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DEFECTIVE CYCLIC ADENOSINE 3′:5′-MONOPHOSPHATE PHOSPHODIESTERASE IN THE *DROSOPHILA* MEMORY MUTANT *dunce*

LAWRENCE M. KAUVAR[1]

Division of Biology, California Institute of Technology, Pasadena, California 91125

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Abstract

A detailed characterization of the cyclic nucleotide phosphodiesterases (PDEs) from normal *Drosophila melanogaster* was made, including purification of the two major enzymes to near homogeneity. A third more labile phosphodiesterase also was identified in crude homogenates. The total activity per fly of one of these three enzymes, PDE-II, is strongly influenced by the *dunce* locus. Two independently derived *dunce* mutants produce variants of PDE-II with modified intrinsic properties: a marked decrease of thermal stability in *dunce*[1] and a 10-fold increase in the Michaelis kinetic constant in *dunce*[2]. These defects, which persisted in purified preparations of PDE-II, were mapped genetically to *dunce*. The results support the identification of *dunce* as the structural locus for PDE-II. The tight connection between the *dunce* gene and the PDE-II enzyme indicates that defective cyclic adenosine 3′:5′-monophosphate metabolism is the primary lesion which leads to failure of *dunce* flies to learn in the olfactory associative conditioning paradigm of Quinn et al. (Quinn, W. G., W. A. Harris, and S. Benzer (1974) Proc. Natl. Acad. Sci. U. S. A. *71:* 708–712).

The use of a single gene mutation to perturb only one component of an interacting network is analogous to the use of a pharmacological agent for the same purpose. Because conclusions derived from pharmacological studies are generally trustworthy only to the extent that the drug can be shown to have a single target, single gene mutations should represent ideal pharmacological agents. In practice, however, it is necessary to distinguish between regulatory and structural gene defects. A regulatory mutant might perturb a particular component only as a side effect of a malfunction many steps removed, or it might regulate a variety of processes other than the one under scrutiny. This caveat is particularly important in the analysis of interactions involving cyclic nucleotides because these processes are largely regulatory in nature. The reciprocal relationships among cyclic nucleotides, calcium, and phosphorylated proteins are so pervasive that perturbation of just one element of the otherwise intact network is unusually difficult (Rasmussen et al., 1975; Sieghart et al., 1979; Greengard, 1978).

The key roles in synaptic modulation postulated for cyclic nucleotide interactions by Nathanson and Greengard (1977), Kandel (1979), Bloom (1974), and others increase the significance of disentangling effects directly caused by experimental manipulations from effects produced as the end result of a cascade of induced abnormalities. An important step in this direction was the discovery by Byers et al. (1981) of a defect in cyclic nucleotide metabolism in the *dunce* mutant of *Drosophila*. This gene is of special interest because *dunce* flies are defective in a variety of associative conditioning paradigms. These include the adult olfactory learning paradigm of Quinn et al. (1974; also Dudai, 1979), the larval olfactory learning paradigm of Aceves-Piña and Quinn (1979), and the leg position conditioning paradigm of Booker and Quinn (1981).

J. A. Kiger's group has contributed a series of papers describing the *dunce* biochemical defect (reviewed in Kiger et al., 1981). They reported that *Drosophila* has two forms of cyclic nucleotide phosphodiesterase (PDE),[2]

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[2] The abbreviations used are: AMP, adenosine 5′-monophosphate; BSA, bovine serum albumin; cAMP, cyclic adenosine 3′:5′-monophosphate; cAMP phosphodiesterase or PDE, 3′:5′-cyclic nucleotide 5′-nucleotidohydrolase (EC 3.1.4.17), cGMP, cyclic guanosine 3′:5′-monophosphate; DEAE, *O*-diethylaminoethyl; DMSO, dimethyl sulfoxide; *dnc*, *dunce*; EDTA, ethylenediaminetetra-acetate; PEI, polyethyleneimine; SDS, sodium dodecyl sulfate.

the enzyme which degredes cAMP. One of these two forms hydrolyzed both cAMP and cGMP, while the second was specific for cAMP. They also provided evidence suggesting that *dunce* codes for the second form. This report provides a more detailed characterization of the cyclic nucleotide phosphodiesterases in normal *Drosophila*. A third, more labile form is identified and purification of the two previously described ones is presented. Investigation of the pronounced effects of partial proteolysis on the activity of the enzymes resolves several substantial inconsistencies in results arising from small variations in methodology. For one of the enzymes, denoted PDE-II, alterations of intrinsic enzymatic properties are demonstrated in two independently isolated *dunce* mutant stocks. Genetic mapping shows that these structural defects are closely linked to *dunce*, confirming the hypothesis that *dunce* codes for PDE-II.

Materials and Methods

Materials

[^{3}H]Cyclic adenosine 3′:5′-monophosphate (cAMP) and [^{3}H]cyclic guanosine 3′:5′-monophosphate (cGMP), obtained from New England Nuclear or Amersham, were dried under nitrogen to remove carrier ethanol and resuspended in water before use. Plastic-backed thin layer chromatography plates coated with polyethyleneimine-cellulose and containing a fluorescent indicator were obtained from E. Merck via MC/B Manufacturing Chemists, Inc., Ohio (PEI-cellulose, F catalog 5504). Trypsin, soybean trypsin inhibitor, cAMP-agarose, caffeine, theophylline, and isobutylmethylxanthine were from Sigma. Samples of the recently developed drugs RO 20-1724 (Hoffmann-LaRoche, Nutley, NJ) and ZK 62-711 (Schering A. G., Berlin) were graciously provided by Kent R. Jennings, who had obtained them from the respective pharmaceutical research laboratories. Acrylamide, hydroxylapatite, blue dextran (Affi-Gel Blue), and protein assay reagent were from Bio-Rad. DEAE-cellulose (DE52) was from Whatman. All other chemicals were of reagent grade and were purchased from Sigma, Schwartz/Mann, Mallinckrodt, or Fisher.

PDE assay

Standard reaction mixtures (0.015 ml) contained 40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1mM $CaCl_2$, 2 mM 2-mercaptoethanol, 75 mM NaCl (AN-75 buffer) plus 1 mg/ml of bovine serum albumin (BSA), 1 mM 5′-AMP, 20 μM [^{3}H]cAMP or [^{3}H]cGMP (80 cpm/pmol), and, where indicated, either 2 mM cAMP or 2 mM cGMP. Samples were incubated at room temperature (21°C) for the times indicated, and the reactions were terminated by the addition of 0.006 ml of a solution containing 50 mM Tris-HCl, pH 7.5, and 200 mM EDTA, to quench the enzymatic activity, and a 10 mM concentration of each of the appropriate cyclic nucleotide and 5′-mononucleotide as markers for chromatography. Aliquots of 0.0035 ml were spotted on strips of fluorescent indicator PEI-cellulose (18 × 66 mm), and ascending thin layer chromatography was performed for 15 min with 50 mM KCl as the developing solution. The strips were dried with a hair dryer. The 5′-nucleotide zone was visualized under ultraviolet light, cut out, and eluted for 5 min in a scintillation vial with 1 ml of 700 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5. Aquasol-2 (10 ml) was added and the radioactivity was measured in a scintillation counter (Rangel-Aldao et al., 1978).

