is not required for survival. If PDE II normally does perform some housekeeping functions, PDE I may be compensating for them, but some defects exist in dunce flies for which PDE I cannot compensate. The two known abnormalities associated with flies lacking PDE II activity are poor learning and female sterility (Kiger, 1977; Byers et al., 1981; Salz et al., 1982), suggesting

a role for the enzyme in some aspect of these functions. Thus, the PDE system in Drosophila provides evidence for functional separation of isozymes sharing a cellular home.

There exists a strong correlation between the PDE II activity and the learning of dunce flies both throughout development (Fig. 4) and in response to genetic modification of the activity of the dunce gene (Fig. 8), but this correlation alone is insufficient to prove a role for this enzyme in learning. As indicated in Fig. 1, both phenotypes could be secondary effects resulting from some as yet unknown primary function of the dunce gene. For this reason it was important to investigate whether PDE II is affected via abnormal regulation in dunce flies, or via a defect in the structural gene for the enzyme.

In other systems, PDE activities are regulated by calmodulin, a heat stable, $Ca^{2+}$-dependent protein that also regulates several other enzyme activities (for review, see Klee et al., 1980). If $dunce^{+}$ encoded calmodulin, for example, then reduced PDE activity would represent only one of many changes that could be responsible for the learning defect in dunce mutants. Yamanaka and Kelly (1981) provided strong evidence for the existence of a calmodulin-like substance in Drosophila, but the heat-stable PDE they found it to regulate appears to correspond to PDE I (Kauvar, 1982), which is normal in dunce flies. Thus, calmodulin itself does not seem a likely suspect in our investigation of the dunce defect. Although calmodulin is the only protein activator of PDE studied to date in other species, the possible role of another regulatory substance affecting PDE II in Drosophila has not been ruled out. The experiments presented in Figs. 5-7 provide strong biochemical and genetic evidence first, that dunce flies do not lack an activator found in normal flies, and second, that they do not produce an abnormal inhibitor of PDE II activity. A regulator that might not be detected by these experiments would be one present in limiting quantity, possibly tightly bound to the enzyme, absolutely required for activity, and acting

stoichiometrically to affect PDE II activity. These are properties more indicative of an enzyme subunit than a less specific regulatory molecule. These experiments make a strong case against the argument that abnormal regulation is the cause of the PDE II defect in dunce files.

Dosage compensation (see Lucchesi, 1978) occurs in both mammals and Drosophila, but via different mechanisms. In mammals, one of the two X chromosomes in each somatic cell of a female becomes inactivated and remains in the cell as a Barr body. Drosophila females, on the other hand, retain two X chromosomes, each of which is half as active as the single X in a male. The results of several studies (for review, see O'Brien and MacIntyre, 1978) indicate that this compensation occurs at the level of transcription, with the transcriptional activity level of genes contained in a male X chromosome being double that of genes in a female X chromosome or in an autosome. Genes contained in small regions from the X chromosome that have been translocated into another chromosome also are dosage compensated according to the sex of the animal.

Duplication or deficiency chromosomes are available in Drosophila for almost any region of the genome. Because of dosage compensation, a duplication for a region of the X chromosome in a female yields an animal with 150% of the normal complement of those genes, while a duplication in a male nearly doubles the dose (see below). Similarly, females with one normal chromosome and one deficiency chromosome exhibit 50% of the usual activity of the affected genes, while males bearing only the deficiency chromosome lack activity for the deleted genes. This type of quantitative dosage compensation can be seen at the level of enzyme activity as well. It has been found in all cases where it has been studied for enzymes with a known structural gene. Regulatory genes, on the other hand, do not appear to give quantitative compensation over several doses of the gene (O'Brien and MacIntyre, 1978).

This mechanism of dosage compensation has had an enormous practical benefit for the biochemical genetics of Drosophila. Lindsley, Sandler and their many coworkers (1972) have developed a set of chromosomal rearrangements that allow the production of flies bearing a duplication or deficiency for almost any region of the genome. They have been used to screen the genome for regions showing dosage compensation for a particular protein of interest, in pursuit of its structural gene. Kiger and Golanty (1977) used this procedure to identify what later proved to be the dunce gene as a region affecting the level of activity of cyclic AMP-PDE. In their original screen they assayed for total cAMP-PDE hydrolytic activity, which presumably represents the summed activities of PDEs I and II. As they suggested, this is the most likely explanation for their finding that female flies carrying only one copy of this region had about 70% of normal cyclic AMP-PDE activity, rather than the value of 50% expected for the activity of the gene's product. Physical separation of the soluble PDE activities from males deficient for the dunce region demonstrated that PDE II activity was greatly reduced in these flies (Kiger and Golanty, 1979). Sucrose gradient fractionation of the soluble activities from females deficient for dunce also revealed no detectable PDE II activity (Davis and Kiger, 1981). In both cases, however, PDE I activity also appeared to be reduced with respect to controls.

Several technical considerations previously made it difficult to draw firm conclusions as to the sensitivity of PDEs I and II to the dosage of this region. In homogenates, separate assay of the two isozymes was not convincing, although attempts were made to separate their activities based on the thermolability and $Mg^{2+}$-dependence of PDE activity (Kiger and Golanty, 1979). Attempts at quantitative analysis based on physical separation of soluble PDE activity also is difficult due to potential trapping of activity in the 100,000 x g pellet, or loss during fractionation, where reported recoveries varied from 49% to 113% (Davis and Kiger, 1981). Thus, it was important to test the dosage sensitivities of both PDEs using the method reported in this paper for separate assay of the PDE isozyme activities in total homogenates. In addition,

the gene dosage analysis has been extended to cover five doses of the normal dunce gene. To allow quantitative comparisons, PDE activity of flies with abnormal doses of the dunce$^+$ gene was assayed in parallel with that of sibling flies (from single pair backcrosses) that carried the normal dose of dunce$^+$. The resulting values show a 1:1 correlation between PDE II activity and the dose of dunce$^+$. The data also show clearly that the levels of cyclic GMP hydrolysis and cyclic AMP hydrolysis by PDE I are not altered by variations in the dose of this region of the chromosome. This had been difficult to demonstrate conclusively before.

Four of the dunce$^+$ doses (0, 0.5, 1, 1.5) correlate closely with the level of PDE II activity. In the fifth case, a male bearing a duplication for dunce$^+$ and therefore carrying a dose of 2.0, the PDE II value was 170% $\pm$ 10% (mean $\pm$ S.D.). While this value may appear to be slightly lower than expected, such a result is reasonably common for duplication-bearing males. Studies of activity levels in males bearing duplications for the structural gene of three other proteins gave values of 129% (G6PD), 135% (6PGD) and 163% (Sgs-4) (see Stewart and Merriam, 1980). There appears to be an undershoot of activity as the dose of the structural gene is increased.

Further biochemical evidence in support of the hypothesis that dunce$^+$ encodes PDE II comes from the work of Davis and Kiger (1981) and Kauvar (1982). Davis and Kiger studied the kinetic properties of PDE II fractionated by gel filtration from supernatants of dunce$^2$ and normal flies. They found anomalous kinetic plots for dunce$^2$ PDE II reproducibly under two different experimental conditions, and suggested that the anomalous kinetics resulted from mutation at the dunce gene. Kauvar also found abnormal kinetics for PDE II in dunce$^2$ homogenates. In addition, he has observed abnormal thermolability in the PDE II activity of dunce$^1$ homogenates, as well as in the partially purified enzyme. Increased thermolability is often interpreted as evidence for an amino acid substitution that results in reduced stability of the protein's tertiary structure. While these altered properties of PDE II in the mutants could result from abnormal postranslational modification of the enzyme's structure,

this explanation seems unlikely based on the evidence presented in this paper that regulation of PDE II activity appears normal in dunce flies.

Taken as a whole, these studies provide strong biochemical evidence that dunce$^+$ encodes PDE II. This indicates that the poor learning associated with mutations at the dunce locus results from a primary defect in PDE II activity, presumably by controlling levels of cyclic AMP.

## Chapter III

## The Relationship Between PDE II, Learning and Female Fertility in *dunce* Mutants

### Introduction

Mutations at the *dunce* locus are associated with three known phenotypes. Two of these, poor learning and a reduction in PDE II activity, have been discussed in some detail in the preceding chapter. It has been shown that the two defects correlate closely both throughout development, and in response to genetic modifications of the activity of the *dunce* gene. The purpose of this chapter is to describe the relationship between these and female sterility, the third phenotype associated with mutations at the *dunce* locus.

In the Benzer laboratory, the first encounter with female sterility was delayed until the isolation of the second allele mutated at the *dunce* locus, $dunce^2$. Female sterility was not being studied for its own sake, but it is a phenotype difficult to ignore when attempting to produce a stock of homozygous flies. $dunce^1$, for reasons that will be discussed below, was successfully established as a homozygous stock soon after its isolation. The $dunce^2$ chromosome, however, carried a recessive female sterile mutation, and could only be maintained as a stock by using females heterozygous for $dunce^2$. Byers (1980) used genetic recombination to map the learning and female sterile phenotypes of $dunce^2$ with respect to one another. He found that they mapped together on the X chromosome between the genes affecting the visible markers *yellow* and *chocolate*, the same region to which the poor learning phenotype of $dunce^1$ previously had been mapped (Dudai et al., 1976). More detailed mapping studies localized *dunce* to a region of the X chromosome that was also being studied by the lab of John Kiger, at the University of California at Davis, for its effects on female sterility and cyclic AMP-PDE (Kiger and Golanty, 1977; Kiger, 1977). Kiger had obtained two female sterile mutants in this region from Dawson Mohler of the University of Iowa (Mohler, 1977). These mutants proved to be allelic with $dunce^1$

and dunce$^2$ (Byers, 1980; Byers et al., 1981). The female sterile mutants isolated by Mohler had poor learning, and did not complement the recessive phenotype of poor learning when heterozygous with either dunce$^1$ or dunce$^2$. They also did not complement the female sterility of dunce$^2$. In addition, the two dunce mutants proved to have elevated cyclic AMP AMP levels as well as reduced PDE II. Because of the evidence that these two female sterile mutants map to the dunce locus, Mohler renamed them dunce$^{M11}$ and dunce$^{M14}$. Thus, the classic technique of genetic mapping led to the discovery of the first biochemical defect to be associated with a Drosophila learning mutant—abnormal regulation of cyclic AMP.

The female sterility phenotype has played an additional role in the analysis of dunce. It has been used to generate suppressor mutations that could affect the function of the dunce gene. As used here, the term suppressor refers to a class of mutations which suppress mutant phenotypes of other genes. The term does not imply knowledge of the mechanism of suppression, which could occur at any level from DNA regulation to behavior. One use for suppressor mutations is to dissect phenotypes genetically, to determine causal relationships between them, and to elucidate mechanisms of their interactions. In the present study, suppressors of dunce female sterility are analyzed to increase our understanding of the relationships among the three phenotypes associated with dunce mutants. In Chapter 2 we dealt only with the two phenotypes of reduced PDE II activity and poor learning. There the analysis was simpler. Either the two phenotypes are related to one another directly, or indirectly via a common intermediary step linking each to the dunce gene (see Fig. 1). An analysis of the potential relationships between all three phenotypes produces several more possibilities (see Fig. 10). In the simplest case, reduced PDE II activity, as the primary defect, would lead to poor learning and female sterility. Alternatively, two of the phenotypes could be closely linked, and indirectly related to the third. In the most extreme case, the three phenotypes could be linked only

by their dependence on normal dunce gene function. Some of these possibilities might be eliminated by analysis of suppressor mutations. Will suppressors of dunce female sterility suppress one or both of the other mutant phenotypes, thus linking them to sterility? Or will they separate fertility from learning and PDE II? These are two of the questions to be addressed in this chapter.

The first allele of dunce, $dunce^1$, gave the earliest indication that female sterility could be separated from the learning and PDE II defects. Female flies homozygous for other dunce alleles (2, M11 and M14) are sterile and show poor learning (Byers, 1980). Homozygous $dunce^1$ females are also learning deficient, but have fertility that is about half that of normal females. Byers (1980) postulated that the original $dunce^1$ X chromosome carried two mutations. One mutation, at the dunce locus, was responsible for reduced learning and PDE II activity, while the second mutation was a suppressor of female sterility. Recently, such a suppressor of female sterility, designated Su(fs), has been separated from $dunce^1$ by genetic recombination to produce a $dunce^1$ chromosome which, when homozygous, causes female sterility (Salz et al., 1982). These authors found that homogenates of $dunce^1$ flies with or without Su(fs) have similar cAMP-PDE activity levels. While PDE II activity was not assayed separately in these studies, their results suggest that it is not dramatically affected by Su(fs). The suppressor of female sterility does not restore normal learning as evidenced by the poor learning of flies bearing the original dunce chromosome that carried Su(fs). This chapter presents results from studies of two new suppressors of dunce female sterility that further demonstrate separate genetic regulation of learning and PDE II activity vs. female fertility.

