dunce Mutants of *Drosophila melanogaster*: Mutants Defective in the Cyclic AMP Phosphodiesterase Enzyme System

RONALD L. DAVIS and JOHN A. KIGER, JR.
Department of Genetics, University of California, Davis, California 95616. Dr. Davis's present address is the Department of Chemistry, California Institute of Technology, Pasadena, California 91125.

ABSTRACT The cyclic AMP and cyclic GMP phosphodiesterase activities present in flies of six mutant strains of the *dunce* gene and in the parent wild-type strains are characterized. All of the mutants exhibit aberrant cyclic AMP metabolism. The mutant strains $dunce^{M14}$, $dunce^{M11}$, and $dunce^{ML}$ appear to be amorphic, because they completely lack the cAMP-specific phosphodiesterase normally present in adult flies. These strains exhibit extremely high levels of cAMP. The mutant strains $dunce^{1}$, $dunce^{2}$, and $dunce^{CK}$ are hypomorphic and exhibit reduced levels of the cAMP-specific phosphodiesterase. These strains exhibit less marked increases in cAMP content compared with the three amorphic strains. The $dunce^{2}$ strain possesses a residual enzyme activity that exhibits anomalous kinetics compared with those of the normal enzyme. The possibility that the *dunce* locus is the structural gene for the cAMP-specific phosphodiesterase is discussed.

Adult flies of the species *Drosophila melanogaster* have two major forms of cyclic nucleotide phosphodiesterase. Form I phosphodiesterase will use either cyclic AMP or cyclic GMP as substrate; form II is specific for cyclic AMP (1). The activity of form II enzyme is controlled in a dosage-dependent manner by chromomere 3D4 of the X chromosome (2). Deletion of chromomere 3D4 produces an associated phenotype of female sterility (3). This has made possible the identification of female-sterile mutations that influence form II activity (H. K. Salz, R. L. Davis, and J. A. Kiger, Jr., unpublished observations).

Several mutants of *D. melanogaster* are known that show deficiency in learning or memory (4–6). The first to be isolated is one named *dunce* (4). Whereas normal flies tend to avoid odorants after training in an apparatus that couples olfactory cues with electrical shock (7), *dunce* mutant flies are defective in associative avoidance and are classed as learning deficient. Recently, allelism between *dunce* mutations and the female-sterile mutations located at chromomere 3D4 was established (8). The allele $dunce^{2}$ is a recessive female-sterile (8), permitting allelism to be determined with the female-steriles in 3D4 through complementation tests. Crude homogenates of flies homozygous for the $dunce^{1}$ or $dunce^{2}$ alleles were shown to have reduced capacity to hydrolyze cyclic AMP, and form II phoshodiesterase was virtually undetectable in homogenates of these mutant flies after velocity sedimentation in sucrose gradients (9).

We describe here a further characterization of the cyclic nucleotide phosphodiesterases in six mutant alleles of the *dunce* locus. Crude homogenates of all six mutants have reduced cyclic AMP, but not cyclic GMP, phosphodiesterase activity. When analyzed by velocity sedimentation, the soluble fractions from all six mutants show form II activity to be either reduced or missing as compared with the appropriate control. Particulate cyclic AMP phosphodiesterase activity is also reduced in the two mutants tested. And, with one possible exception, all of the mutants have elevated cyclic AMP levels.

MATERIALS AND METHODS

Materials

Two of the *dunce* alleles analyzed here were produced in a Canton-S stock by S. Benzer's group (4). These have been named $dunce^{1}$ and $dunce^{2}$. The mutants $dunce^{M14}$ and $dunce^{M11}$ were isolated by J. Dawson Mohler (10) on the basis of female sterility and were subseqently shown to be allelic to $dunce^{1}$ and $dunce^{2}$ (8; Salz et al., unpublished observations). These two mutants were induced in an X chromosome carrying the visible markers *yellow* (*y*, 1-0.0), *crossveinless* (*cv*, 1-13.7), *vermilion* (*v*, 1-33.0), and *forked* (*f*, 1-56.7). Two other *dunce* mutants were generated in a chromosome carrying the visible mutations *y*, *white* (*w*, 1-1.5), and f^{36a}. The latter two were selected in our laboratory. The mutant $dunce^{CK}$ was selected on the basis of female sterility (Salz et al., unpublished observations), and $dunce^{ML}$ by virtue of low cyclic AMP phosphodiesterase activity (M. L. Kipps and J. A. Kiger, Jr., unpublished observations). All of the mutants were induced with ethylmethane sulfonate except $dunce^{CK}$, which was induced with x rays. The six *dunce* mutants fail to complement one another with respect to

THE JOURNAL OF CELL BIOLOGY · VOLUME 90 JULY 1981 101-107

female sterility (Salz et al., unpublished observations), indicating that they all affect the same gene. Moreover, they have similar biochemical anomalies (this report). However, *dunce*^{ML} and *dunce*^{CK} have not yet been tested for learning ability. The unmutagenized parent stock, either Canton-S, *y cv v f*, or *y w f*^{36a}, serves as the control for the corresponding *dunce* mutants. Adult female flies, 0–4 d posteclosion, were used here. The two X-chromosome deficiencies, *Df(1)dm*^{75e19} and *Df(1)N*^{64i16}, are described in detail in reference 2.

The sources for most of the materials used in this study are described in reference 1. Cyclic AMP radioimmunoassay kits were purchased from New England Nuclear, Boston, Mass. Ultrapure sucrose was obtained from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.

Preparation of Crude Homogenates and Soluble and Particulate Fractions

Flies were homogenized in one of three buffers given below at 0°C in a glass mortar with 10–15 strokes of a motor-driven Teflon pestle. The homogenate was used directly for cyclic nucleotide phosphodiesterase assay or was separated into soluble and particulate fractions as previously described (1).