Excessive dilution of the sample, leading to the loss of PDE activity, was largely overcome by the inclusion of 1 mg/ml of BSA in the assay buffer. The inclusion of 5′-AMP (1 mM) in the mixture, to eliminate the small but variable conversion of the enzyme product to free nucleoside by action of endogenous phosphatases, has no effect on enzyme activity (S. L. Shotwell, personal communication).

Purification procedures

Homogenization. For small scale work, 5 flies were homogenized by hand in 0.04 ml of the appropriate buffer using a Kontes microtissue grinder which consists of a small glass tube and fitted glass pestle. For large scale work, 10^3 to 10^5 male flies were homogenized using a Brinkmann Polytron with a PT10ST or PT35/4 generator using 5 ml of buffer/gm of flies (1 fly weighs about 0.8 mg).

High speed supernatant. For small scale work, a supernatant protein fraction was prepared using a Beckman Airfuge at 100,000 × *g* at 22°C for 5 min. For medium scale work, centrifugation was performed at 100,000 × *g* at 4°C for 1 hr in Beckman SW60 or SW41 rotors. For large scale preparations, a Beckman type 19 rotor was used at 35,000 × *g* at 4°C for 3 hr. The supernatant was freed of any floating debris by filtration through 100-μm-mesh nylon screen.

Polyethyleneimine (PEI) precipitation. The high speed supernatant was slowly brought to 0.15% PEI at 0°C with stirring. The precipitate was collected by centrifugation for 15 min at 15,000 × *g* in a Sorvall GSA or SS-34 rotor and resuspended by low speed Polytron homogenization in the standard buffer described above supplemented with NaCl to 600 mM using 1.5 ml/gm of flies in the original homogenate. After salt extraction, the sample was substantially depleted of nucleic acid as measured by the ratio of absorbances at 280 and 260 nm; before the PEI step, $A_{280/260}$ = 0.63, while afterward, $A_{280/260}$ = 1.10. Optimal recovery from the PEI precipitation required both the correct final PEI concentration and the correct ratio of PEI to homogenate.

Ammonium sulfate precipitation. The NaCl eluate of the PEI precipitate was precipitated with $(NH_4)_2SO_4$ at a final concentration of 50% of saturation, mixed with inert carrier diatomaceous earth (Celite) using 0.3 gm/gm of flies homogenized, and packed into a column (King, 1972). After washing with one bed volume of 55% $(NH_4)_2SO_4$, the column was eluted at 1 ml/min with a gradient of Tris-buffered $(NH_4)_2SO_4$, decreasing from 55% to 5% saturation (0.05% saturation decrease per ml). Peak fractions were pooled and concentrated by reprecipitation with $(NH_4)_2SO_4$ and centrifugation at 15,000 × *g* at 4°C. This step provided separation of the two main PDE activities which then were processed in parallel.

Ion exchange chromatography. The samples obtained after $(NH_4)_2SO_4$ fractionation were diluted with 40 mM

Tris-HCl, pH 7.5, until the conductivity was below 4 mS, loaded on DEAE-cellulose columns (5 × 75 cm), and washed with the standard AN-75 buffer. For each 10 gm of flies, 3 ml of DEAE-cellulose was used (i.e., ~7.5 mg of protein/ml of DEAE-cellulose) and the column was eluted with a NaCl gradient, AN-75 to AN-275. Peak fractions were concentrated by $(NH_4)_2SO_4$ precipitation.

Gel filtration. A 1.8-liter Sephadex G-150 column (5 × 90 cm) equipped with flow adapters was used at 4°C. The buffer was the standard AN-75.

Hydroxylapatite. Peak fractions from the gel filtration step were concentrated by binding to a 2-ml column of hydroxylapatite freshly equilibrated with AN-75 and eluted with a sodium phosphate gradient, 0 to 100 mM, pH 7.5, containing 2 mM mercaptoethanol at 4°C.

Nondenaturing electrophoresis. Acrylamide gels were prepared in slabs (0.75 mm thick) containing a stacking gel of 5% acrylamide, 0.13% bisacrylamide, 100 mM Tris-HCl, pH 6.8, on top of a resolving gel of 7.5% acrylamide, 0.2% bisacrylamide, 350 mM Tris-HCl, pH 8.8, with 10% DMSO included throughout. The running buffer, pH 8, contained 50 mM Tris base, 185 mM glycine, and 1.5 mM mercaptoacetic acid. Electrophoresis was performed at a constant current of 7.5 mA at 4°C with bromophenol blue as the tracking dye. Peak fractions from the hydroxylapatite fractionation were loaded onto two gels.

After electrophoresis, the gel was cut into transverse slices (2 or 3 mm long); two guide strips (3 mm wide) were removed down the length of the gel and the slices were assayed for PDE activity. For one guide strip, the substrate was added to the gel slices in 50 μl of AN-75 buffer and incubated for 5 min, and the product was analyzed by PEI-cellulose chromatography. For a more accurate estimation of the activity recovered, the second guide strip was eluted overnight into AN-75 buffer supplemented with serum albumin. Peak fractions indicated by the rapid assay were the same as by the more careful procedure. In this manner, the choice of slices for pooling was accomplished in 1.5 hr during which time diffusional spreading of Coomassie-stainable bands was not significant. The peak fractions from the whole gel were pooled and eluted by diffusion at 4°C into a solution containing 40 mM Tris-HCl, pH 7.5, 10% DMSO, 2 mM 2-mercaptoethanol with repeated changes over 24 hr.

Protein assay. Quantitative protein assays were performed using the protein dye-binding assay (Bradford, 1976) from Bio-Rad with carbonic anhydrase as the standard. To follow bulk protein concentrations in the various chromatographic steps, absorbance at 280 nm was used. At the end of the purification procedure, the protein content in the peak fractions from the nondenaturing electrophoresis was roughly estimated by comparing Coomassie Blue staining of an aliquot of enzyme with various amounts of carbonic anhydrase marker run in parallel on an analogous gel.

Abbreviated purification. For rapid processing of smaller amounts of material (10 to 20 gm of flies), a simplified version of the full purification was used. The initial steps were as just described. In place of $(NH_4)_2SO_4$ gradient resolubilization, the salt eluate of PEI-precipitated material was simply precipitated with 50% saturated $(NH_4)_2SO_4$, resuspended in 40 mM Tris-HCl, pH 7.5, 2 mM 2-mercaptoethanol, and dialyzed 3 hr at 4°C against the same buffer. The sample then was loaded on DEAE-cellulose using 0.6 ml of packed resin/10 gm of flies homogenized. The column was washed with AN-75 and step-eluted with AN-275. After brief dialysis to reduce the NaCl concentration, the sample was loaded on a native gel and processed as described above.