The relationship between the learning, PDE II activity and female fertility phenotypes was also studied using a deficiency chromosome that affects all three phenotypes. Chromosome $Df(1)N^{64j15}$ is deficient for the region of the X-chromosome from 3C4 to 3D3, just distal to 3D4 (the band to which the dunce gene has been mapped; see Fig. 2). $Df(1)N^{64j15}$ seems to reduce, but not remove, PDE II activity

(Kiger and Golanty, 1977). Df(1)N$^{64j15}$/Dp(1;Y)w$^{+}$ males are deficient for band 3D3. In homogenates, cyclic AMP AMP hydrolysis (representing the summed activities of PDE I and PDE II) in these males was intermediate between that of normal males and males deficient for dunce [Df(1)N$^{71h24-5}$/Dp(1;Y)w$^{+}$]. The effect of Df(1)N$^{64j15}$ on learning is straightforward—it makes flies stupid (Byers, 1980). Df(1)N$^{64j15}$ has a more complex effect on female sterility. Heterozygous female flies carrying this chromosome and any of the dunce mutant alleles or deficiencies show variable fertility. Some of the females are sterile, while some are completely fertile (Kiger, 1977; Salz et al., 1982). Byers established several lines starting with single fertile Df(1)N$^{64j15}$/Df(1)dm$^{75e19}$ females. In each case the females of successive generations showed the same variable fertility. This ruled out one possible explanation, which was that the Df(1)N$^{64j15}$ stock had been contaminated with another X chromosome (Byers, personal communication). Just as with suppressor mutations, there are several possible mechanisms whereby a deficiency chromosome could have variable effects on mutant phenotypes (see Discussion). In this chapter, I will present the results of studies that analyze the relationship between PDE II activity and the variable female fertility associated with Df(1)N$^{64j15}$. The aim in studying both this deficiency chromosome and the suppressor mutations is similar—to understand the relationships between the three phenotypes associated with dunce.

## Materials and Methods

Stocks. The duplication and deficiency chromosomes and mutant alleles used in these experiments are described in Chapter 2, with one exception. The deficiency chromosome Df(1)N$^{64j15}$ described by Kiger and Golanty (1977) also has been employed.

Fertility tests. To test female fertility, individual 0-1 day old females were placed in a vial with three 1-3 day old CS males. After six days at 25°C, the parent flies were removed, and the adult progeny emerging by day 17 were counted. (The second generation of normal flies can begin to emerge as early as day 18 at 25°C).

Behavioral testing. Tests of learning were performed as described in Chapter 2.

cAMP phosphodiesterase assay. PDE activities were assayed as described in Chapter 2, with the following exception: Single flies were homogenized in 150 µl of buffer A. Homogenization of more than one fly was carried out at a concentration of 1 fly per 100 µl, as described previously.

## Results

I have studied two second chromosome suppressors of female sterility in $dunce^2$ isolated by Madeline Crosby (personal communication). The first is a recessive mutation that was segregating in the $dunce^2$ stock. It has been named su(fs)C67. The second was induced by ethylmethanesulfonate mutagenesis. This proved to be a dominant mutation, and was named Su(fs)C119. I tested the effect of these mutations on PDE II activity and learning. Neither suppressor seems to increase PDE II activity. In one experiment, the PDE II activities of $dunce^2$ females with or without su(fs)C67/su(fs)C67 were 27% and 25%, respectively, of control values. I also found similar PDE II activity in $dunce^2$ females with or without Su(fs)C119/+ (26% and 20%, respectively, of control values). At least one of the suppressors does not improve the learning ability of the flies. The learning index of $dunce^2$ females was 0.02 with Su(fs)C119/+, and 0.03 without; su(fs)C67 was not tested for its effect on learning. These two suppressors for $dunce^2$ separate the phenotype of fertility from PDE II activity and, in at least one case, from learning.

In order to study the deficiency chromosome $Df(1)N^{64j15}$, I obtained from Byers one of the "clonal" lines begun from a single $Df(1)N^{64j15}$ chromosome. I tested for female fertility using the same procedure he and Kiger had used (see Materials and Methods). In my hands, also, most of the females with one $Df(1)N^{64j15}$ chromosome and one dunce deficiency [$Df(1)dm^{75e19}$] were fertile, while about 10-15% were sterile. To demonstrate that this variability in female fertility is a function of the genotype of the flies, and not the testing conditions, it is important to note that in parallel tests, none of the control flies [$Cs/Df(1)dm^{75e19}$] was sterile, while all of the females deficient for dunce [$Df(1)N^{71h24-5}/Df(1)dm^{75e19}$] were sterile

(Table 6, and unpublished observations). I assayed the PDE activities of 9 sterile and 11 fertile females bearing Df(1)N$^{64j15}$. The flies were homogenized and assayed individually at 6-7 days of age, after their rather important role in the fertility test was completed. The results presented in Table 6 show that female flies with one normal X chromosome (CS) and one deficient for dunce [Df(1)dm$^{75e19}$ have half the PDE II specific activity of females with two copies of dunce$^{+}$ (CS/CS). Females with two overlapping deficiencies for dunce [Df(1)N$^{71h24-5}$/Df(1)dm$^{75e19}$] have essentially no PDE II activity. These results follow the dosage sensitivity described in Chapter 2. In agreement with the conclusion of Kiger and his coworkers, however, the chromosome Df(1)N$^{64j15}$ (in the combination Df(1)N$^{64j15}$/Df(1)dm$^{75e19}$) appears to only partially reduce PDE II activity. The present finding is that PDE II activity does not covary with fertility in these flies. Both fertile and sterile females of the genotype Df(1)N$^{64j15}$/Df(1)dm$^{75e19}$ have about 50% of the PDE II specific activity that would be expected if Df(1)N$^{64j15}$ carried a normally functioning dunce$^{+}$ gene (i.e., 25% of the activity found in a normal female). I have also tested the thermostability of PDE II from females bearing this chromosome and the dunce deficiency chromosome Df(1)dm$^{75e19}$. All of the PDE-II activity in such females results from Df(1)N$^{64j15}$, and appears to have normal thermostability (see Fig. 11).

## Discussion

The presence of PDE II activity associated with Df(1)N$^{64j15}$ demonstrates that this chromosome does carry a copy of the dunce gene. Kiger and his coworkers (1981) speculated that Df(1)N$^{64j15}$ deletes a regulatory element that affects the function of the dunce gene, based on the observations that PDE II activity and female fertility are reduced but not eliminated. Several other possible explanations exist, however. For example, this alteration in dunce gene function could be a position effect (Lewis, 1950) in which a rearrangement breakpoint affects the activity of nearby genes. In this case, the right breakpoint of the deficiency chromosome

*Df(1)N*$^{64j15}$ could be affecting the function of *dunce*, which is in the next band. Another, simpler explanation is that the chromosome is a complex rearrangement rather than a simple deletion, and contains a second defect in the *dunce* gene itself. In fact, recent examination of the DNA sequence organization of *Df(1)N*$^{64j15}$ has shown aberrations relative to *CS* in the sequence organization proximal to the deficiency breakpoint at 3D3 (Ron Davis, personal communication). The relationship between these additional aberrations and the *dunce* gene is not known, however.

The deficiency chromosome *Df(1)N*$^{64j15}$ and the three suppressors studied to date divide the *dunce* phenotypes into two groups, one that contains all three phenotypes, and one that contains only reduced PDE II activity and poor learning. The observation that the suppressors appear to suppress only one of the three mutant phenotypes indicates that the primary defect in the mutated *dunce* gene is still being expressed and, therefore, that the suppression is acting at some secondary level. Fertility is an important phenotype, and it can be affected by mutations at several loci (see Mohler, 1977). As there are many ways to disrupt fertility, there are probably many ways to rescue it when it goes awry. Therefore it is not surprising to find that the female sterility associated with *dunce* mutations can be regulated by the activity of several other genes, and that this regulation leaves the other mutant phenotypes intact. It simply indicates that female sterility represents a separate part of the pathway(s) linking *dunce* gene activity to its associated phenotypic defects.

The failure to separate the *dunce* phenotypes of reduced PDE II activity and poor learning is consistent with the hypothesis discussed in Chapter 2, that reduced PDE II activity is the primary defect in *dunce* mutants, and that the reduction in its activity leads to poor learning.

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| Ratio PDE I/PDE II | PDE Sample | Percent of substrate hydrolyzed | |
|---|---|---|---|
| | | $^{3}$H-cAMP | $^{3}$H-cAMP + unlabeled cGMP |
| 5.0 | I | 17.2 | 1.8 |
| | II | 3.8 | 4.1 |
| | I + II | 21.0 (predicted) | 5.9 (predicted) |
| | I + II | 20.5 (measured) | 4.8 (measured) |
| 1.0 | I | 11.7 | 0.1 |
| | II | 10.1 | 9.0 |
| | I + II | 21.8 (predicted) | 9.1 (predicted) |
| | I + II | 22.2 (measured) | 9.4 (measured) |
| 0.2 | I | 4.9 | 0.0 |
| | II | 8.7 | 7.1 |
| | I + II | 13.6 (predicted) | 7.1 (predicted) |
| | I + II | 13.5 (measured) | 6.9 (measured) |

Table 1

# Phosphodiesterase Distribution in Normal Adult Flies

| Sample | | PDE(substrate) | | |
|---|---|---|---|---|
| | | II (cAMP) | I (cAMP) | (cGMP) |
| Female | whole fly | 1800 | 700 | 1200 |
| | head | 2900 | 2500 | 6600 |
| | thorax | 1200 | 110 | 420 |
| | abdomen | 2000 | 750 | 860 |
| | brain | 4700 | 3700 | 9300 |
| | gut | 3400 | 730 | 1200 |
| | ovary | 250 | 200 | 270 |
| Male | whole fly | 1900 | 560 | 1400 |
| | head | 3200 | 2800 | 6900 |
| | thorax | 2000 | 130 | 510 |
| | abdomen | 1700 | 630 | 950 |

**Enzyme specific activity is expressed in picomoles of substrate hydrolyzed per minute – milligram protein.**

Table 2

## Phosphodiesterase Activities in Clonal Drosophila Cell Lines

| Cell line | PDE (substrate) | | |
|---|---|---|---|
| | II (cAMP) | I (cAMP) | (cGMP) |
| CL 7 | 150 | 30 | 120 |
| CL 12 | 130 | 18 | 160 |
| CL 14 | 58 | 36 | 84 |

Enzyme specific activity is expressed in picomoles of substrate hydrolyzed per minute - milligram protein.

Table 3

| Effective Dose of dunce$^{+}$ | Sex | Genotype |
|---|---|---|
| 0 | male | $\frac{Df(1)N^{71h24-5}}{Dp(1;Y)w^{+}}$ ; $\frac{+}{+}$ |
| 0.5 | female | $\frac{Df(1)N^{71h24-5}}{FM7^{a}}$ ; $\frac{+}{+}$ |
| 1.0 | female | $\frac{FM7^{a}}{FM7^{a}}$ ; $\frac{+}{+}$ |
| 1.0 | male | $\frac{FM7^{a}}{Y}$ ; $\frac{+}{+}$ |
| 1.5 | female | $\frac{w}{w}$ ; $\frac{Dp(1;2)w^{+51b7}}{+}$ |
| 2.0 | male | $\frac{w}{w}$ ; $\frac{Dp(1;2)w^{+51b7}}{+}$ |

Table 4

| Genotype | PDE II * Specific Activity (avg. SEM) | PDE II % of Control (avg. SEM) | n |
|---|---|---|---|
| CS / CS | 1419 ± 10 | 100% ± 5.3% | 10 |
| CS / $Df(1)dm^{75e19}$ | 710 ± 44 | 50% ± 3.1% | 9 |
| $Df(1)N^{71h24-5}$ / $Df(1)dm^{75e19}$ | 52 ±6.7 | 3.7% ± 0.5% | 9 |

* Specific activity is expressed in picomoles of substrate hydrolyzed per minute - milligram protein.