Cyclic Nucleotide Phosphodiesterase Assay

The two assay methods for cyclic nucleotide phosphodiesterase activity used here are described in reference 1. The first employs chromatography on Whatman 3MM paper for the separation of substrate from the 5′-nucleoside monophosphate product of the phosphodiesterase reaction. In the second, the 5′-nucleotide product of phosphodiesterase is converted to adenosine or guanosine by 5′-nucleotidase activity present in snake venom added before a second incubation step. The nucleoside is then separated from remaining cyclic nucleotide substrate by batchwise treatment with anion exchange resin.

The second assay was used for gel chromatography and velocity sedimentation experiments, and the first assay for all others.

Cyclic Nucleotide Phosphodiesterase Activity in Crude Homogenates and Particulate Material

To measure phosphodiesterase activity in crude homogenates, adult flies were homogenized in groups of eight, in 1 ml of a solution containing 80 mM Tris·HCl, pH 8.0, 20 mM $MgCl_2$, and 4 mM 2-mercaptoethanol. A 0.05-ml volume of each homogenate was assayed with 0.05 ml of 0.2 mM substrate prepared in water. Thus, the reaction mixture came to 0.10 ml and contained all components listed above at half their concentrations.

Particulate material was prepared using 40 mM Tris·HCl, pH 8.0, 10 mM $MgCl_2$, and 2 mM 2-mercaptoethanol as buffer. Flies were homogenized in 25-mg groups. 0.05 ml of each particulate preparation was assayed with 0.05 ml of substrate prepared in buffer.

Velocity Sedimentation

Linear 5–20% sucrose gradients with a total volume of 4.8 ml were prepared in 40 mM Tris·HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM $CaCl_2$, and 2 mM 2-mercaptoethanol in cellulose nitrate centrifuge tubes. Frozen (−80°C) flies were homogenized in the same buffer at 100 mg/ml, the 105,000-*g* supernate was prepared, and 0.10 ml of the supernate was layered on the preformed gradients. The gradients were centrifuged at 50,000 rpm in a Beckman 50.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 12 h at 1°–3°C. After centrifugation, the gradients were fractionated into equal-volume fractions (usually 36–37) by pumping the gradient from the bottom of each tube. A 0.03-ml volume of each appropriate fraction was assayed against 0.01 ml of substrate prepared at 0.2 mM in 40 mM Tris·HCl, pH 8.0, 10 mM $MgCl_2$, and 2 mM 2-mercaptoethanol. Recoveries of enzyme activities were determined from the soluble supernate diluted to 4 mg/ml.

Gel Filtration

For chromatography on Sephadex G-150, 2.9 ml of a soluble supernate in 40 mM Tris·HCl, pH 8.0, 10 mM $MgCl_2$, and 2 mM 2-mercaptoethanol from about 1 g of flies was applied to a 2.2 × 97 cm column equilibrated in the same buffer. 2.8-ml fractions were collected with a flow rate of 6 ml/h. A 0.05-ml aliquot from each appropriate fraction was assayed with 0.05 ml of substrate at 0.2 mM prepared in equilibration buffer. The recoveries of enzyme activities were determined from the activity in the starting material diluted to ~4 mg/ml. Fractions indicated in the text have been pooled, concentrated by Amicon ultrafiltration (CF 25; Amicon Corp., Scientific Systems Div., Lexington, Mass.), and stored in convenient aliquots at −80°C.

Cyclic AMP Radioimmunoassay

Cyclic AMP levels were determined by radioimmunoassay developed by New England Nuclear as previously described (11).

Protein Determination

Protein was assayed with a modified Lowry procedure (12).

RESULTS

Cyclic Nucleotide Phosphodiesterase Activity in Crude Homogenates

The rates of cyclic AMP and cyclic GMP hydrolysis by crude homogenates of the six *dunce* alleles and their respective controls are displayed in Table I. Each mutant homogenate hydrolyzes cyclic AMP at a rate significantly slower than that of the appropriate control homogenate. Some mutants are more severely affected than others with respect to cyclic AMP hydrolysis. The mutants *dunce*^{1}, *dunce*^{2}, and *dunce*^{CK} show 73, 62, and 57% of the control activity, confirming previously reported estimates for the first two (9). These mutants appear mildly affected compared with the mutants *dunce*^{ML}, *dunce*^{M14} and *dunce*^{M11}, which, in order, have only 35, 26, and 28% of the cyclic AMP phosphodiesterase activity found in the appropriate control.

In contrast, cyclic GMP phosphodiesterase activity in the same homogenates is unaffected. Under the conditions employed, none of the mutants has a rate of cyclic GMP hydrolysis different from the control at the 5% significance level.

We conclude from these data that total cyclic AMP phosphodiesterase activity is reduced in *dunce* fly homogenates. To determine which, if either, of the two soluble forms of cyclic nucleotide phosphodiesterase is affected by mutation of the *dunce* gene, we have subjected mutant and control supernates to velocity sedimentation in sucrose gradients.

TABLE I

Rate of Cyclic Nucleotide Hydrolysis by Crude Homogenates

Genotype	Cyclic AMP hydrolysis	Cyclic GMP hydrolysis
Canton-S	165 ± 4	93 ± 4
dunce^1	120 ± 3	104 ± 3
dunce^2	102 ± 2	86 ± 2
y w f^{36a}	179 ± 2	114 ± 4
dunce^{ML}	62 ± 4	101 ± 3
dunce^{CK}	102 ± 2	117 ± 4
y cv v f	196 ± 6	95 ± 5
dunce^{M14}	50 ± 2	80 ± 4
dunce^{M11}	54 ± 4	93 ± 3

Rates are expressed as picomoles of cyclic nucleotide hydrolyzed per minute of incubation per milligram of fly plus or minus one standard error of the mean. Six homogenates of each genotype were assayed at 100 μM substrate. Data for each of the three groups, representing three different genetic backgrounds, have been evaluated statistically by one-way analysis of variance with subsequent computation of contrasts by Tukey's method as described by Scheffé (13). None of the mutants under investigation show mean values significantly different from the appropriate control at the 5% level with respect to cyclic GMP hydrolysis. All of the mutant strains have rates of cyclic AMP hydrolysis significantly different from their control at the 1% level. In addition, *dunce*^{1} and *dunce*^{2} have significantly different rates of cyclic AMP hydrolysis at the 1% level, as do *dunce*^{ML} and *dunce*^{CK}.