A number of other purification procedures were attempted but were not found to be useful; either recovery was very low or no significant purification was obtained. These included ion exchange chromatography on carboxymethyl cellulose or phosphocellulose, isoelectric focusing, and affinity chromatography on cAMP-agarose or blue dextran.

Other procedures

SDS electrophoresis. Slab gels containing 0.1% sodium dodecyl sulfate were run following the method of Laemmli (1970) using 10% acrylamide, 0.25% bisacrylamide. Proteins were visualized by the silver staining procedures of Switzer et al. (1979) or Merril et al. (1981). The latter procedure also was used as further modified by George Gaines (personal communication); in place of potassium dichromate and nitric acid, sodium periodate was used (1% $NAIO_4$ in 3% acetic acid). Some gels were stained with 0.1% Coomassie Blue in 50% methanol, 10% acetic acid and destained in 10% ethanol, 7.5% acetic acid.

Partial trypsin digestion. For studies on the trypsin activation of PDE, 5 to 15 flies were homogenized, freshly prepared trypsin was added to a final concentration of 10 μg/ml, and the mixture was incubated at 21°C for 5 min. The digestion was stopped by the addition of soybean trypsin inhibitor to a final concentration of 25 μg/ml.

Genetics. Drosophila oocytes contain large quantities of yolk proteins which are prone to aggregate nonspecifically, causing difficulties for the purification of other proteins (Kauvar, 1978). To avoid yolk protein contamination, only adult males were used. Mass preparation of males was accomplished by using an attached X-homozygous *shibire*^{ts} stock (Poodry et al., 1973) from which viable female larvae were eliminated by shifting the larvae from 21°C to 29°C for 48 hr after hatching.

Flies were grown on standard medium (Lewis, 1960). The genetic markers used are described in Lindsley and Grell (1968). Mutant stocks were from the collection of Seymour Benzer (California Institute of Technology) as previously described (Byers et al., 1981).

Results

Identification of three phosphodiesterase activities in Drosophila. Kiger and Golanty (1977, 1979) identified two major forms of *Drosophila* PDE by sucrose gradient sedimentation analysis. Davis and Kiger (1980) subsequently showed that the larger form, PDE-I, was about equally efficient at hydrolyzing cGMP as cAMP but that the smaller form, PDE-II, was active only on cAMP. They further showed that cGMP acted as a competitive inhibitor of cAMP hydrolysis by PDE-I. Shotwell (1981) extended these results by showing that PDE-II can be assayed independently of PDE-I by using tritium-labeled cAMP as the substrate in the presence of a large excess

of unlabeled cGMP to inhibit PDE-I. The same logic in reverse, using labeled cGMP as the substrate and unlabeled cAMP to inhibit PDE-I, has now allowed identification of a third PDE, PDE-III, which is specific for cGMP and markedly more heat labile than PDE-I or PDE-II (Fig. 1). PDE-III activity found in crude homogenates is 99% inactivated within 1 to 2 min at 45°C; PDE-II requires 15 min at the same temperature. After the loss of PDE-II and -III, the remaining activity against both cAMP and cGMP decays in parallel at 52°C to ~50% of the initial activity in 30 min.

Ca^{2+} stimulation of *Drosophila* PDE was variable and the variability was traced to changes in the homogenization conditions. Homogenization performed by hand, using a Kontes microtissue grinder with 5 flies in 0.04 ml of buffer, allowed the demonstration of Ca^{2+} sensitivity for PDE-I but not for PDE-II or -III. However, when a Polytron motorized homogenizer was used with 500 flies in 4.0 ml of buffer, no Ca^{2+} sensitivity was demonstrable for any of the phosphodiesterases (Table I). Yamanaka and Kelly (1981) showed that Ca^{2+} activation of PDE-I is mediated by calmodulin. The total PDE activity per fly was 3- to 4-fold higher with the motor-driven homogenizer, whereas the total protein released was not increased significantly. Some factor apparently is released by the vigorous homogenization which can activate PDE without exogenous Ca^{2+}.

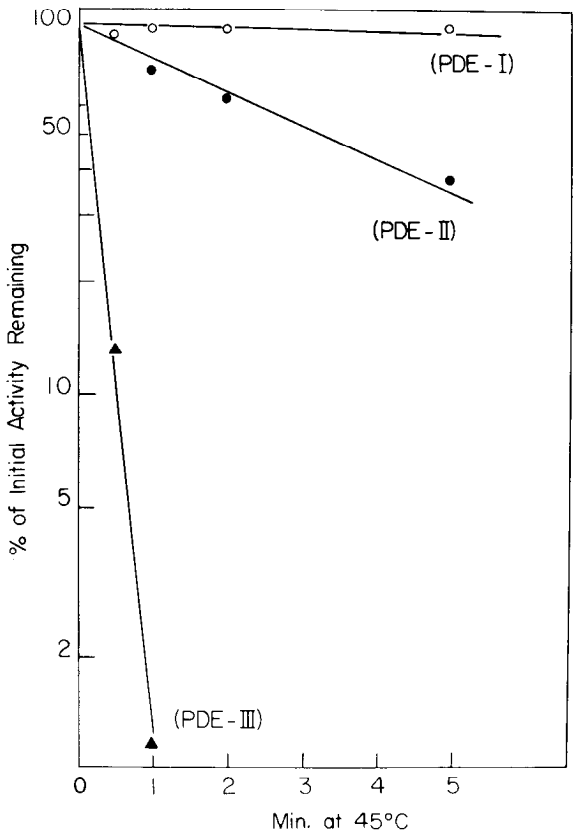

Figure 1. The thermal stabilities of PDE-I, -II, and -III differ widely. Crude homogenates prepared from 5 normal males were heated in a water bath at 45°C. At various times, samples were chilled to 0°C and then assayed at 21°C. The activity remaining for each PDE is expressed as a percentage of the initial activity for that enzyme. PDE-III (▲——▲) is defined as the activity found against [^{3}H]cGMP (20 μM) in the presence of a 50-fold excess of unlabeled cAMP. Similarly, PDE-II (●——●) is the activity against [^{3}H]cAMP in the presence of excess cGMP. PDE-I (○——○) is defined as the residual activity which is not specific for only one of cAMP and cGMP.

TABLE I

Effects of assay conditions on enzyme activities

Protocols were carried out on aliquots of single batches of crude homogenate initially containing no divalent cations and 1 mM EDTA. With no added Mg^{2+}, activity was below the detection limit. PDE-II and -III require higher Mg^{2+} for maximal activity than PDE-I (lines 1 and 2), but only PDE-I is stimulated by Ca^{2+} (lines 2 and 3). This Ca^{2+} effect on PDE-I is not seen after limited trypsin digestion (lines 4 and 5) or after vigorous machine homogenization (lines 6 and 7). Trypsin treatment of hand-homogenized extracts, or machine homogenization, increases the activity, measured at a substrate concentration of 20 μM, for both PDE-I and -II but not PDE-III (lines 2, 4, and 6).