Table 5

| Genotype | Dose of dunce+ | Learning Index[a] | Fertility[b] | PDE II[c] | PDE I[c] |
|---|---|---|---|---|---|
| CS / CS | 1.0 | 0.33±0.01 (n=463) | 19±1.7 (n=10) | 1419±10 (n=10) | 513±30 (n=10) |
| CS / Df(1)dm$^{75e19}$ | 0.5 | 0.26±0.03 (n=5) | 24±3.9 (n=9) | 710±44 (n=9) | 480±44 (n=9) |
| Df(1)N$^{64j15}$ / Df(1)dm$^{75e19}$ | ? | 0.04±0.02 (n=12) | 13±1.8 (n=11) | 361±24 (n=11) | 485±28 (n=11) |
| | | | 0±0 (n=9) | 322±24 (n=9) | 421±14 (n=9) |
| Df(1)N$^{71h24-5}$ / Df(1)dm$^{75e19}$ | 0.0 | 0.03±0.03 (n=6) | 0±0 (n=9) | 52±6.7 (n=9) | 380±19 (n=9) |

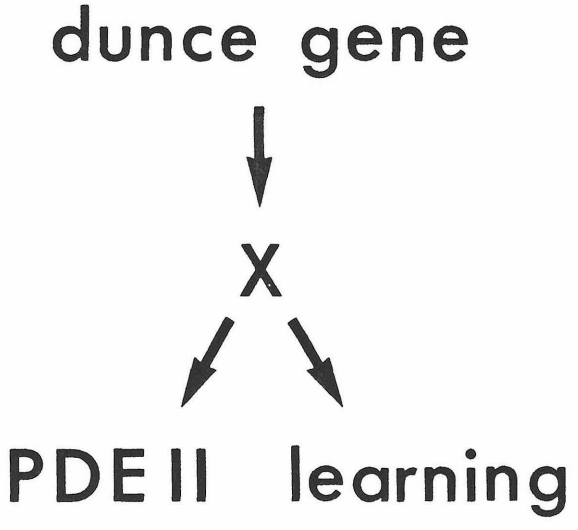
a Data of Duncan Byers (1980, and unpublished; mean±S.E.M.)

b Number of progeny per female in 17 days (mean±S.E.M.)

c Specific activity expressed in picomoles of cyclic AMP hydrolyzed per minute per mg protein at 30°C (mean±S.E.M.)

Table 6

**Figure 1.** These logic diagrams represent two possible relationships between the reduced PDE II activity and the learning disability associated with the dunce gene. In the first case (diagrammed on the left), the product of the dunce gene is unknown. A defect in this gene product (X) would lead to reduced PDE II activity and poor learning through mechanisms that may be shared or separate. In this scenario, the involvement of PDE II in learning would remain obscure. In the second case (diagrammed on the right) the dunce gene codes directly for PDE II. The learning disability then would result from the primary defect in PDE II activity. A role for PDE II in learning would be demonstrated.



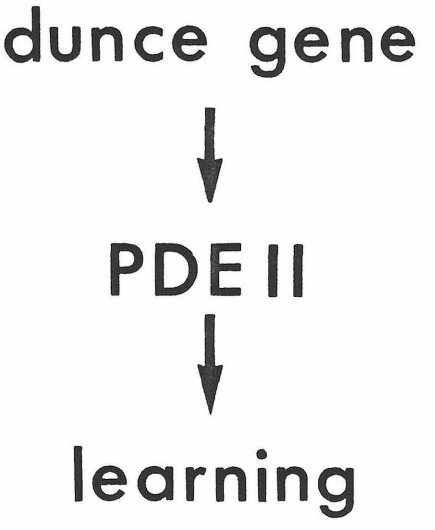




Figure 1

**Figure 2.** This drawing of a segment of the Drosophila X chromosome shows the location of deficiency and duplication chromosomes used in genetic studies of the dunce gene. The break points of these chromosomes were determined by Lefevre (see Kiger and Golanty, 1977). They are illustrated in the upper portion of the figure. Solid bars represent regions of chromosomal material; dotted lines indicated their continuation. Gaps indicate the absence of chromosomal material. The sketched region represents approximately 3% of the X chromosome. The locations of the genes white, Notch and diminuitive are shown for reference.

Explanation of terminology. The numbers below the figure represent regions of the X chromosome as mapped by Bridges (1935). The X chromosome is divided into 20 regions (numbered 1 to 20 from the distal tip to the centromere) each of which contains 5 subdivisions (lettered A to F). The meaning of the names assigned to duplication or deficiency chromosomes is best illustrated by an example. Duplication $(1;2)w^{+51b7}$ is a chromosome containing duplicated material from the X (1) chromosome translocated into the second (2) chromosome. The duplicated region contains a normal copy of the white gene ($w^{+}$). Numbers or letters in superscript identify a specific chromosome. They often refer to the initials of the person who isolated it, the year of isolation, the bottle it came from, or the score of the latest Dodgers-Yankees game.

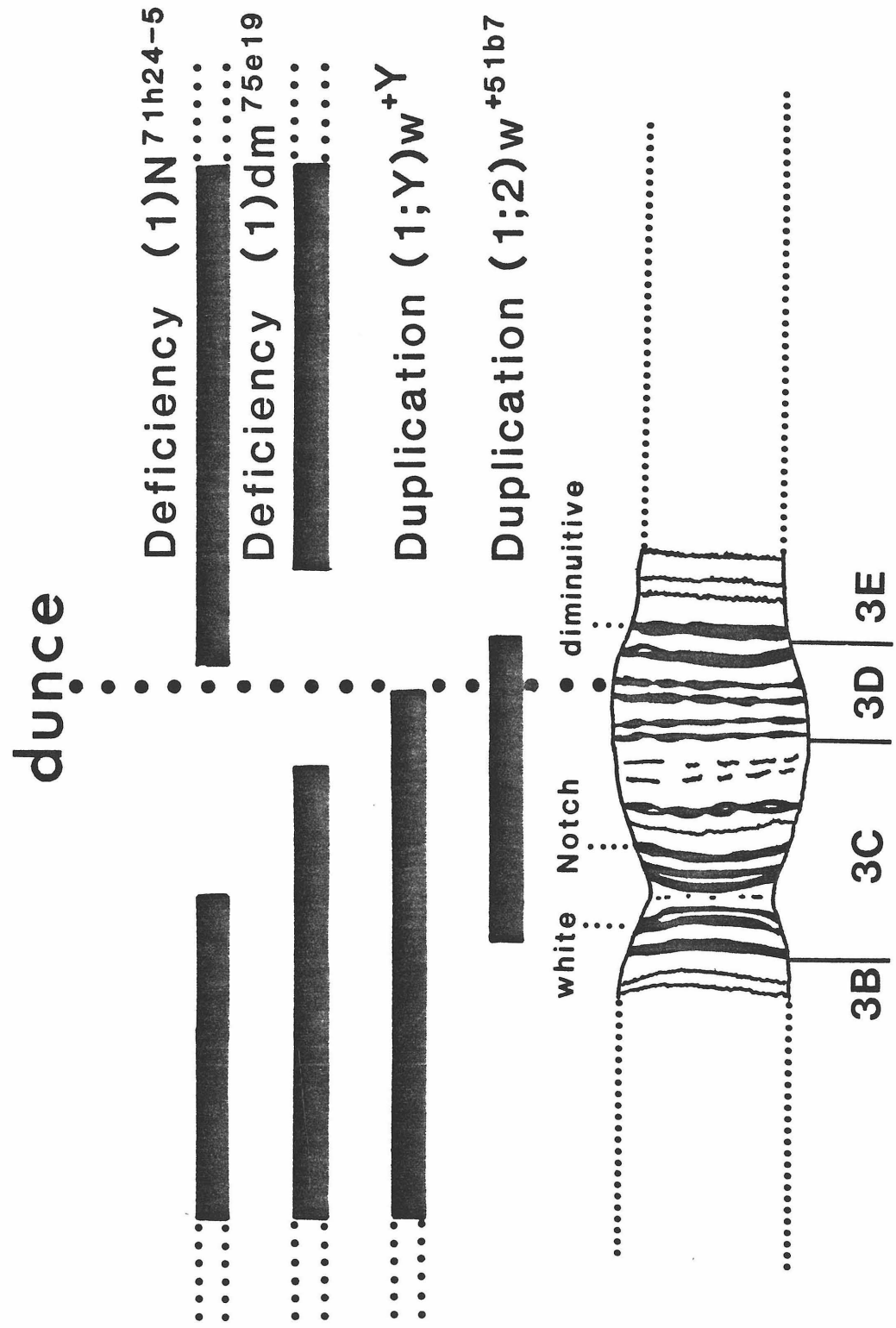

dunce
Deficiency (1)N 71h24-5
Deficiency (1)dm 75e19
Duplication (1;Y)w+Y
Duplication (1;2)w+51b7
diminuitive
Notch
white
3B
3C
3D
3E

Figure 2

**Figure 3.** Ion exchange column chromatography of normal Drosophila PDE. The sample (dialyzed 100,000 x g supernatant from CS adults) was loaded onto a 30 x 1 cm column of pre-cycled DE52 cellulose equilibrated with buffer B. After washing with 90 ml of buffer B, bound material was eluted with a 90 ml gradient of NaCl (100-300 mM) in buffer B. 2 ml fractions were collected, equilibrated with buffer A containing 1 mg/ml BSA, and assayed for PDE as described (see Materials and Methods). There was no PDE activity in the pre-gradient fractions (not shown). Two peaks of activity eluted in the gradient, one at 170 mM NaCl (fx 28) and one at 220 mM NaCl (fx 38). The first peak contained hydrolytic activity against both cyclic AMP and cyclic GMP. Hydrolysis of $^{3}$H-cAMP by this peak was eliminated by the inclusion of 3 mM cyclic GMP. The second peak of enzyme hydrolyzed only cyclic AMP, and its activity was not affected by cyclic GMP.

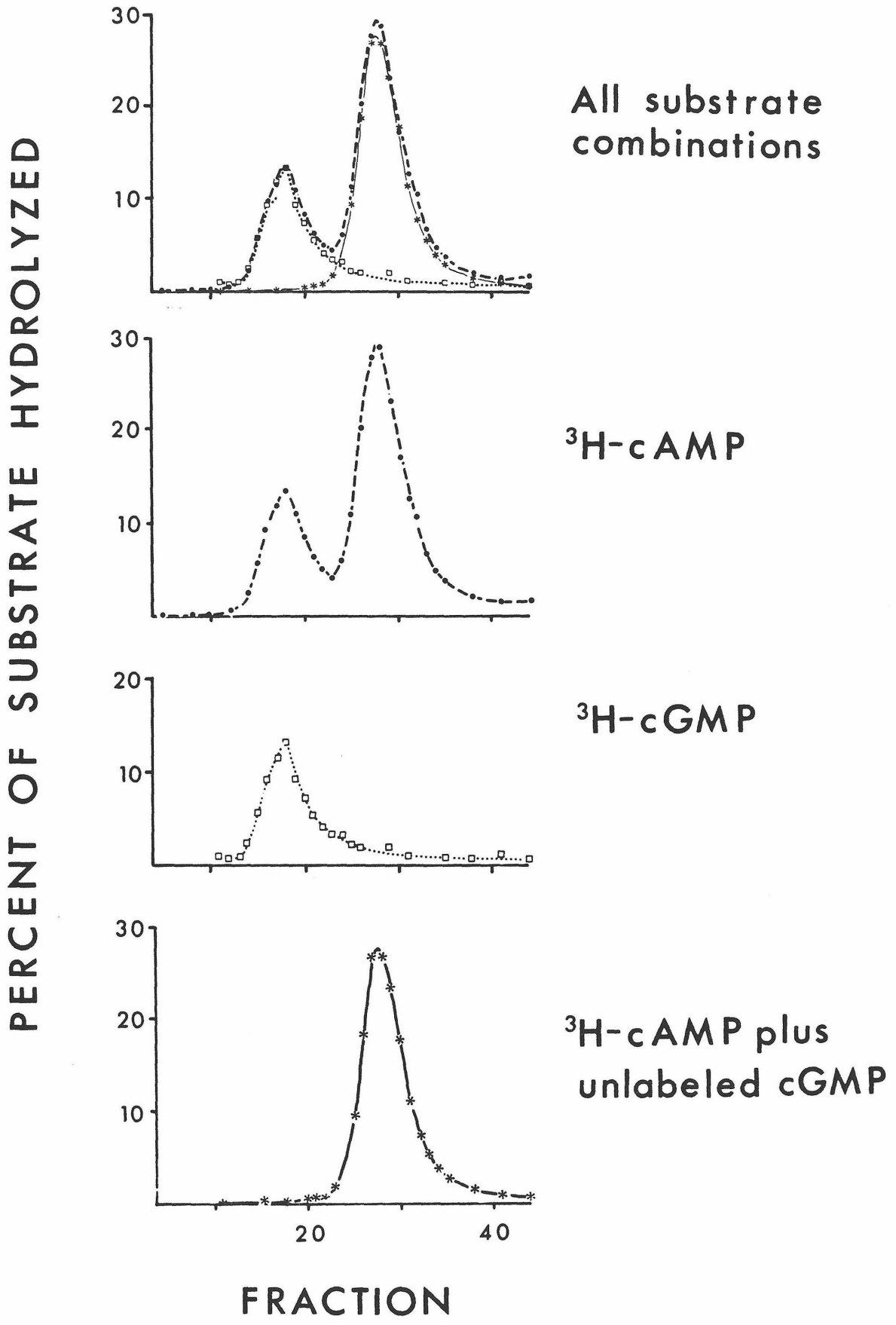

PERCENT OF SUBSTRATE HYDROLYZED
30
20
10
All substrate combinations
30
20
10
$^3$H-cAMP
20
10
$^3$H-cGMP
30
20
10
$^3$H-cAMP plus unlabeled cGMP
20
40
FRACTION

Figure 3

**Figure 4.** Correlation between reduced PDE II activity and learning disability through development. Drosophila demonstrate associative learning both as adults (Quinn et al., 1974) and as larvae (Aceves-Piña and Quinn, 1979). The learning index used by these authors indicates the fraction of flies selectively avoiding the odor that was previously paired with shock (see Materials and Methods). Aceves-Piña and Quinn (1979) have compared the learning ability of CS and $dunce^1$ flies at three developmental stages. Learning in $dunce^2$ is greatly reduced in each case. Their data are graphed in the left column for comparison with PDE activities. The PDE activities of these animals were assayed as described in Materials and Methods. For each homogenate, the values from duplicate determinations of each PDE activity were averaged and then normalized to CS PDE activity assayed in parallel. Values are presented as mean $\pm$ S.E.M. for n experiments.