Velocity Sedimentation of Soluble Cyclic Nucleotide Phosphodiesterase Activity

GENERAL COMMENTS: We have previously analyzed the soluble cyclic nucleotide phosphodiesterase activity from flies deficient for salivary gland chromosome bands 3D3-3D4 (2) and from the two alleles *dunce*^{1} and *dunce*^{2} (9) by the technique of velocity sedimentation in sucrose gradients. Deficiency flies and the two *dunce* alleles show a reduction or absence in form II cyclic AMP phosphodiesterase activity relative to their respective control genotypes. This section presents sucrose gradient profiles of soluble cyclic nucleotide phosphodiesterase activity from the six mutants under investigation and their controls, and from flies lacking X-chromosome bands 3C12–3D4.

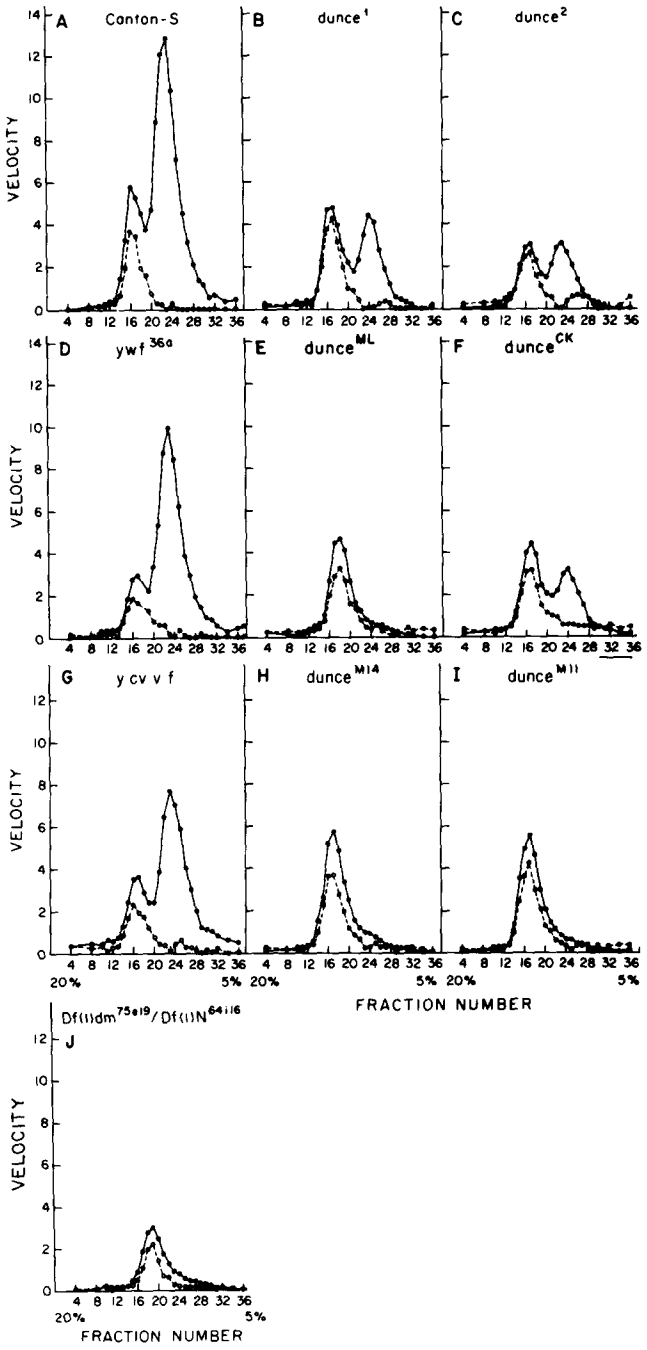

FIGURE 1 Velocity sedimentation of soluble cyclic nucleotide phosphodiesterase from *dunce* flies and their controls. (*A–C*) Control Canton-S, *dunce*^{1}, and *dunce*^{2}. (*D–F*) Control *y w f* ^{36a}, *dunce*^{ML}, and *dunce*^{CK}. (*G–I*) Control *y cv v f*, *dunce*^{M14}, and *dunce*^{M11}. *J* is a profile of phosphodiesterase activity from adult flies deficient for salivary gland chromosome bands 3C12-3D4, made with the overlapping deficiencies *Df(1)dm* ^{75e19} and *Df(1)N* ^{64i16}. Fractions of the gradients were assayed at 50 μM cAMP (●) and 50 μM cGMP (○). Velocities are expressed as picomoles of cyclic nucleotide hydrolyzed per minute of incubation per milligram of fly loaded on the gradient per fraction. The total number of units of cAMP phosphodiesterase activity in the supernates and the recovery of

The sucrose density gradient profiles (Fig. 1) from the three control genotypes, Canton-S, *y w f*^{36a}, and *y cv v f*, show two prominent forms of cyclic nucleotide phosphodiesterase. The larger of the two forms (form I) appears to hydrolyze both cyclic AMP and cyclic GMP. The smaller (form II) is specific for cyclic AMP. We have previously provided evidence that form I phosphodiesterase hydrolyzes both cyclic AMP and cyclic GMP at the same active site (1). The existence of a minor form is suggested by the small peak of cyclic GMP phosphodiesterase activity between fractions 24–28 and the usual trail of cyclic AMP phosphodiesterase activity present in some of the gradients. This activity may correspond to the minor form previously noted on some occasions (1).