	PDE-I (cGMP)	PDE-II (cAMP)	PDE-III (cGMP)
	pmol/min/fly[a]		
Kontes hand homogenizer			
1. 1 mM Mg^{2+}	43	7	<2
2. 20 mM Mg^{2+}	42	47	26
3. 20 mM Mg^{2+}, 1 mM Ca^{2+}	63	45	27
4. Trypsin, then 20 mM Mg^{2+}	91	72	26
5. Trypsin, then 20 mM Mg^{2+}, 1 mM Ca^{2+}	85	71	23
Polytron motorized homogenizer			
6. 20 mM Mg^{2+}	80	112	15
7. 20 mM Mg^{2+}, 1 mM Ca^{2+}	81	115	15

[a] Measured at a substrate concentration of 20 μM and at 21°C.

Epstein et al. (1978) have reported that limited trypsin digestion of calmodulin-activated PDE from BHK fibroblasts results in a permanent activation of the PDE, which is accompanied by a loss of Ca^{2+} sensitivity. Analogously, I found that a hand-homogenized sample of *Drosophila* PDE, partially digested with trypsin (see "Materials and Methods"), showed an increase in the activity of PDE-II of about 150% and PDE-I of about 200 to 300%, with a concomitant loss of PDE-I's Ca^{2+} sensitivity; trypsin had no effect on PDE-III. The proteolytic action was as effective on a 100,000 × *g* supernatant as on the crude homogenate. Motorized homogenization may be acting via the release of endogenous proteases from the fly; standard inhibitors of trypsin-like proteases, such as phenylmethylsulfonyl fluoride, did not eliminate the activity difference resulting from the two homogenization protocols. Since insect proteases are known to differ from their mammalian counterparts (Kikkawa, 1968), the protease inhibitor studies are not definitive.

The degree of endogenous activation of PDE is difficult to control and results in variability of the measured total activity per fly. Another variable parameter in published reports on *Drosophila* PDE is the Michaelis kinetic constant, K_m. In particular, nonlinear Lineweaver-Burke double reciprocal plots have been reported for PDE kinetics in crude homogenates or after limited purification (Davis and Kiger, 1980; Yamanaka and Kelly, 1981). These nonlinearities also can be mimicked by trypsin treatment of hand-homogenized flies. The apparent activation of both PDE-I and PDE-II occurs through low-

ering the K_m and increasing the V_{max} as illustrated for PDE-I in Figure 2. Incomplete activation, through shorter trypsin treatment, resulted in a mixed population of enzymes differing in K_m, a circumstance known to produce nonlinear kinetics (Cleland, 1970).

Table I summarizes the measured PDE activities found after varying the assay conditions as just described. In brief, *Drosophila* contains three different PDE activities, each of which can be assayed independently of the other two.

Various inhibitors of mammalian PDE were tested for differential effects on the three *Drosophila* enzymes in small scale crude homogenates. PDE-I and -III are somewhat more sensitive to the presence of theophylline and papaverine than is PDE-II. For example, for PDE-II assayed under standard conditions, 50% inhibition was produced by papaverine at ~5 mM, while PDE-I was comparably inhibited at ~0.5 mM. Highly potent inhibitors of mammalian PDE, such as RO 20-1724 and ZK 62-711, gave only 30 to 50% inhibition of *Drosophila* PDE even at concentrations near saturation; however, some specificity for PDE-I relative to PDE-III was found with RO 20-1724 and vice versa for ZK 62-711. None of the drugs tested was exclusively active against just one of the three PDE enzymes.

Purification of PDE-I and -II. In order to understand more fully the relationship between the three activities observed in crude homogenates, purification of the enzymes was undertaken. In this report, the properties of PDE-I and -II are described. PDE-III is very unstable and no further information is presently available on its physical properties. The details of the purification scheme used are presented under "Materials and Methods" and in the legend to Figure 3 which shows enzyme profiles for the major steps. Table II summarizes the enrichment factor and Figure 4*A* illustrates the degree of purity achieved in the most successful large scale preparations. There are uncertainties of ~25% in the measurement of the small amounts of protein obtained at the end of the purification and more significant uncertainties of ~50% in the appropriate definition of enzyme activity because of the proteolysis effects discussed above. Nevertheless, the final enrichment factors given for PDE-I and -II, approximately 20,000-fold for each, are useful for estimating the abundance of PDE in the crude homogenate.

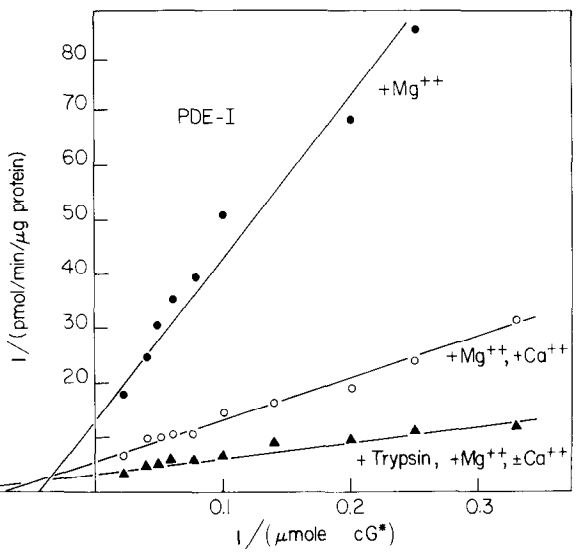

Figure 2. Kinetic parameters of PDE-I vary with assay conditions. One batch of homogenate from 15 normal males was heated at 45°C for 1 min to inactivate PDE-III, divided into three aliquots, and assayed against [^{3}H]cGMP (*cG**) with 10 mM Mg^{2+} (●——●), 10 mM Mg^{2+} + 1 mM Ca^{2+} (○——○), and 10 mM Mg^{2+} ± 1 mM Ca^{2+} after a limited trypsin digestion (▲——▲). Data are graphed as a Lineweaver-Burke plot showing the reciprocal of the rate of product formation versus the reciprocal of the concentration of substrate initially added.

The full purification scheme (Table II) is a long procedure with low yield. The previous experience of other investigators in purifying enzymes from *Drosophila*, such as DNA polymerase (Banks et al., 1979), has indicated that control over proteolysis effects is difficult to achieve with the available protease inhibitors but that rapid processing helps. Accordingly, an adaptation of the full purification scheme for PDE-I and -II, which requires only 2 days instead of 8 days was made ("Materials and Methods"). Although cumbersome for processing large amounts of material through the final gel electrophoresis step, this procedure was quite valuable on an analytical scale. The purification achieved was not as complete as for the full procedure, but it did make possible a comparison of extensively purified PDE from normal and mutant flies. The degree of purity for the most enriched fractions is shown in Figure 4*B*, and a summary of the purification is presented in Table III.