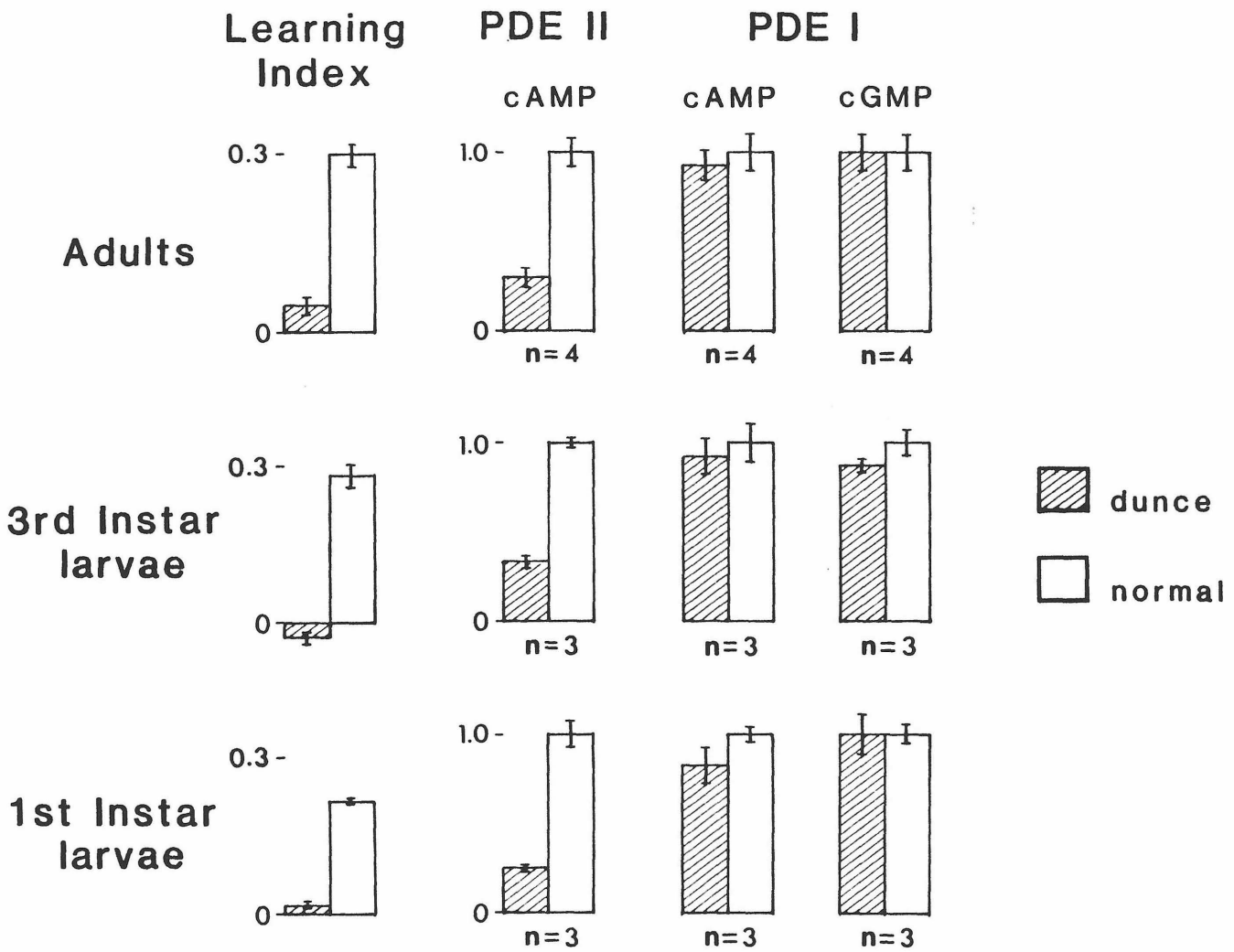

Learning Index
PDE II
PDE I
cAMP
cAMP
cGMP
Adults
3rd Instar larvae
1st Instar larvae
0.3
1.0
0
n=4
n=3
dunce
normal

Figure 4

**Figure 5.** Tests for abnormal biochemical regulation of PDE II activity in dunce flies. a) Flies deficient for dunce [Df(1)N$^{71h24-5}$/Dp(1;Y)w$^{+}$] were homogenized at a concentration of one fly per 100 µl buffer A. Aliquots of the dunce homogenate were mixed with homogenates of normal (CS) flies. Volumes were varied to give ratios of CS:dunce material of 3:1, 1:1, and 1:3. The PDE II activity measured in each mixture was normalized to the level of PDE II activity in the CS homogenate assayed in parallel. Each point represents the average of duplicate assays from a separate experiment. b) Flies deficient for dunce [Df(1)N$^{71h24-5}$/Dp(1;Y)w$^{+}$] were cohomogenized with CS flies at a concentration of one fly per 100 µl buffer A. The number of flies of each genotype was varied to yield CS:dunce ratios ranging from 4:1 to 1:4. The PDE II activity measured in each cohomogenate was normalized to the level of PDE II activity in a CS homogenate assayed in parallel. Each point represents the average of duplicate assays from a separate experiment.

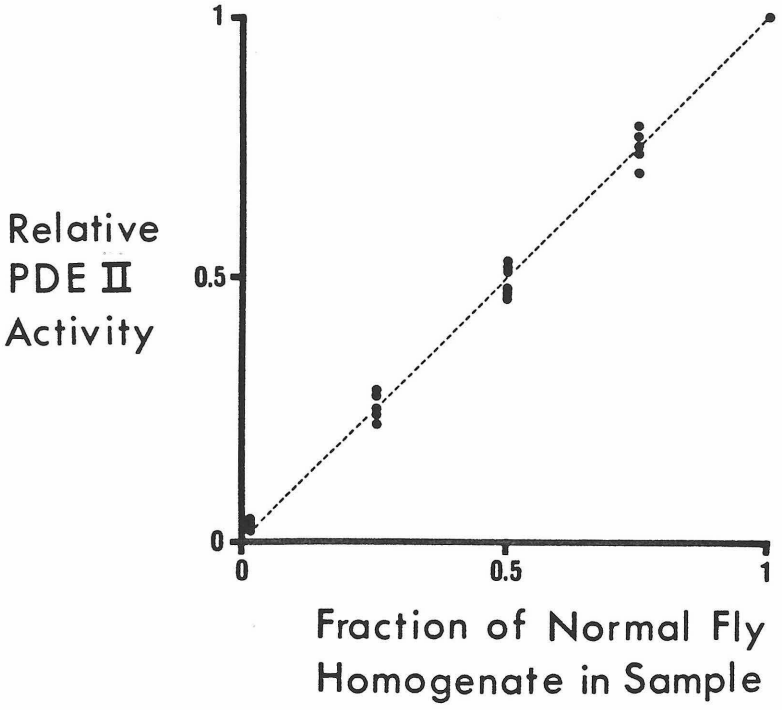



Figure 5a

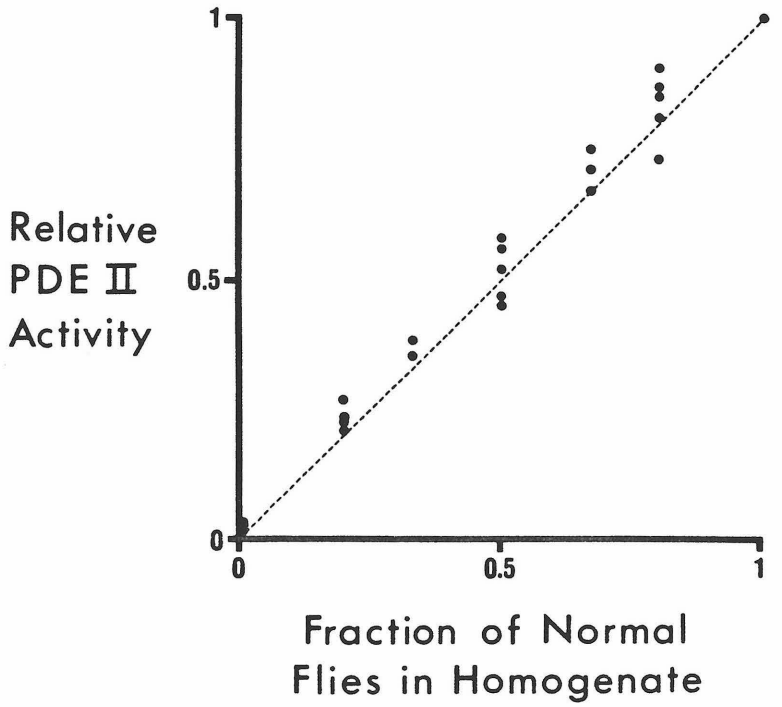



Figure 5b

**Figure 6.** Tests for abnormal genetic or biochemical regulation of PDE II in dnc males. The PDE II activity in homogenates of male flies bearing a dnc mutation (y dnc $^{M11 \text{ or } M14}$ cv v f/Y) was compared with the PDE II activity of control males (y cv v f) and mutant males carrying an additional copy of the normal dnc gene [y dnc $^{M11 \text{ or } M14}$ cv v f/Y; Dp(1;2)w$^{+51b7}$/+]. Three 4-5 day old males of a given genotype were homogenized in 300 µl buffer A and assayed for PDE and protein as described in Materials and Methods. The average of duplicate determinations of PDE II specific activity was normalized to the value obtained for control flies assayed in parallel. The height of each bar represents the mean, and the error bars the S.E.M., for 4 to 6 experiments. Abbreviations: cv - crossveinless, dnc - dunce, f - forked, v - vermilion, y - yellow.