The recovery of enzyme activities from the gradients has been disappointingly variable, even though all experiments were done in identical fashion. For instance, the recovery of cyclic GMP phosphodiesterase activity varies between 40 and 65% for the three control genotypes. Nevertheless, because the mutants under study drastically affect cyclic AMP phosphodiesterase activity, reliable conclusions can be drawn from the gradient profiles.

CANTON-S, *dunce*^{1} AND *dunce*^{2}. The amount of soluble cyclic nucleotide phosphodiesterase activity found in supernates of Canton-S, *dunce*^{1}, and *dunce*^{2} before fractionation is presented in the legend of Fig. 1. The supernates contain 100, 38, and 43 U of cyclic AMP phosphodiesterase activity, respectively. Thus, cyclic AMP phosphodiesterase activity appears reduced in supernates of the mutants as well as crude homogenates. The mutants do not have detectably altered soluble cyclic GMP phosphodiesterase activity.

The velocity sedimentation profiles of soluble cyclic nucleotide phosphodiesterase activity from the three stocks are shown in Fig. 1*A–C*. It is evident that both mutants have two major cyclic AMP phosphodiesterase activities. However, the activity of form II from *dunce*^{1} and from *dunce*^{2} is clearly reduced compared with the Canton-S control. Differential recovery of cyclic AMP phosphodiesterase activity from the gradients cannot account for the reduction of form II activity from these mutants. Indeed, even if the 18–28% loss of cyclic AMP phosphodiesterase activity found after fractionation of *dunce*^{1} and *dunce*^{2} supernates is attributable only to a loss in form II activity, the mutants still have no more than 40% of the same activity found in the Canton-S control. The obvious presence of form II activity in *dunce*^{1} and *dunce*^{2} supernates as displayed in Fig. 1*B* and *C*, is in contrast to the virtual absence of this

cAMP phosphodiesterase activity from the gradients (in parentheses) were determined to be (for *A–J*, respectively), 100 (96%), 38 (82%), 43 (72%), 93 (77%), 36 (80%), 45 (96%), 61 (113%), 48 (76%), 49 (68%), and 26 (79%). Similarly, the values for cGMP phosphodiesterase activity are 27 (59%), 28 (80%), 30 (64%), 33 (40%), 29 (78%), 28 (95%), 26 (65%), 35 (66%), 39 (65%), and 24 (49%). 1 U of activity is defined to be 1 pmol of cyclic nucleotide hydrolyzed/min of incubation per mg of fly.

activity from other gradients of these mutant supernates (9). Apparently, the increased supernate concentration and shorter centrifugation times used here are important factors for recovering this relatively unstable (1, 2) form of activity.

It is difficult to make accurate quantitative comparisons of form I phosphodiesterase activities in the three stocks. Note that the ratio of cyclic AMP to cyclic GMP hydrolysis by this activity is reduced in the mutants relative to Canton-S. This could be a function of mutation but could also be attributed to aggregation of the form II Canton-S enzyme and cosedimentation of this aggregate with the form I phosphodiesterase activity. The form I activity does sediment at a position corresponding to that of about twice the molecular weight of form II (1). Consequently, the apparent reduction in form I cyclic AMP phosphodiesterase activity in *dunce*^{1} and *dunce*^{2} relative to Canton-S need not be an effect of mutation of the *dunce* gene on form I activity. Cyclic GMP phosphodiesterase activity is a better marker for this enzyme than is cyclic AMP phosphodiesterase activity. However, the occasional appearance of a low molecular weight cyclic GMP phosphodiesterase activity in *dunce*^{1} and *dunce*^{2} profiles between fractions 24–28 complicates the use of cyclic GMP phosphodiesterase activity as a specific marker for quantity and recovery of the large form of enzyme. This minor cyclic GMP phosphodiesterase is presumably not the result of mutation at the *dunce* locus, because *y cv v f* flies also show this form (Fig. 1 *G*). We conclude that mutation at the *dunce* locus probably does not affect the large form of activity, because cyclic GMP phosphodiesterase activity in *dunce* crude homogentates and supernates are not significantly different from the control.

y w f^{36a}, *dunce*^{ML}, AND *dunce*^{CK}: The sucrose gradient profiles of cyclic nucleotide phosphodiesterase activity from the soluble fraction of the mutants *dunce*^{ML} and *dunce*^{CK}, and their control *y w f*^{36a}, are shown in Fig. 1 *D–F*. The *y w f*^{36a} control and *dunce*^{CK} show two separable forms of activity. The activity of form II from *dunce*^{CK} is clearly reduced, compared with *y w f*^{36a}. This form of activity from *dunce*^{CK} can account for no more than 35% of the recoverable low molecular weight activity from *y w f*^{36a}. The mutant *dunce*^{ML} shows only one form of cyclic nucleotide phosphodiesterase activity, that being form I, as evidenced by its sedimentation position and substrate specificity. Thus, *dunce*^{ML} appears to represent the amorphic state with respect to form II cyclic nucleotide phosphodiesterase activity, whereas *dunce*^{1}, *dunce*^{2}, and *dunce*^{CK} represent hypomorphic states.

Like *dunce*^{1} and *dunce*^{2}, the supernates of *dunce*^{ML} and *dunce*^{CK} have reduced cyclic AMP phosphodiesterase activity. Cyclic GMP phosphodiesterase activity in the supernates of the mutants and control are comparable. The relatively small peak height of *y w f*^{36a} form I activity can be accounted for by low recovery.

y cv v f, dunce^{M14}, AND *dunce*^{M11}: Fig. 1 *G–I* shows the sucrose gradient profiles of soluble cyclic nucleotide phosphodiesterase activity from *y cv v f, dunce*^{M14}, and *dunce*^{M11}. Neither mutant shows any form II activity. Both appear to be amorphs.