After the full purification procedure, both PDE-I and -II migrated as single peaks on nondenaturing gels. Electrophoresis of crude homogenates, however, resulted in severely broadened peaks or, in some instances, the appearance of two peaks of activity due to variable degrees of proteolysis. The profile obtained is influenced both by the homogenization protocol and by treatment with trypsin, just as was found for the total activity in crude homogenates. At the final step of the short purification, some of the activity for both PDE-I and -II migrated as observed after the full purification, while some was present as a broad peak migrating more slowly. The molecular weight of the enzyme migrating as a sharp peak was determined by gel filtration and agrees well with the value derived from SDS-gel electrophoresis of the purified enzymes: 35,000 for PDE-II and 120,000 for PDE-I. More slowly migrating PDE from the short purification had a larger apparent size by gel filtration, broadly distributed from ~50,000 to 90,000 for PDE-II and ~150,000 for PDE-I. For each of the peaks from the nondenaturing gel, re-electrophoresis shows no interconversion of the peaks. The larger, more slowly migrating forms of PDE activity therefore do not appear to arise from reversible association of smaller subunits. After purification, Ca^{2+} and calmodulin had no effect on the enzymes. Both PDE-I and -II are tentatively identified as monomeric enzymes whose apparent size is dependent on degree of proteolysis during purification.

Comparison of PDE from normal and dunce flies. Davis and Kiger (1981) have presented a kinetic analysis of two dunce alleles that retain some PDE-II activity

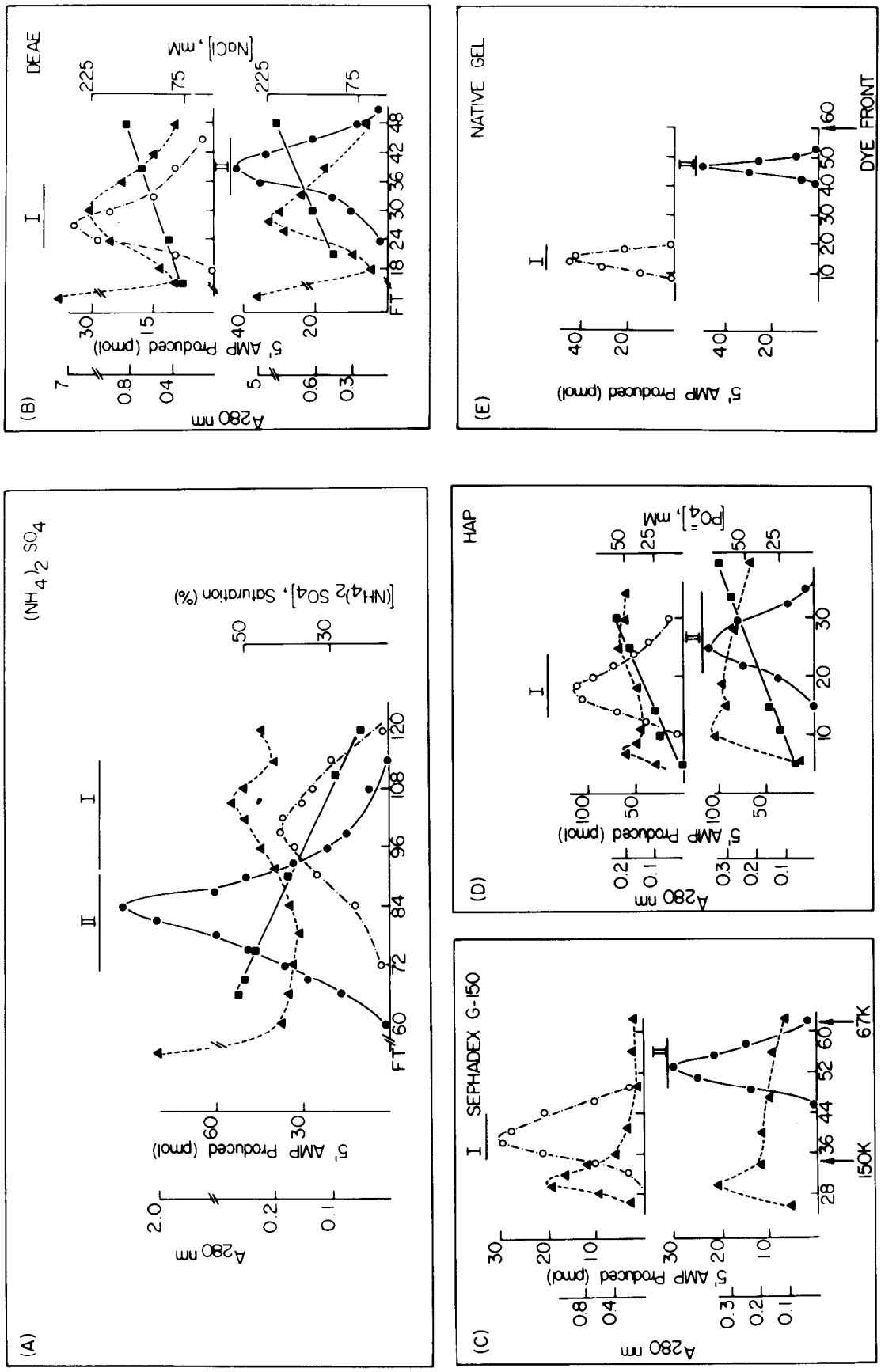
(A)
(NH4)2 SO4
[(NH4)2 SO4], Saturation (%)
A280 nm
5' AMP Produced (pmol)
FT
(B)
DEAE
[NaCl], mM
A280 nm
5' AMP Produced (pmol)
FT
(C)
SEPHADEX G-150
A280 nm
5' AMP Produced (pmol)
150K
67K
(D)
HAP
[PO4], mM
A280 nm
5' AMP Produced (pmol)
(E)
NATIVE GEL
5' AMP Produced (pmol)
DYE FRONT