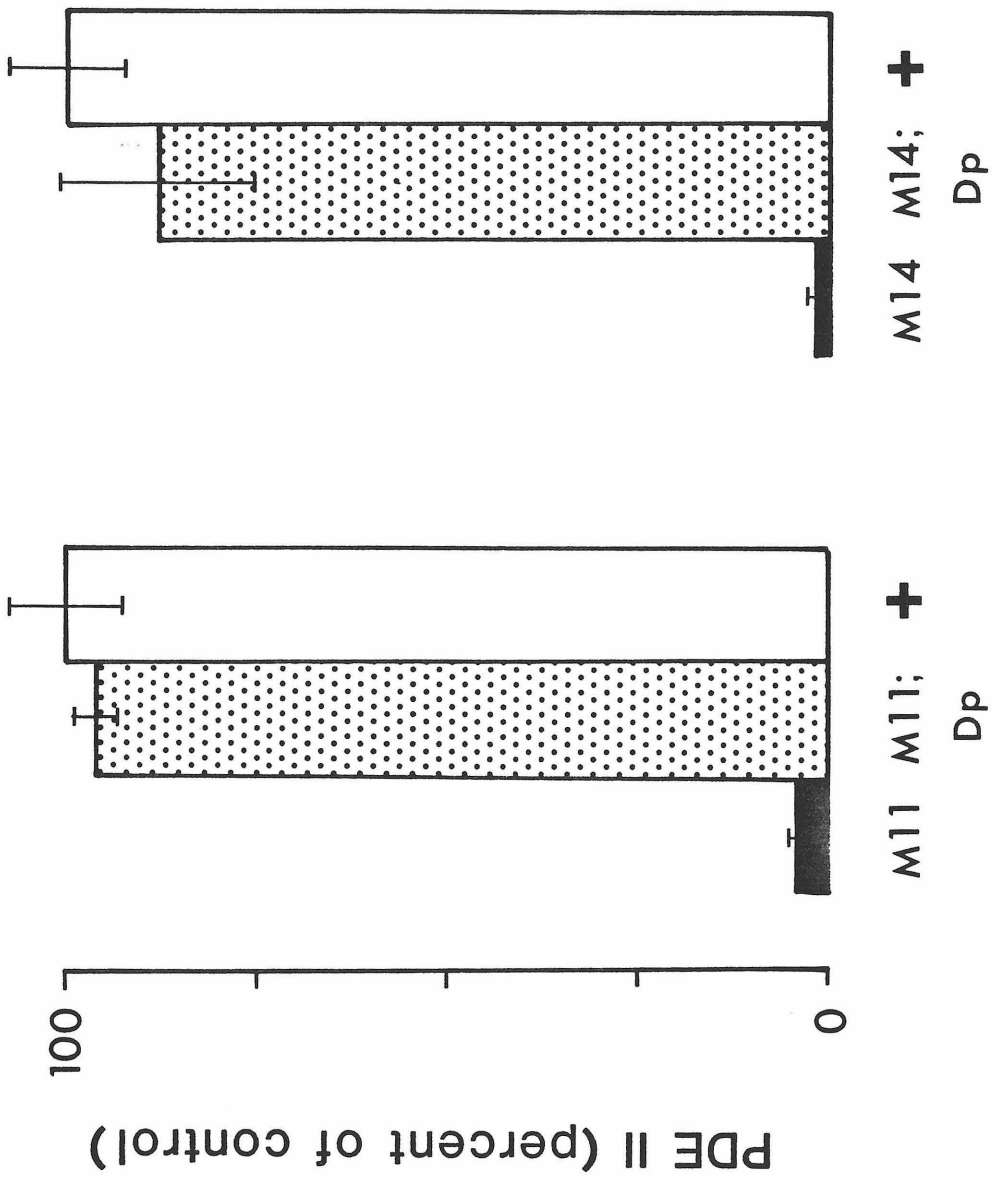



Figure 6

**Figure 7.** Tests for abnormal genetic or biochemical regulation of PDE II in dunce females. PDE II activities were compared for control females (y cv v f/FM7b), females heterozygous or homozygous for dunce (y $dnc^{M11 \text{ or } M14}$ cv v f), and heterozygous dunce females with an additional copy of the normal dunce gene [$Dp(1;2)w^{+51b7}$]. Three 4-5 day old females of a given genotype were homogenized in 300 µl of buffer A and assayed for PDE and protein as described in Materials and Methods. The average of duplicate determinations of PDE II specific activity was normalized to the value obtained for control flies assayed in parallel. The height of each bar represents the mean, and the error bars the S.E.M., for 4 to 6 experiments. Abbreviations: see the figure legend for Figure 6.

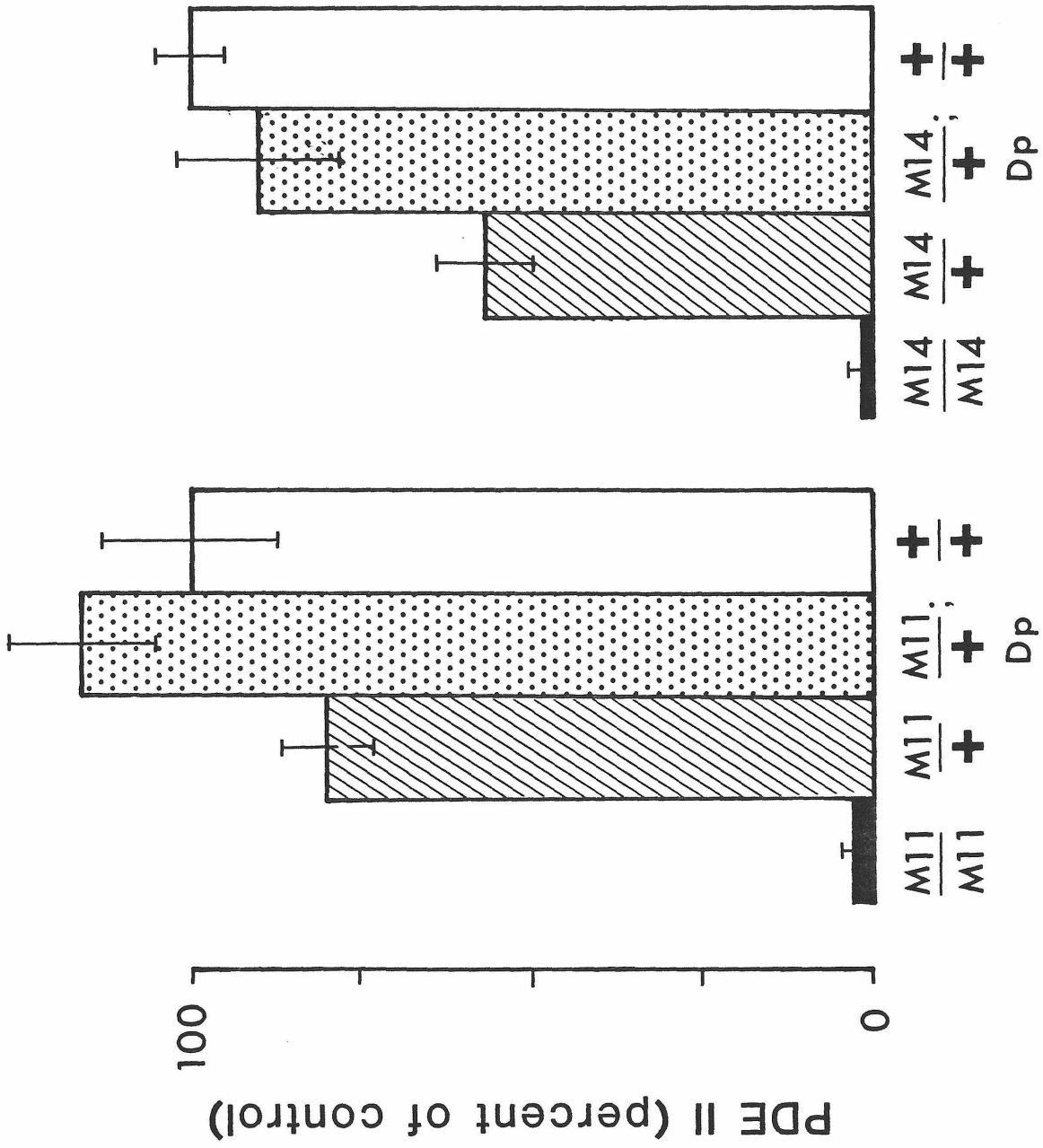

PDE II (percent of control)
100
0
M11/M11
M11/+
M11/+
Dp/+
M14/M14
M14/+
M14/+
Dp/+

Figure 7

**Figure 8.** The effect on learning of mutant and normal dunce genes, either separately or in combination. The learning of male flies with a dunce mutation (y dnc$^{M11 \text{ or } M14}$ cv v f/Y) was compared with that of control males (y cv v f/Y) and mutant males carrying an additional copy of the normal dunce gene [y dnc$^{M11 \text{ or } M14}$ cv v f/Y; Dp(1;2)w$^{+51b7}$]. Learning tests were carried out as described in Materials and Methods. Learning scores were normalized to those of control flies. Bars represent the mean, and error bars the S.E.M., of 3 experiments. Abbreviations: see the figure legend for Figure 6.

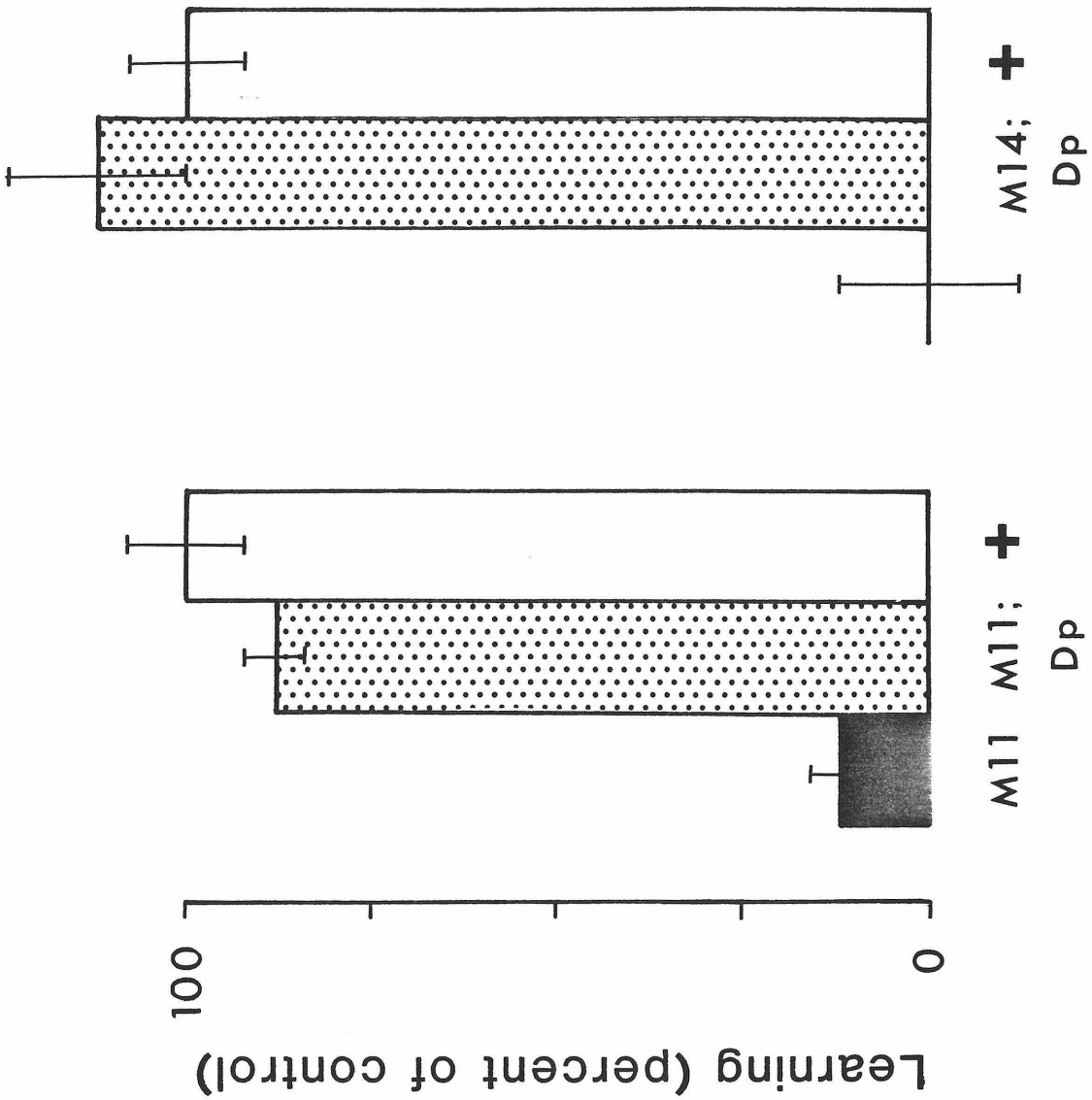



Figure 8

**Figure 9.** Gene dosage studies. The data presented here demonstrate the effect on PDE activities of varying the dosage of a small region of the X chromosome that contains the normal dunce gene, dunce$^+$. The genotypes of the flies used in these experiments are presented in Table 4. Sibling flies of the same genotype and age (3 to 5 days) were homogenized in groups of 2 or 3 and assayed in duplicate as described in Materials and Methods. PDE specific activities were compared to those of control siblings (dose of dunce$^+$ = 1.0) that were assayed in parallel. The results are presented as the mean $\pm$ the S.E.M. for n experiments. n = 4 for dose of dunce$^+$ = 0.0. n = 9 for dose of dunce$^+$ = 0.5. n = 7 for dose of dunce$^+$ = 1.5 or 2.0. The dashed lines indicate the PDE activity levels expected if dunce$^+$ encodes PDE II, but not PDE I.