The *dunce*^{M14} and *dunce*^{M11} supernates have only slightly reduced cyclic AMP phosphodiesterase activity and increased cyclic GMP phosphodiesterase activity relative to *y cv v f*. However, this is a result of underestimating the amount of activity in the *y cv v f* supernate in the experiment presented. In a separate experiment, we have determined soluble cyclic AMP and cyclic GMP phosphodiesterase activity from *y cv v f* to be 101 and 41 U, respectively. If these numbers are used, the data become consistent with the other data. Soluble cyclic AMP phosphodiesterase activity is clearly reduced in *dunce*^{M14} and *dunce*^{M11} but cyclic GMP phosphodiesterase activity is about the same as in the control.

Df(1)dm^{75e19}*/Df(1)N*^{64i16}: The amorphic state of a locus occurs upon its deletion from the genome. Flies deficient for chromomeres 3D3-3D4, that region of the X chromosome that is responsible for the presence of form II phosphodiesterase (2), can be constructed using the overlapping deficiencies *Df(1) dm*^{75e19} and *Df(1)N*^{64i16}. Flies heterozygous for these two deficiencies are missing chromomeres 3C12-3D4 (3). The profile of cyclic nucleotide phosphodiesterase activity after sedimentation of the soluble fraction of these flies is shown in Fig. 1 *J*. Only the large form of cyclic nucleotide phosphodiesterase activity is obviously present. Cyclic AMP phosphodiesterase activity in the deficiency supernate may be lower than that found in the amorphic *dunce* alleles, but it is not clear whether this is attributable to the deficiency, genetic background, or some other cause.

ADDITIONAL COMMENTS: The results of sucrose gradient sedimentation studies are consistent with the measurement of cyclic nucleotide phosphodiesterase activity in crude homogenates. The mutants *dunce*^{ML}, *dunce*^{M14}, and *dunce*^{M11} have severely reduced cyclic AMP phosphodiesterase activity in their crude homogenates. These three mutations show only one form of cyclic AMP phosphodiesterase activity in the sucrose gradient profiles, and represent the amorphic, or near-amorphic, condition. In contrast, *dunce*^{1}, *dunce*^{2}, and *dunce*^{CK} show the presence of some form II phosphodiesterase activity in velocity sedimentation profiles and are much less affected in crude homogenate cyclic AMP phosphodiesterase activity than the previous three.

Cyclic Nucleotide Phosphodiesterase Activity in Particulate Fractions

The previous sections make it clear that all six mutants under investigation are deficient in total cyclic AMP phosphodiesterase activity and that one soluble form of cyclic AMP phosphodiesterase activity is either reduced or eliminated in mutant supernates. However, as in most mammalian tissues, adult *Drosophila* have a considerable portion of activity associated with particles (1). We have previously defined the particulate fraction as a once-washed 105,000-*g* pellet of a crude homogenate (1). The amount of particulate cyclic AMP phosphodiesterase activity in normal flies is usually between 15 and 25% of that found in crude homogenates. The question arises whether *dunce* flies also exhibit reduced particulate activity.

We have investigated this question by measuring the amount of cyclic nucleotide phosphodiesterase activity in particulate fractions obtained from Canton-S, *dunce*^{1}, and *dunce*^{2} flies. Table II shows that particulate preparations from Canton-S homogenates have ~23 U of cyclic AMP phosphodiesterase activity. *dunce*^{1} and *dunce*^{2} particulate fractions show reduced cyclic AMP phosphodiesterase activity compared with the Canton-S control. The mutants do not have any detectable difference in the amount of cyclic GMP phosphodiesterase activity in particulate fractions relative to Canton-S.

These results suggest a genetic relationship between soluble and particulate cyclic AMP phosphodiesterase. However, it is not known how well our particulate preparations represent in vivo particles and their associated enzyme activities.

TABLE II

Rate of Cyclic Nucleotide Hydrolysis by Particulate Material

Genotype	Cyclic AMP hydrolysis	Cyclic GMP hydrolysis
Canton-S	23 ± 2(10)	7 ± 1(10)
dunce^1	17 ± 1(6)	8 ± 1(6)
dunce^2	8 ± 1(6)	6 ± 1(6)

Rates are expressed as picomoles of cyclic nucleotide hydrolyzed per minute of incubation by particulate material from one milligram of adult fly. Assays were performed at 100 μM substrate. Numbers in parentheses indicate the number of particulate fractions assayed. Confidence intervals given are one standard error of the mean. The velocity values of cyclic nucleotide hydrolysis have been analyzed statistically by one-way analysis of variance with subsequent computation of contrasts by the method of Scheffé (13). Both *dunce*^{1} and *dunce*^{2} show significantly altered particulate cyclic AMP phosphodiesterase activity; *dunce*^{1} is significant from Canton-S at the 5% level and *dunce*^{2} at 1%. The values for cyclic GMP hydrolysis by mutant particulate fractions are not significantly different from the control at the 5% level.

Approximately 100% of the Canton-S and *dunce*^{2} particulate cyclic GMP phosphodiesterase activity was found in each respective particle wash. With regard to cyclic AMP phosphodiesterase, 100 and 160% of the particulate activity was recovered in the Canton-S and *dunce*^{2} particulate washes, respectively. The higher cyclic AMP phosphodiesterase activity found in the *dunce*^{2} particle washes cannot account for the significantly lower enzyme activity found in washed *dunce*^{2} pellets.

Velocity data have also been normalized with respect to protein concentration in each particulate fraction. The relative velocity values remain the same after this normalization.

Gel Filtration of Soluble Cyclic Nucleotide Phosphodiesterase Activities

For further analytical and preparative purposes, we have fractionated the two major phosphodiesterase activities found in Canton-S, *dunce*^{1}, and *dunce*^{2} soluble supernates by gel filtration on Sephadex G-150. The profile of activities eluting from the column for each of the strains is shown in Fig. 2.