Figure 3. Stages in the purification of PDE-I and PDE-II. Conductivity measurements, ■——■; protein determined by absorbance at 280 nm, ▲- - -▲; PDE-I assayed with 20 μM [^{3}H]cGMP, ○·-·-·-○; PDE-II assayed with 20 μM [^{3}H]cAMP plus 2 mM cGMP, ●——●. Standard buffer, AN-75, consisted of 40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM 2-mercaptoethanol, and 75 mM NaCl. Comparable buffers with higher concentrations of NaCl, such as 275 mM, are designated AN-275. Aliquots of various fractions were diluted, as described below, in AN-75 buffer supplemented with 1 mg/ml of bovine serum albumin and assayed at 21°C for 5 min; the maximum substrate hydrolyzed per assay was below 30%. All procedures were performed at 0 to 4°C. The following dilutions were used at each step for PDE-I and PDE-II, respectively: *A*, 1:75 and 1:75; *B*, 1:125 and 1:150; *C*, 1:100 and 1:150; *D*, 1:200 and 1:200; *E*, 1:250 and 1:250. *A*, $(NH_4)_2SO_4$ fractionation. From a homogenate of 450 gm of flies, a 100,000 × *g* supernatant was prepared. Most of the PDE-I and PDE-II activity was recovered in the 100,000 × *g* supernatant fraction; repeated washing of the 100,000 × *g* pellet left only a small percentage of the PDE in the particulate fraction. PDE-III appears to be more tightly membrane associated, but the data are not very reliable as recoveries were low. Following precipitation with polyethyleneimine and NaCl extraction of the precipitate, the sample was mixed with 150 gm of diatomaceous earth and solid $(NH_4)_2SO_4$ was added slowly to 50% saturation at 0°C. This mixture was packed into a column, giving a bed volume of 500 ml, and washed with 500 ml of 55% saturated $(NH_4)_2SO_4$ in AN-75 buffer (flow-through, *FT*). The graph shows the elution profile with a 1000-ml gradient (0.5 ml/min) of decreasing $(NH_4)_2SO_4$ concentration, from 55 to 5% saturation, collected in 7-ml fractions. Separation of PDE-I and -II was achieved at this point, and each enzyme was processed in parallel for the remaining steps. Activity lost at this step was not restored by recombining the separated fractions, suggesting that the decrease did not reflect the loss of an activating subunit. The loss may be due to this being the first step involving substantial dilution of the enzymes. *B*, DEAE-cellulose chromatography of pooled peak fractions I and II from *A*. Samples were precipitated with 50% $(NH_4)_2SO_4$ and resuspended in 500 ml of 40 mM Tris-HCl, pH 7.5 Samples then were loaded on a 150-ml bed volume column of DEAE-cellulose equilibrated in AN-75 and washed with 100 ml of AN-75 (flow-through, *FT*). The graph shows the fractions that were eluted with a NaCl gradient (500 ml, 1.5 ml/min), AN-75 to AN-275; 7-ml fractions were collected assay. *C*, Gel filtration of peak fractions I and II from *B*. Pooled fractions were concentrated by 50% $(NH_4)_2SO_4$ precipitation and resuspended in 5 ml of AN-75. The graph shows chromatography on a 1.8-liter column of Sephadex G-150. Using AN-75 as a buffer, fractions of 16.5 ml were collected at a flow rate of 1 ml/min. The molecular weight markers used were bovine serum albumin (67,000) and IgG (150,000). *D*, Hydroxylapatite (*HAP*) chromatography of the peak fractions from the Sephadex G-150 step of *C*. These were pooled and loaded directly onto 2-ml columns of hydroxylapatite freshly equilibrated in AN-75. The graph shows elution (0.25 ml/min) with a gradient (20 ml) of sodium phosphate, pH 7.5, 0 to 75 mM; fractions of 0.67 ml were collected. *E*, Native gel electrophoresis of peak fractions from the hydroxylapatite columns of *D*. These were pooled, mixed with glycerol, and loaded directly onto two nondenaturing acrylamide slab gels (165 × 200 × 0.75 mm) as described under "Materials and Methods." The graph shows enzyme activity eluted from the gel slices in 0.1 ml of assay buffer for 12 hr at 4°C.

after limited purification. They found that, at low substrate concentrations, the *dunce*^{2} allele gave a complex nonlinear Lineweaver-Burke plot for the hydrolysis of cAMP. Because PDE-II can be assayed independently, without any purification, by using tritiated cAMP at the substrate in the presence of a large excess of unlabeled cGMP (Shotwell, 1981), it has been possible to find a simpler expression of the PDE-II kinetic abnormality in *dunce*^{2}. Figure 5 shows the kinetics of the *dunce*^{2} cAMP-specific activity. The K_m for the *dunce*^{2} PDE-II, 60 μM, is about 10-fold higher than the normal value of 5 μM. In contrast, homogenates of flies carrying the *dunce*^{1} allele showed normal kinetics. Limited trypsin digestion of the *dunce*^{2} homogenate produced an apparent activation of PDE-II measured at a substrate concentration of 20 μM, an effect due both to lowering the K_m and to increasing the V_{max}.

Because the total activity of various gene dosages and in point mutants reflects the product of both the number of enzyme molecules and the intrinsic activity per molecule, no direct information is provided on which parameter is varying. By contrast to the effects of the *dunce*^{2} mutation, changes in PDE-II activity per fly in females with a duplication or deficiency for the normal *dunce* gene are solely due to changes in the V_{max} with no apparent change in the K_m (data not shown).

An additional abnormality of PDE-II from *dunce*^{2} was observed in crude homogenates but has not been characterized in detail. The sensitivity of the mutant enzyme to inhibition by papaverine and theophylline is much greater than normal. Fifty per cent inhibition was achieved at ~50 μM theophylline instead of at ~5 mM for the normal enzyme. Thus, the *dunce*^{2} mutant enzyme, which has an altered affinity for the substrate also has an altered affinity for inhibitors. No such effect was seen in *dunce*^{1}.

As presented above, PDE-I, -II, and -III differ in thermal stability, a parameter that is widely employed in population genetics research to detect structural gene polymorphisms (Trippa et al., 1976). Using substrate specificity to define PDE-II in crude homogenates, a marked difference in the thermal stability of the enzyme from the *dunce*^{1} strain is evident (Fig. 6). This property is not affected by partial trypsin digestion ("Materials and Methods"). The PDE-II activity in *dunce*^{2} flies shows normal heat inactivation

Mutant defects persist after purification. The defects of PDE-II observed in crude homogenates of *dunce*^{1} and *dunce*^{2} appear to reflect differences in the enzymes themselves; they also are seen after the short purification presented above. Using this procedure, PDE-II from normal and mutant flies was purified 1,000- to 2,000-fold. PDE-II from normal flies at this stage of purification is somewhat more labile than in the crude homogenate, but the PDE-II from *dunce*^{1} remains markedly less stable to heating than are the normal or *dunce*^{2} enzymes (Fig. 7*A*).

Observation of the *dunce*^{2} kinetic defect after purification is complicated by the effects of partial proteolysis. After homogenization of *dunce*^{2} flies with a motorized Polytron, assay of the crude homogenate yields biphasic kinetics in contrast to the linear kinetics from hand homogenates of 5 flies. One component of the biphasic

TABLE II

Purification of major Drosophila cyclic nucleotide phosphodiesterases

Enzymes were assayed at substrate concentrations of 20 μM. [^{3}H]cAMP plus excess unlabeled cGMP was used to define PDE-II. PDE-I is defined as the total activity against [^{3}H]cGMP minus the contribution of PDE-III, which was defined as the activity against [^{3}H]cGMP in the presence of excess unlabeled cAMP. PDE-III activity was unmeasurable after the PEI precipitation step. Protein was assayed by the Bio-Rad dye-binding procedure with carbonic anhydrase as the standard except for the last step, where protein was estimated from Coomassie-stained gels including carbonic anhydrase standards. Activity measurement at the last step is based on the elution of an aliquot of the nondenaturing gel into optimal assay buffer, which includes 1 mg/ml of bovine serum albumin. Enzyme profiles for each step are presented in Figure 3.