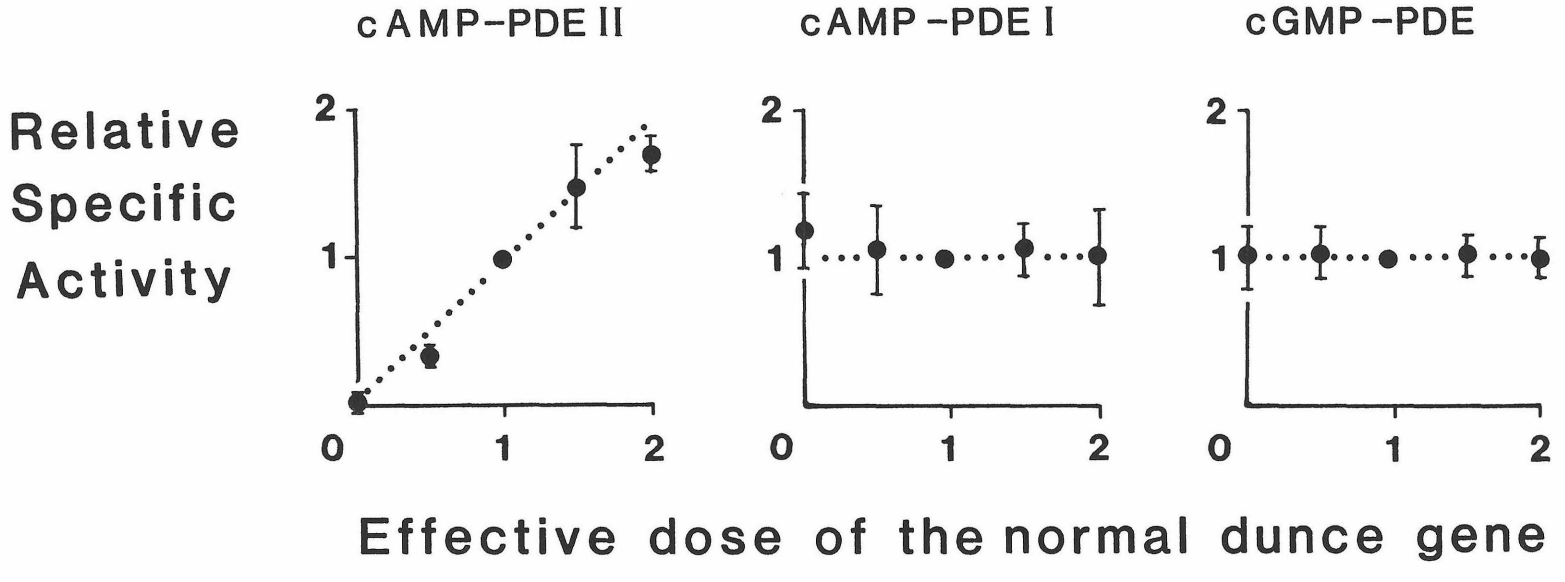
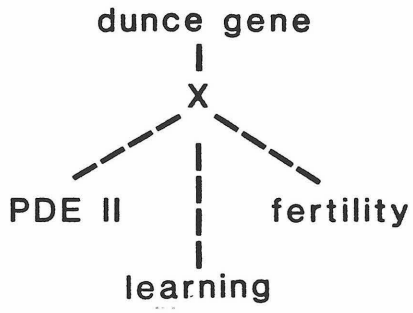

cAMP-PDE II
cAMP-PDE I
cGMP-PDE
Relative Specific Activity
2
1
0
1
2
Effective dose of the normal dunce gene

Figure 9

**Figure 10.** These logic diagrams represent several possible relationships between the three phenotypes associated with mutations in the dunce gene (reduced PDE II activity, poor learning, and female sterility). X and Y represent unknown activities. Dashed lines are used to indicate possible intermediate steps.


dunce gene
X
PDE II
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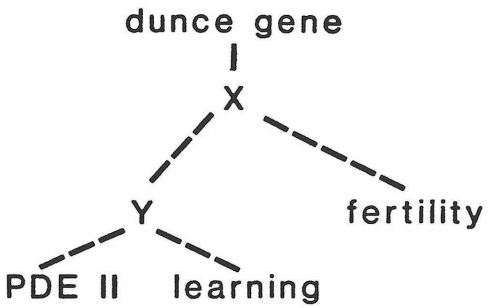
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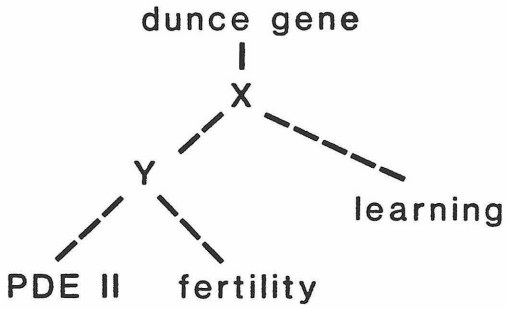
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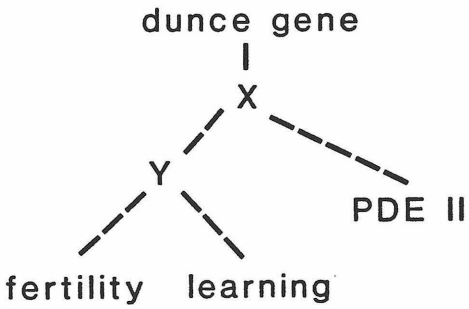
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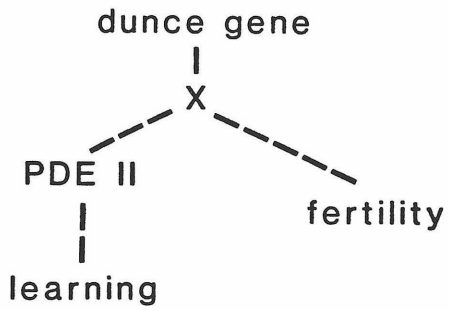
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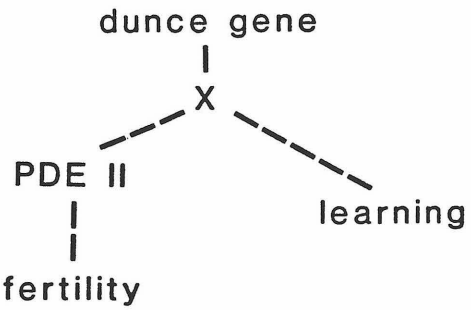
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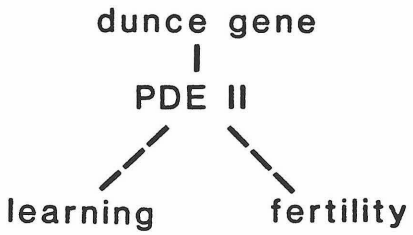
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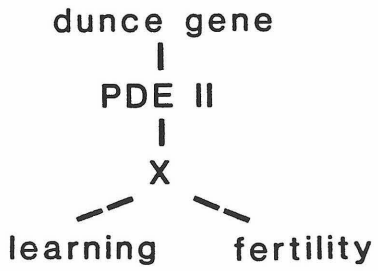
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Figure 10

**Figure 11.** Thermolability of PDE II activity. Three 4 to 5 day old flies of a given genotype were homogenized as described in Materials and Methods, and aliquots were incubated in a 43°C water bath for the indicated length of time. Aliquot tubes then were rapidly cooled in an ice water bath, and assayed at 30°C for PDE II activity as described. Each point represents the average of duplicate assays.

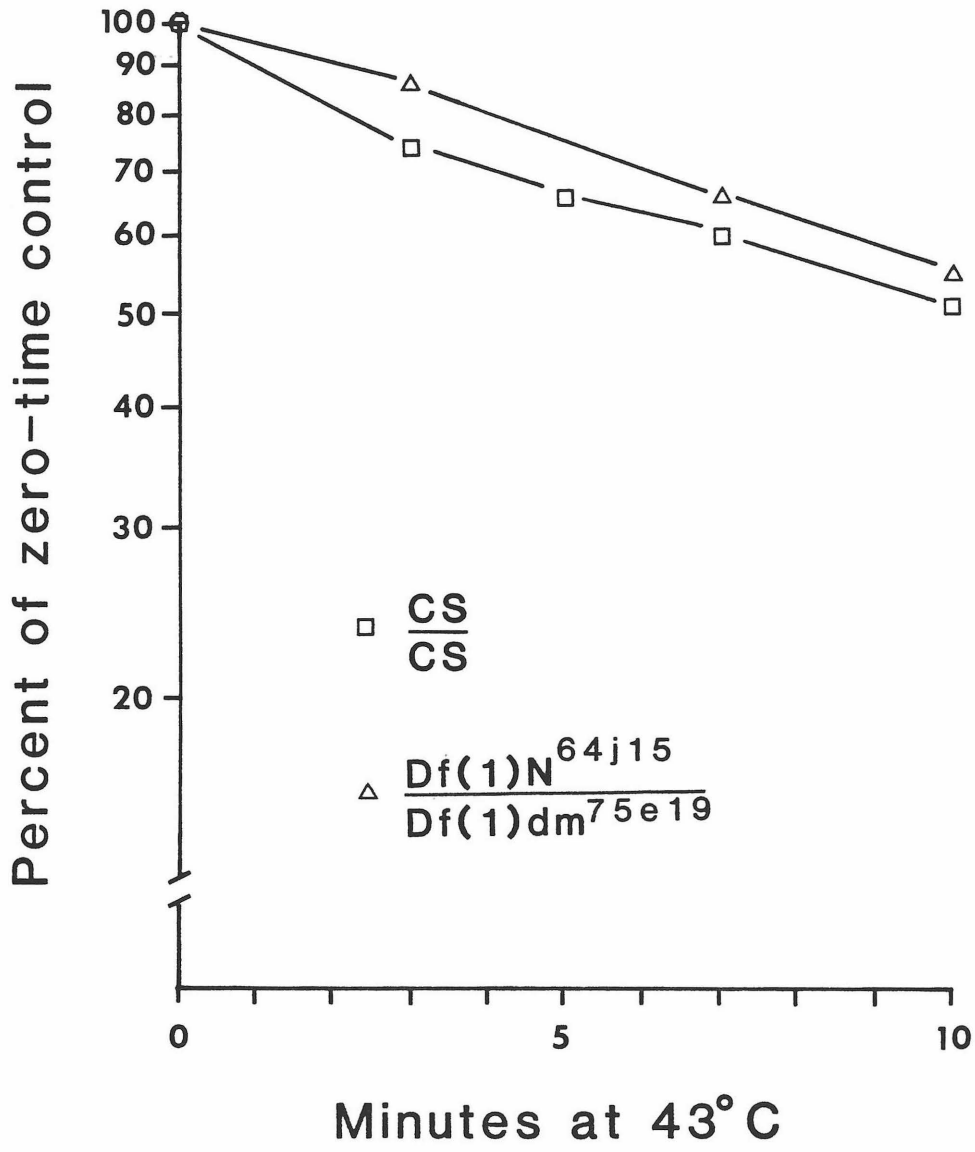
Percent of zero-time control
100
90
80
70
60
50
40
30
20
CS / CS
Df(1)N64j15 / Df(1)dm75e19
0
5
10
Minutes at 43°C

Figure 11

# Appendix

## DIRECTIONAL SENSITIVITY OF INDIVIDUAL VERTEBRATE HAIR CELLS TO CONTROLLED DEFLECTION OF THEIR HAIR BUNDLES*

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### INTRODUCTION

Among the most striking and consistent morphological features of vertebrate hair cells is the geometrical arrangement of their mechanosensitive organelles, the hair bundles. These structures each consist of 30–200 microvilluslike stereocilia and a single, eccentrically placed, axonemal kinocilium. Three geometrical features of hair bundles are widespread, if not universal. First, the stereocilia and kinocilium are inserted into the cellular apex in a regular, hexagonal array. Second, the lengths of stereocilia increase monotonically from one edge of the hair bundle to the other, but are approximately equal within a row of stereocilia across the hair bundle. Finally, the kinocilium is located at the edge of the hair bundle at which the longest stereocilia occur. Distortions of these features may occur; for example, the spacing of stereocilia is not absolutely uniform in some hair cells, but varies from one edge of the hair bundle to the other. Hair bundles become progressively distorted and asymmetrical as the apex of the mammalian cochlea is approached.[1] The kinocilium, although present in ontogeny, is lost from some hair cells in mammalian cochleas.[2] Even in these exceptional cases, however, the general pattern of arrangement of the hair bundle is evident.