The gel filtration activity profiles are similar to the sucrose density gradient profiles in that two forms of activity are clearly present in all three stocks. A cyclic AMP phosphodiesterase activity and a parallel cyclic GMP phosphodiesterase activity are first in elution order from the column, followed by a cyclic AMP-specific activity. The two separation techniques yield dissimilar results with respect to the ratio of activity found in the two forms. The form II activity in the Canton-S sucrose gradient profile represents about two to three times the activity of form I. After separation by gel filtration, there is only 15% more form II activity.

The gel filtration profiles confirm that *dunce*^{1} and *dunce*^{2} have reduced form II activity. Differential recovery cannot account for the reduction in the form II activity in the two mutants.

Kinetics of Cyclic AMP Hydrolysis by Canton-S, *dunce*^{1}, and *dunce*^{2} Form II Phosphodiesterases

The form II phosphodiesterases (Fig. 2) from the three stocks have been analyzed for their kinetic properties after partial purification by gel filtration on Sephadex G-150. Double reciprocal plots of cyclic AMP hydrolysis by these activities are presented in Fig. 3. The Canton-S enzyme preparation hydrolyzes cyclic AMP with linear kinetics and an apparent K_m of ~2 μm ($\bar{X} = 2.2 \pm 0.5$; $n = 4$). This is in agreement with results obtained for the same enzyme from *y cv v f* flies (1). The *dunce*^{1} enzyme shows a similar K_m ($\bar{X} = 2.5 \pm 0.4$; $n = 3$), but the V_{max} for cyclic AMP hydrolysis is reduced relative to the Canton-S enzyme by ~70%. The *dunce*^{2} enzyme preparation displays anomalous kinetic plots that are not readily interpretable.

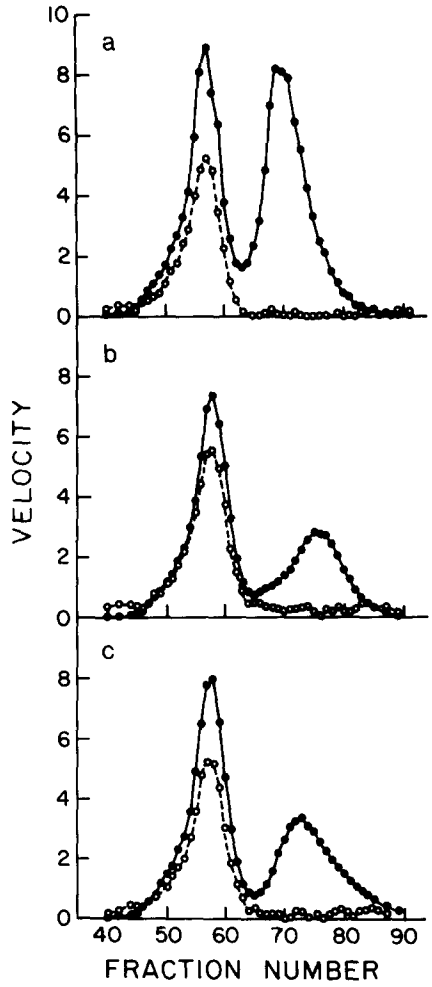

FIGURE 2 Gel filtration of soluble cyclic nucleotide phosphodiesterase activity from (*a*) Canton-S, (*b*) *dunce*^{1}, and (*c*) *dunce*^{2} adult flies. The soluble supernates were chromatographed individually on a Sephadex G-150 column as described in Materials and Methods. Fractions were assayed at 100 μM cAMP (●) and 100 μM cGMP (○). Enzyme activity is expressed as picomoles hydrolyzed per minute of incubation per milligram of fly loaded on the column per fraction. The supernates of Canton-S, *dunce*^{1}, and *dunce*^{2}, contained, respectively, 155, 75, and 96 U of cAMP phosphodiesterase activity and 65, 66, and 68 U of cGMP phosphodiesterase activity. 1 U is defined as 1 pmol of cyclic nucleotide hydrolyzed/min of incubation per mg of fly. The recovery of cAMP phosphodiesterase activity was 88, 115, and 105% respectively. Recovery of cGMP phosphodiesterase activity was 64, 81, and 70%, respectively. Fractions 66–75, 70–82, and 68–80 have been pooled for the Canton-S, *dunce*^{1}, and *dunce*^{2} gel filtration exxperiments respectively, concentrated by Amicon ultrafiltration (CF 25), and stored frozen at −80°C.

Results like those displayed in Fig. 3 have been obtained in three different experiments, although we have noted biphasic kinetic plots of cyclic AMP hydrolysis by the form II enzyme from *dunce*^{1} preparations. Biphasic plots are those that show two distinct kinetic components (two linear portions of the double reciprocal plot with different slopes); such plots are common for mammalian phosphodiesterase preparations (14).

The results shown were obtained after adjusting each enzyme preparation to the same *Drosophila* protein concentration. Similar results are obtained if the preparations are adjusted to the same initial velocity at 1 μM cyclic AMP. This indicates that the anomalous kinetic plots observed for the form II enzyme