Step	PDE-I				PDE-II			
	Protein	Activity	Specific Activity	Enrichment	Protein	Activity	Specific Activity	Enrichment
	mg	*nmol/min*	*nmol/min/mg*	*-fold*	*mg*	*nmol/min*	*nmol/min/mg*	*-fold*
Crude extract	96,000	31,000	0.32	1.0	96,000	52,000	0.55	1.0
100,000 × *g* supernatant	37,000	28,000	0.77	2.4	37,000	47,000	1.27	2.3
NaCl extract of PEI precipitate	8,700	24,000	2.82	8.8	8,700	41,000	4.75	8.6
$(NH_4)_2SO_4$ gradient solubilization	900	5,500	6.11	19	670	9,400	14.0	25
DEAE-cellulose chromatography	170	4,100	24	76	44	5,600	127	230
Sephadex G-150	23	2,400	104	320	9	3,000	330	610
Hydroxylapatite	4.7	1,600	340	1,000	2.3	2,200	950	1,700
Native gel electrophoresis	<0.2	1,200	6,000	~18,000	<0.1	1,200	12,000	~22,000

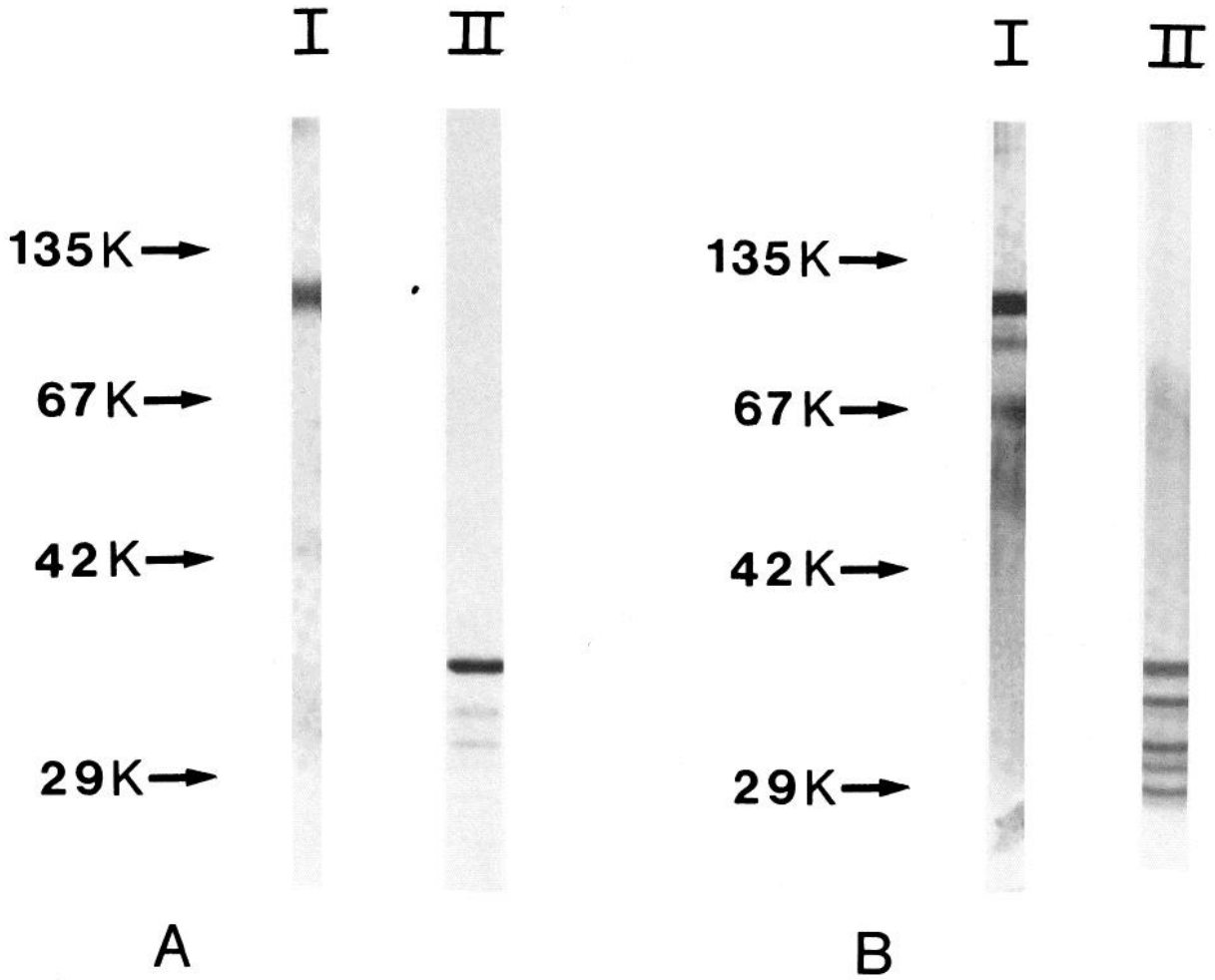

Figure 4. SDS-acrylamide gel electrophoresis shows the degree of purity of PDE-I and PDE-II achieved. Preparation of the samples is described in the legends to Tables II and III and Figure 3. Gels were stained by the reduced silver method as described under "Materials and Methods." *A*, Results of the full purification procedure for PDE-I and PDE-II. *B*, Results of the short purification for PDE-I and PDE-II. The molecular weight markers used were carbonic anhydrase (29,000), ovalbumin (42,000), bovine serum albumin (67,000), and *Escherichia coli* β-galactosidase (135,000).

TABLE III

Short purification of PDE-I and -II

Procedures were as described under "Materials and Methods."

Step	Protein	Specific Activity	
		PDE-I	PDE-II
	mg	*nmol/min/mg*	
Crude homogenate	2,130	0.3	0.7
100,000 × *g* supernatant	890	0.7	1.5
NaCl extract of PEI precipitate	190	2.5	3.8
0 to 50% $(NH_4)_2SO_4$ precipitate	78	6	5.4
DEAE-cellulose eluate	10	28	34
Native gel electrophoresis	~0.2	850	1,300