A consequence of this organization is that the hair bundle possesses a plane of mirror symmetry. This plane is perpendicular to the apical surface of the hair cell and runs through the kinocilium and along the cell from the edge with the longest stereocilia to that with the shortest. It is parallel with one of the three axes of hexagonal symmetry defined by the insertions of the stereocilia and kinocilium at the hair cell's apex. Lowenstein and Wersäll suggested a coincidence of the plane of symmetry and the axis of sensitivity of hair cells;[3] movement of the hair bundle's distal tip toward the kinocilium was found to produce increased firing in the eighth nerve, while oppositely directed motion inhibited firing. A similar correspondence was found during extracellular recordings of microphonic potentials in other acousticolateralis organs in which the orientation of stimulation was reasonably well known.[4,5] Movement of the hair bundle's distal tip toward the kinocilium was shown by intracellular recordings[6] to produce a depolarizing receptor potential, while opposite movement elicited a small hyperpolarization. The response of hair cells to stimuli directed at various angles to the plane of mirror symmetry was inferred from microphonic recordings in the lateral-line organ;[7] for relatively small stimuli, the response declined from its

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maximal value with on-axis stimulation as the cosine of the angle by which the stimulus orientation deviated from that axis.

We report here observations on the directional sensitivity of the response to hair bundle displacement. Our results confirm the cosine relationship for responses to stimuli of small amplitude and show this to be a consequence of the hair cell's general insensitivity to displacements of its hair bundle perpendicular to the plane of symmetry.

## Materials and Methods

All experiments were performed with hair cells in saccular maculi from bullfrogs (*Rana catesbeiana*) of both sexes. Organs dissected from animals 90–150 mm in snout-vent length were maintained at pH 7.2–7.3 in a saline solution containing 113 mM $Na^+$, 2 mM $K^+$, 4 mM $Ca^{2+}$, 123 mM $Cl^-$, 3 mM *D*-glucose, and 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid. The otolithic membrane overlying the hair bundles was removed by dissection, usually after loosening by mild proteolysis.[8] Experiments were done under a Zeiss WL microscope with differential interference contrast (Nomarski) optics and at a total magnification of 800×. Preparations maintained at 22°C in room air yielded satisfactory responses for at least four hours.

Mechanical stimuli were usually applied directly to hair bundles by fine glass capillaries slipped over their distal ends (FIGURE 1). Ideal stimulus probes had inner diameters of 1.5–2.5 μm and encompassed the most distal 1–2 μm of the hair bundles. A few experiments were done using blunt, adhesive stimulus probes attached directly to kinocilia.[9] This method produced generally similar results. Probes were oriented such that their tips moved in a plane parallel with the epithelium's apical surface. They were displaced with micromanipulators consisting of two perpendicularly mounted piezoelectric bimorph elements linked by balsa-wood struts.[10] The axis along which a probe moved could be continuously varied through 360° by control of the fraction of the stimulus voltage applied to each of the bimorphs. Stimulus amplitudes were calibrated against an eyepiece micrometer; stroboscopic illumination was sometimes employed to sharpen the image of an oscillating stimulus probe. For amplitudes up to ±4 μm and within an observational uncertainty of about ±0.1 μm, the stimulus amplitude was found to vary linearly with the driving voltage and to be independent of angular orientation. With the optical system employed, the angular orientations of stimuli with respect to the planes of symmetry of hair cells could be estimated to about ±5°. The data presented herein were taken using static displacements and 10-Hz, triangle-waveform oscillatory stimuli.

Intracellular recordings were made with 3 M KCl-filled microelectodes approximately 250 MΩ in resistance. The electrodes were bent[11] to allow their introduction under the short-working-distance, 40×, water-immersion objective lens. Cells from which data were analyzed had resting potentials of −45 to −60 mV that were stable for at least 10 minutes; receptor potentials elicited by saturating stimuli were 5–24 mV in peak-to-peak amplitude. Data taken from a total of 51 hair cells in 13 animals were stored with an FM tape recorder. The records shown in FIGURES 3, 4, 5, and 7 each represent averages of 8–32 consecutive responses. The sensitivities of hair cells to various stimuli were determined by plotting each receptor potential against the deflection producing it and measuring the greatest slope of the resultant curve.

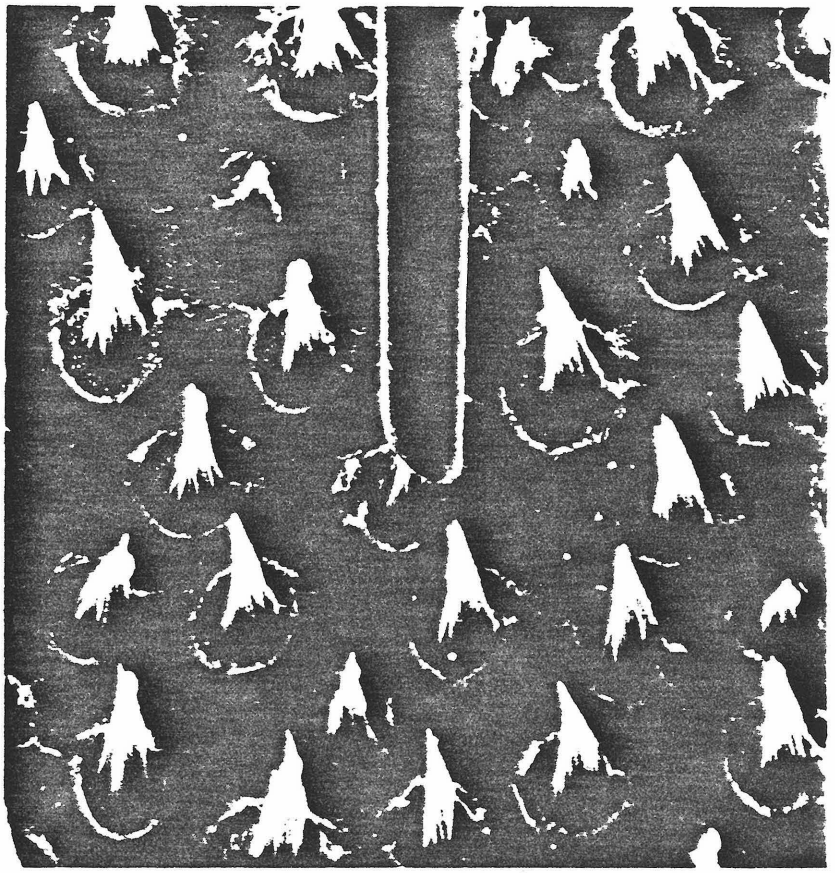

FIGURE 1. Scanning electron micrograph of the apical surface of a bullfrog's saccular epithelium. The hair bundles of several hair cells are shown, one of which is engaged by a capillary-type stimulus probe about 2 μm in internal diameter. The supporting cells between the hair cells are overlain by matted remnants of the otolithic membrane in this preparation, which was not subjected to proteolytic digestion. ×2300.

In order to describe the responses of hair cells to various stimuli and to investigate the geometrical arrangement of their hair bundles, it is convenient to define a coordinate system in which stimuli occur (FIGURE 2). Because every vertebrate hair cell ordinarily possesses only one kinocilium (or its remnant after regression), a convenient and unambiguous origin for the coordinate system is the site of insertion of the kinocilium into the apical cellular surface. The x-axis of the coordinate system runs along the hair cell's apical surface in the plane of mirror symmetry of the cell; the positive x-direction (in Cartesian coordinates) or 0° (in polar coordinates) is that in which the kinocilium stands with respect to the rest of the hair bundle. This polarity convention accords with that of other investigators.[7] The y-axis lies within the plane of the cellular apex and perpen-

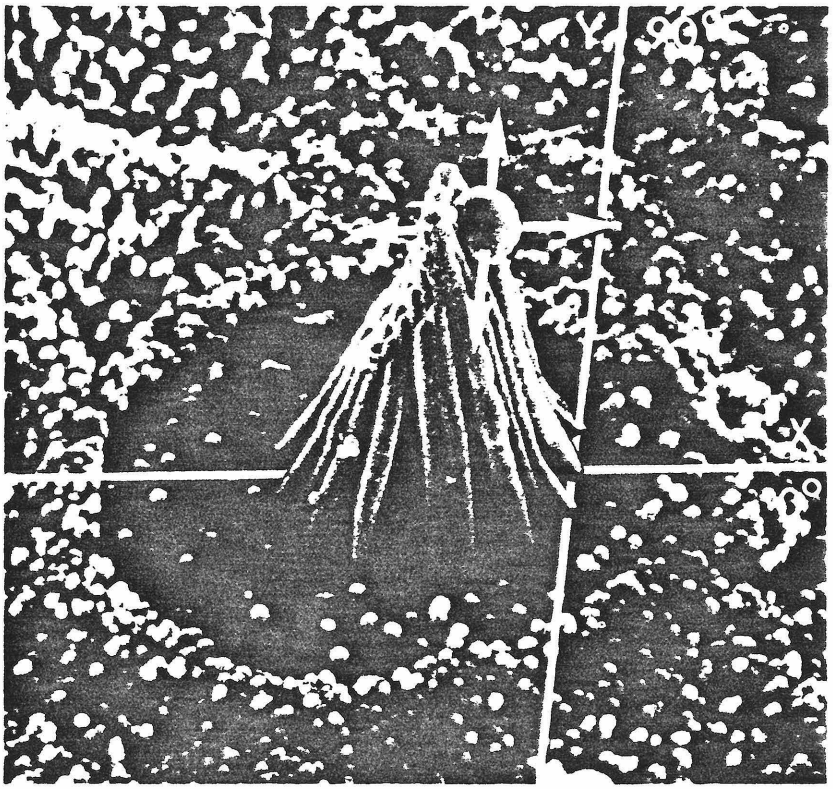


FIGURE 2. A scanning electron micrograph of the hair bundle on the apical surface of a large hair cell. Note that the stereocilia grow progressively longer from the left edge of the hair bundle to the right extreme, at which the kinocilium occurs. The superimposed lines represent the x-axis (0°), which lies at the intersection of the plane of mirror symmetry of the cell and the plane of the apical epithelial surface, and the y-axis (90°), which runs perpendicular to the x-axis and within the plane of the epithelial surface. The origin of the coordinate system is at the kinocilium's base; the z-axis runs through this origin and perpendicular to the cell's surface. Arrows emanating from the bulbous tip of the kinocilium represent positive and negative deflections parallel with the x- and y-axes. The otolithic membrane was removed from this preparation by proteolytic digestion. ×9000.

dicular to the x-axis; by the standard Cartesian-to-polar transformation, the positive y-axis corresponds to 90°. The z-axis is perpendicular to the other axes, and the positive z-direction is from the base toward the apex of the hair bundle.

The distal end of the hair bundle, at which stimuli are applied, is conveniently described during experiments by the position of the kinocilium's bulbous tip. For large hair cells in the bullfrog's sacculus, the center of this bulb lies about 6.9 $\mu$m above the epithelial surface and approximately 1.2 $\mu$m in the negative x-direction. Since the stereocilia and kinocilium are rather stiff elements that pivot near their basal insertions, the tip of each process actually sweeps through an arc as the hair bundle is deflected. The amplitudes of the stimuli employed in the present study—less than 1.5 $\mu$m along any axis—involve motions of the

kinocilium through an angular range of under ±12°. The height of the kinociliary bulb above the epithelial surface therefore changes by only 0.1 μm, or under 2%, for the largest stimuli; the motion of the hair bundle's distal tip thus essentially lies within a plane.

Sacculi destined for histological study were dissected, mounted, and maintained as those used in electrophysiology. They were then fixed for 60 minutes at 4°C in 40 mM $OsO_4$ and 10 mM $CaCl_2$ buffered to pH 7.3 with 80 mM sodium cacodylate.[9] After dehydration in ethanol, critical-point drying from liquid $CO_2$, and gold sputter-coating, specimens were observed and photographed in a scanning electron microscope operated at 20 keV.

## Results

Deflection of the distal tip of a hair bundle within the hair cell's plane of mirror symmetry produces receptor potentials of a form reported previously[6] (Figure 3). Motion in the positive x-direction produces a depolarization whose amplitude is graded with stimulus strength and reaches 4–20 mV. Deflection in the negative x-direction yields a graded hyperpolarization of about one-fifth this amplitude. Much of the variability in the peak amplitude of responses from cell to cell stems from damage sustained upon microelectrode penetration; hair cells studied with favorable electrodes consistently yield responses about 15 mV in peak-to-peak amplitude. Moreover, the displacement-response curve, which relates the instantaneous receptor potential with stimulus probe position, is highly consistent from cell to cell. This relationship is roughly sigmoidal, with the displacements encompassing 90% of the full response range characteristically separated by about 0.3 μm.