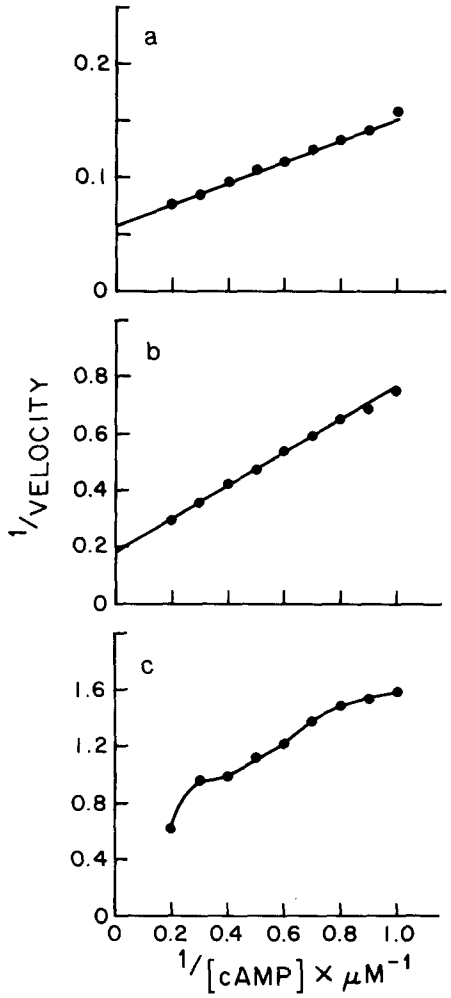

FIGURE 3 Kinetics of cAMP hydrolysis by (*a*) Canton-S, (*b*) *dunce*^{1}, and (*c*) *dunce*^{2}, form II phosphodiesterases. Aliquots of the three enzyme preparations as purified by Sephadex G-150 chromatography (Fig. 2) were thawed at 4°C, and diluted with 40 mM Tris·HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and 1 mg/ml bovine serum albumin, to the same fly protein concentration. Final reaction mixtures contained 40 mM Tris·HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 0.5 mg/ml bovine serum albumin, cAMP substrate at the appropriate concentration, and 0.4 μg of fly protein. Data for Canton-S and *dunce*^{1} were analyzed by weighted least squares regression as previously described (1). For Canton-S, the apparent K_m was ~1.7 × 10^{-6} M and V_{max} was ~17.4; for *dunce*^{1}, they were ~3.2 × 10^{-6} M and ~5.5, respectively. Velocity is expressed as picomoles of cAMP hydrolyzed per minute of incubation per microgram of fly protein.

from *dunce*^{2} and the biphasic plots from *dunce*^{1} are probably not the results of mere differences in enzyme concentration relative to the control. Enzyme concentration has pronounced effects upon the kinetics of cyclic nucleotide hydrolysis by mammalian phosphodiesterases (14).

We conclude that the anomalous kinetic plots produced by *dunce*^{2} form II enzyme probably result from mutation at the *dunce* gene. This conclusion must be tentative because the enzyme preparations are quite crude. Hence, one cannot rule out the possibility that some unknown factor in the *dunce*^{2} preparations is responsible for this observation. Definitive evidence will come only after the form II enzymes from Canton-S and *dunce*^{2} have been purified to homogeneity. It is less likely that the shift between simple, linear and biphasic kinetic plots observed for the *dunce*^{1} preparations result from the *dunce*^{1} mutation. This shift has been previously observed with mammalian phosphodiesterases, and can result from changes in enzyme concentration (14) and possibly from other unknown factors.

Cyclic AMP Levels in the Mutants

Cyclic AMP levels in Canton-S, *dunce*^{1}, and *dunce*^{2} flies have been previously determined (9). Females of these three genotypes have 1.6 ± 0.1, 2.3 ± 0.2, and 2.6 ± 0.1 pmol of cyclic AMP/mg of fly, respectively. The cyclic AMP levels found in females homozygous for the other four *dunce* alleles and in their respective control females are given in Table III. With one possible exception, cyclic AMP levels are elevated in mutants. The most extreme increases in cyclic AMP content are observed in *dunce*^{M14}, *dunce*^{M11}, and *dunce*^{ML}, which show increases of ~600, 500 and 300%, respectively. The possible exception is *dunce*^{CK}, which does not have a significantly higher cyclic AMP level than is found in the *y w f*^{36a} control. An approximate inverse relationship exists between the amount of crude homogenate cyclic AMP phosphodiesterase activity (Table I) and the level of cyclic AMP.

DISCUSSION

Our results confirm and extend a previous finding (9) that *dunce* mutants have aberrant cyclic AMP metabolism. Crude homogenates of the mutants have decreased cyclic AMP phosphodiesterase activity. Particulate cyclic AMP phosphodiesterase activity is decreased in the two mutants tested. A soluble cyclic AMP phosphodiesterase activity is reduced or missing in mutant supernates, as evidenced by gel-filtration and velocity-sedimentation profiles. Moreover, the mutants in general have elevated cyclic AMP content.

The data suggest that cyclic GMP phosphodiesterase is not affected by mutation at the *dunce* locus. Crude homogenates of the mutants and their controls have about the same amount of cyclic GMP phosphodiesterase activity. This is also true of mutant and control supernates and of mutant and control particulate preparations.

The mutants *dunce*^{ML}, *dunce*^{M14}, and *dunce*^{M11} appear to be amorphic with respect to the form II phosphodiesterase activity. This is shown by a severe reduction of cyclic AMP phosphodiesterase activity in their crude homogenates, the apparent absence of the soluble form II phosphodiesterase after supernate fractionation by velocity sedimentation, and extreme increases in cyclic AMP content. The three mutants *dunce*^{1},

TABLE III

Cyclic AMP Levels in dunce Females

Genotype	Cyclic AMP level
y w f^{36a}	1.5 ± 0.2(6)
dunce^{ML}	4.4 ± 0.2(6)
dunce^{CK}	1.6 ± 0.1(4)
y cv v f	1.5 ± 0.1(6)
dunce^{M14}	9.9 ± 0.9(4)
dunce^{M11}	8.0 ± 0.6(4)

Levels are expressed as picomoles of cyclic AMP per milligram of adult fly. Confidence intervals are one standard error of the mean. Statistical analysis has been performed by one-way analysis of variance for each group of data. Contrasts were computed by the method of Scheffé (13). With the exception of *dunce*^{CK}, the data for each mutant show a significant increase over the respective control at the 1% level.

dunce^2, and *dunce*^{CK} are hypomorphs; they show intermediate reductions in crude homogenate cyclic AMP phosphodiesterase activity, some soluble form II phosphodiesterase activity, and relatively slight increases in cyclic AMP levels.