Stimulation parallel with the y-axis, at 90° to the hair cell's plane of mirror symmetry, produces little or no receptor potential (Figure 3). If the hair bundle is

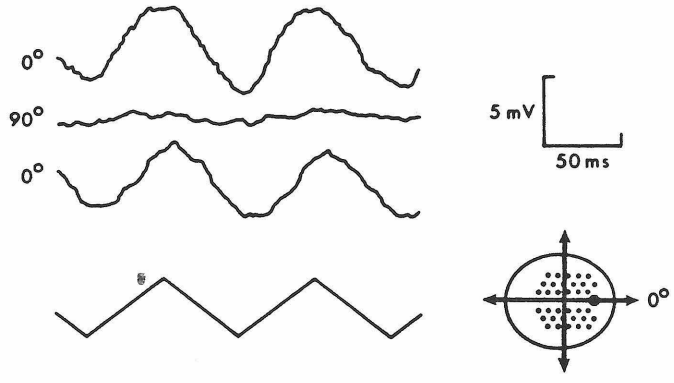


Figure 3. Averaged intracellular potentials from a hair cell stimulated by deflections of its hair bundle parallel with the x-axis (0°) and perpendicular to it (90°). The former yield responses that saturate in both the depolarizing and hyperpolarizing directions; the latter, no significant receptor potential. The responses have been vertically offset for clarity. A schematic representation of stimulus motion with respect to the hair bundle's orientation is shown at the lower right of this and of most subsequent figures. The peak-to-peak amplitude of the 10-Hz, triangle-waveform stimulus is 0.6 μm.

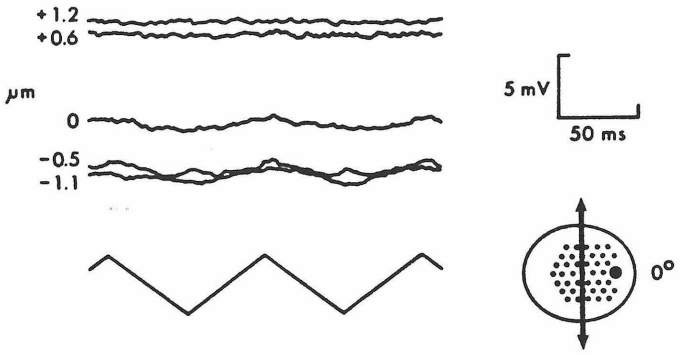


FIGURE 4. Intracellular responses from a cell whose hair bundle is moved parallel with the x-axis by the amounts indicated to the left of the responses, then held there while an oscillatory stimulus parallel with the y-axis is applied. The static deflections along the x-axis produce receptor potentials manifested by the offsets among the responses and totaling 13 mV. Movement of the hair bundle parallel with the y-axis by ±0.5 μm produces little (lower traces) or no response.

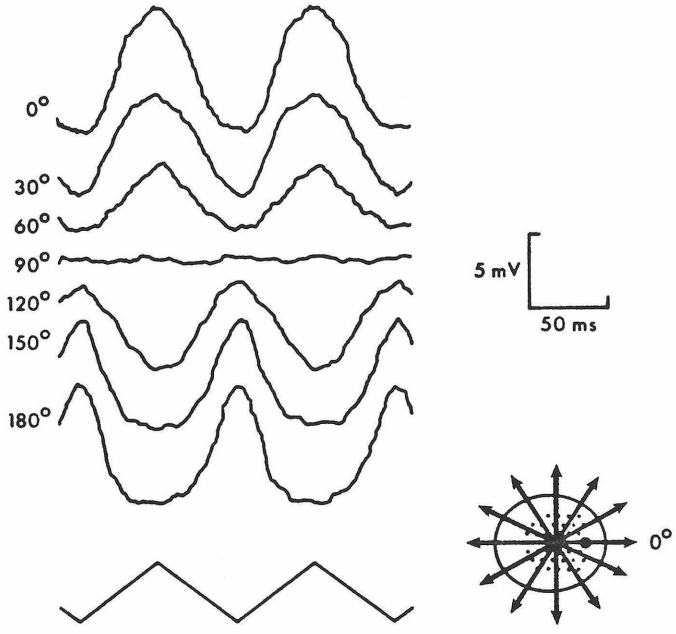


FIGURE 5. Receptor potentials elicited by 0.5-μm (peak-to-peak) stimuli directed at various angles with respect to the x-axis (0°). The traces have been vertically displaced for clarity. Note that both the peak response amplitude and the sensitivity of the cell, or greatest slope of the receptor-potential trace, decline from a maximum for stimulation parallel with the x-axis to zero at 90° to it.

pushed some distance in the positive x-direction and held there, a depolarizing receptor potential arises, then declines to a steady-state level after an adaptation process lasting a few hundred milliseconds.[12] If back-and-forth motion of the hair bundle parallel to the y-axis is now added to the sustained deflection, little additional response occurs (FIGURE 4). An analogous result ensues if a cell's hair bundle is first deflected in the negative x-direction, then stimulated parallel to the y-axis.

A small receptor potential at twice the stimulus frequency occasionally occurs in response to stimulation parallel with the y-axis (FIGURE 4, $-0.5$-$\mu$m trace). This is seen frequently when a derivatized glass probe is used to move the hair bundle, but rarely when the bundle is displaced with a hollow capillary tube

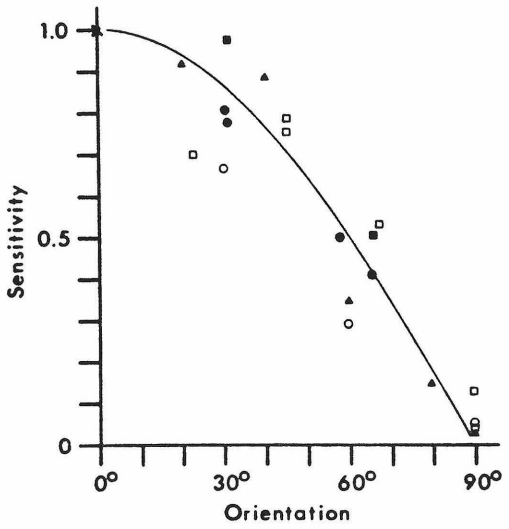


FIGURE 6. Plot of the sensitivities of hair cells to stimulation at various angles with respect to the plane of cellular mirror symmetry. For comparison among five cells (different symbols), the largest sensitivity of each cell has been normalized to unity, and other sensitivities are expressed as a fraction of this value. The continuous curve is the cosine of the angle by which stimuli differ from parallel with the cells' x-axes.

engaging the hair bundle's tip. Visual observation suggests that the hair bundle twists slightly about the vertical (z) axis when the bulbous tip of the kinocilium is pulled sideways by an adhesive probe. The component of motion along the x-axis could elicit a receptor potential, and should do so for movements in either direction, resulting in small responses at twice the stimulus frequency. Capillary stimulus probes, which apply force to a broad surface on the side of the hair bundle when driven parallel with the y-axis, presumably produce less torque than do adhesive probes, and accordingly seldom induce responses.

As the orientation of stimulus motion is varied from parallel with the x-axis to perpendicular to the x-axis, the amplitude of the receptor potential declines continuously from its maximum to essentially zero (FIGURE 5). At the same time, the sensitivity of the cell, measured as the greatest slope of the displacement-

response curve, declines from a maximum to a minimum. A plot of this slope (FIGURE 6) indicates that sensitivity decreases roughly as the cosine of the angle by which the stimulus orientation deviates from the x-axis.

The shape of the displacement-response curve is little affected by movements of the hair bundle perpendicular to the x-axis (FIGURE 7). The receptor potential

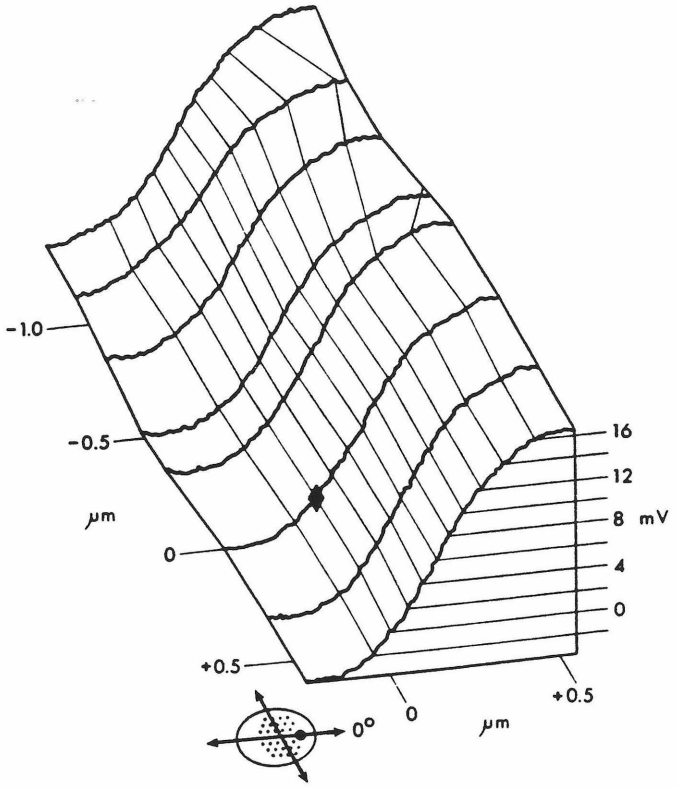


FIGURE 7. A family of displacement-response curves from a cell whose hair bundle is statically displaced by the various distances indicated parallel with the y-axis, then oscillated back and forth through 0.8 μm parallel with the x-axis. The rhomboid at the lower center of the figure represents the rest position of the kinociliary bulb before stimuli are applied. Note that the displacement-response relationships for stimulation parallel with the x-axis are similar to one another despite the fact that the distal end of the hair bundle is bent by as much as 11° to the side, parallel with the y-axis.

evoked by an oscillatory stimulus parallel with the x-axis is unaltered by additional, static displacements in either direction parallel with the y-axis.

## DISCUSSION

The relationship between the displacement of the tip of a hair bundle and the cellular receptor potential is largely described by FIGURE 7. The result shown in

this figure is implicit in the data of FIGURES 4 and 5: a similar displacement-response surface is defined by appropriate combinations of static and/or oscillatory stimuli along the x- and y-axes. Within the range of amplitudes tested, stimuli parallel with the x-axis produce displacement-response curves of similar amplitudes and slopes. Stimulation parallel with the y-axis has little or no effect on the response.

The portion of the displacement-response surface near the rest position of the kinociliary bulb (rhomboid in FIGURE 7) is essentially a flat sheet of constant slope. Stimuli confined to this region would be expected to yield responses whose amplitudes scale with the cosine of the angular deviation of the stimulus from parallel with the x-axis, in agreement with a previous study on the microphonic response of a lateral-line organ.[7] For stimuli of all sizes, even those producing saturation of the response, the greatest slope of the displacement-response curve should respect a cosine relationship with angular orientation; this prediction is supported by the data of FIGURE 6.

It should be noted that several factors act to make the apparent sensitivity of the hair cell—the slope of the displacement-response surface of FIGURE 7—less than it actually is. Since the time constant of bullfrog hair cells is about 10 mseconds in the present recording circumstances, responses to 10-Hz stimuli are significantly delayed and broadened. An adaptation process in the transduction mechanism[12] operates rapidly enough to diminish the size of responses. The coupling between stimulus probes and hair bundles is not perfect and doubtlessly exhibits some hysteresis between opposite phases of stimulation. Stimuli of saturating intensity significantly fatigue the transduction process. Finally, slow drift in the position of the stimulus probe and in the timing of the stimulating and recording apparatus acts to broaden the averaged receptor potentials displayed in the figures.

The present results demonstrate that vertebrate hair cells are not only very sensitive to displacement of their hair bundles within the plane of mirror symmetry, but also remarkably insensitive to stimulation orthogonal to this plane. The morphological feature of the hair bundle most conspicuously aligned with the cell's axis of sensitivity, the kinocilium, is not itself the source of the receptor potential.[9] Since the individual stereocilia appear to lack any structural element of polarization along the axis of sensitivity, it seems most likely that it is the tapered array of stereocilia of differing heights that confers directional sensitivity upon the hair bundle. Whether this comes about through the mechanics by which forces are transmitted to a transducer located at the cellular apex, or by interactions among the distal ends of the tapered stereocilia themselves, remains to be determined.

## ACKNOWLEDGMENT

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