In addition to their similar effects on cyclic AMP metabolism, the mutants share at least two other phenotypes. The first is female sterility or semisterility (8; Salz et al., unpublished observations). In addition, the phenotype of learning disability in an olfactory paradigm is shared by the four mutants that have been tested so far (9). These findings compel the conclusion that aberrant cyclic AMP metabolism, female sterility, and learning disability are the result of mutation in the same gene.

The product of the *dunce*$^+$ gene has not yet been identified. The data so far are consistent with the hypothesis that *dunce*$^+$ is the structural gene for the form II phosphodiesterase. However, we cannot yet rule out the possibility that *dunce*$^+$ affects the activity of form II phosphodiesterase in a regulatory manner. Such regulation could be at transcriptional, translational, or posttranslational levels. If the role of the *dunce*$^+$ product is regulatory, then the data favor the last alternative. The kinetics of cyclic AMP hydrolysis by form II from the mutant *dunce*^{2} appear anomalous, suggesting a structural defect in the enzyme. This would not be observed if the *dunce*$^+$ gene serves only to regulate the quantity of enzyme in the fly, by affecting the efficiency of transcription of the structural gene or translation of the message. If the regulatory theory is correct, then the regulatory molecule is probably an absolute requirement for activity of form II phosphodiesterase, as some of the mutants appear to be amorphic with respect to this activity and the activity is proportional to the dosage of the *dunce*$^+$ gene. The identification of the *dunce*$^+$ gene product is now a major goal.

If *dunce*$^+$ is the structural gene for form II phosphodiesterase, then it is extremely likely that aberrant cyclic AMP levels are the cause of the learning defect and female sterility. Greengard (15) has formulated the hypothesis that many, if not all, of the intracellular actions of cyclic AMP are brought about by the phosphorylation of specific substrates by cyclic AMP–dependent protein kinases. Specific roles for cyclic AMP and cyclic AMP–dependent protein kinases in the generation of postsynaptic potentials by certain neurotransmitters (16) and in amphibian oocyte maturation (17) have been proposed. The study of cyclic AMP–dependent protein kinases and their substrates is the most reasonable direction in which to proceed to uncover how the physiological defects might be caused by aberrant cyclic AMP levels.

Several other mutants of *Drosophila melanogaster* have been isolated that show deficiency in learning ability or memory (5, 6). Whether some or all of these mutants have an associated perturbation of cyclic nucleotide metabolism is a question for future research.

This work was supported by grants GM 21137 and GM 07467 from the U. S. Public Health Service; R. L. Davis was a predoctoral National Institutes of Health Trainee.

Received for publication 2 September 1980, and in revised form 5 March 1981.

REFERENCES

1. Davis, R. L., and J. A. Kiger, Jr. 1980. A partial characterization of the cyclic nucleotide phosphodiesterases of *Drosophila melanogaster*. *Arch. Biochem. Biophys.* 203:412–421.
2. Kiger, J. A., Jr., and E. Golanty. 1979. A genetically distinct form of cyclic AMP phosphodiesterase associated with chromomere 3D4 in *Drosophila melanogaster*. *Genetics.* 91:521–535.
3. Kiger, J. A., Jr. 1977. The consequences of nullosomy for a chromosomal region affecting cyclic AMP phosphodiesterase activity in *Drosophila*. *Genetics.* 85:623–628.
4. Dudai, Y., Y. Jan, D. Byers, W. Quinn, and S. Benzer. 1976. *dunce*, a mutant of *Drosophila* deficient in learning. *Proc. Natl. Acad. Sci. U. S. A.* 73:1684–1688.
5. Quinn, W. G., P. P. Sziber, and R. Booker. 1979. The *Drosophila* memory mutant *amnesiac*. *Nature* (*Lond.*) 277:212–214.
6. Aceves-Piña, E. O., and W. Quinn. 1979. Learning in normal and mutant *Drosophila* larvae. *Science* (*Wash. D. C.*). 206:93–96.
7. Quinn, W. G., W. A. Harris, and S. Benzer. 1974. Conditioned behavior in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* 71:708–712.
8. Byers, D. 1979. Studies on learning and cyclic AMP phosphodiesterase of the *dunce* mutant of *Drosophila melanogaster*. PhD Thesis. California Institute of Technology, Pasadena, California.
9. Byers, D., R. L. Davis, and J. A. Kiger, Jr. 1981. Defect in cyclic AMP phosphodiesterase due to the *dunce* mutation of learning in *Drosophila melanogaster*. *Nature* (*Lond.*). 289:79–81.
10. Mohler, J. D. 1977. Developmental genetics of the *Drosophila* egg. Identification of 59 sex-linked cistrons with maternal effects on embryonic development. *Genetics.* 85:259–272.
11. Davis, R. L., and J. A. Kiger, Jr. 1978. Genetic manipulation of cyclic AMP levels in *Drosophila melanogaster*. *Biochem. Biophys. Res. Commun.* 81:1180–1186.
12. Markwell, M. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane or lipoprotein samples. *Anal. Biochem.* 87:206–210.
13. Scheffé, H. 1959. The Analysis of Variance. John Wiley & Sons, Inc., New York.
14. Pichard, A. L., and W. Y. Cheung. 1976. Cyclic 3':5'-nucleotide phosphodiesterase: interconvertible multiple forms and their effects on enzyme activity and kinetics. *J. Biol. Chem.* 251:5726–5737.
15. Greengard, P. 1978. Phosphorylated proteins as physiological effectors. *Science* (*Wash. D. C.*). 199:146–152.
16. Greengard, P. 1976. Possible role for cyclic nucleotides and phosphorylated membrane proteins in postsynaptic actions of neurotransmitters. *Nature* (*Lond.*). 260:101–108.
17. Bravo, K., C. Otero, C. Allende, and J. E. Allende. 1978. Amphibian oocyte maturation and protein synthesis: related inhibition by cyclic AMP, theophylline, and papaverine. *Proc. Natl. Acad. Sci. U. S. A.* 75:1242–1246.