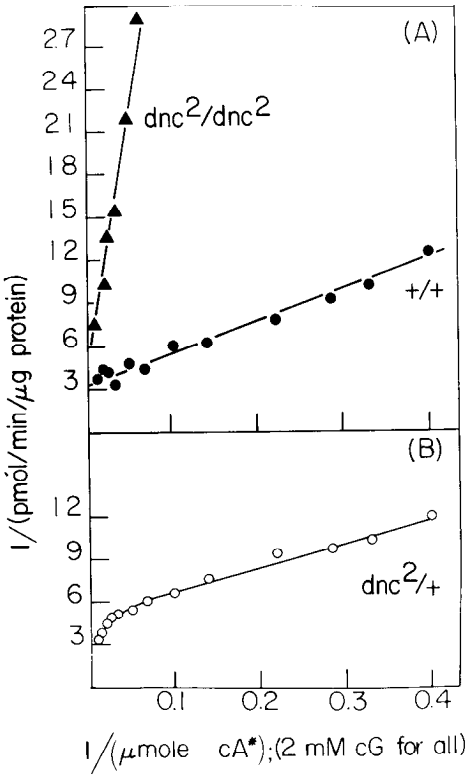

Figure 5. Kinetic abnormality of PDE-II in the *dunce*^{2} allele is revealed in crude homogenates by assaying the activity against [^{3}H]cAMP (*cA**) in the presence of excess unlabeled cGMP (*cG*). Data are graphed as a Lineweaver-Burke double reciprocal plot. The times of reaction at each concentration were adjusted to produce less than 10% hydrolysis of the substrate. Even at the highest cAMP substrate concentration, a sufficient excess of cGMP was present such that PDE-I's contribution to the hydrolysis of cAMP did not exceed 3% of the PDE-II's contribution. *A* shows the activity from a homogenate of 5 females homozygous for *dunce*^{2} (▲——▲) or for the normal (*dunce*$^+$) X chromosome (●——●) containing the markers *sc* w^{bl} *ec cv* used for genetic mapping. The PDE-II from the marker stock was indistinguishable from that in the standard Canton-S wild-type stock used for the other experiments. *B* shows biphasic activity from heterozygous females, *dunce*^{2}/*sc* w^{bl} *ec cv*. Lines drawn are hand-fitted approximations.

kinetics has an apparent K_m in the range of 50 μM, the same value as derived from the small scale homogenate's linear kinetics; the other component has an apparent K_m in the range of 10 to 20 μM. Since trypsin treatment of either normal or *dunce*^{2} small scale homogenates results in a reduction of the K_m, it is most reasonable to attribute the biphasic kinetics of the large scale *dunce*^{2} homogenate to partial proteolysis. After deliberate partial trypsinization, small scale *dunce*^{2} homogenates also showed graded degrees of biphasic kinetics. The nonlinearity seen in the large scale crude homogenate of *dunce*^{2} was seen after each step of purification, including the final native gel electrophoresis. PDE-II activity was present as a broad peak at the last step as also was seen in electrophoretic analysis of normal fly small scale crude homogenates treated with trypsin. Extrapolation of the biphasic kinetics of purified *dunce*^{2} PDE-II (Fig. 7*B*) yields K_m values of ~50 and ~10 μM. The protease-induced change in K_m for PDE-II from normal flies is not as pronounced as for *dunce*^{2} and approximately linear kinetics were found at all stages of purification, with the K_m ranging from 5 to 10 μM.

Tests using heterozygous flies. Because the *dunce*^{1} and *dunce*^{2} defects which were seen in crude homogenates persist in highly purified preparations, it is most reasonable to attribute them to structural alterations of the enzyme itself and not to other components present in the homogenate. However, it is possible that the differ-

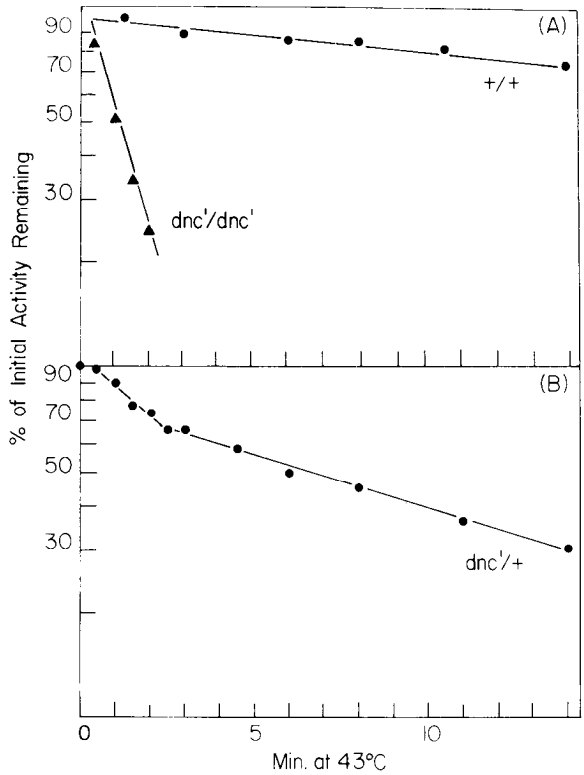

Figure 6. A compares the thermal instability of PDE-II from the *dunce*^{1} allele (▲——▲) to normal PDE-II from a stock carrying X chromosome markers *sc* w^{bl} *ec cv* (●——●). Aliquots of a homogenate of 5 females were heated in a water bath at 43°C for various times, rapidly cooled to 0°C, and assayed at 21°C. The activity remaining after different times at 43°C is expressed as a percentage of the initial activity for each stock. The initial activity in *dunce*^{1} is about 70% that of normal. *B* illustrates results from heterozygous females, *dunce*^{1}/*sc* w^{bl} *ec cv*. The data approximate the linear sum of half of the pure normal and half of the pure mutant activities.

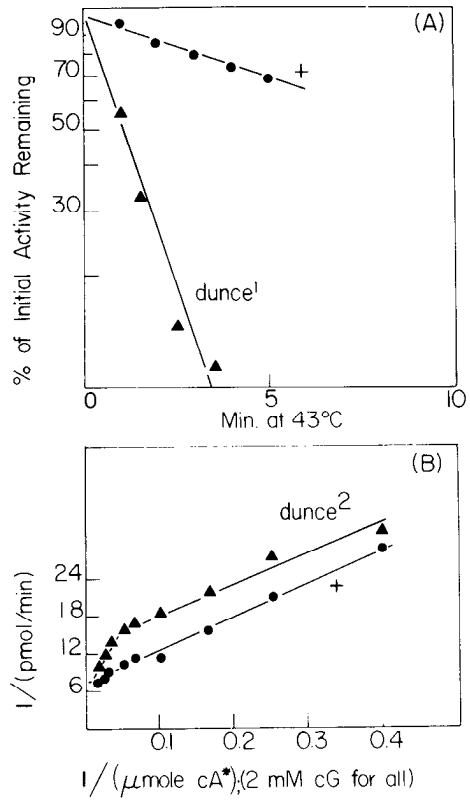

Figure 7. Defects in PDE-II from *dunce*^{1} and *dunce*^{2} persist after extensive purification. Enzymes were purified as described in the legend to Table III. *A* shows the thermal lability defect of *dunce*^{1} assayed as described for crude homogenates in Figure 6. *B* shows, at high substrate concentrations, the presence of a high K_m component in purified enzyme from *dunce*^{2}; a lower K_m component, coincidentally similar in K_m to the normal enzyme, arises from proteolysis of the mutant PDE-II. The data shown is from PDE-II migrating in the middle of the broad peak obtained at the last step of purification, the native gel. Faster migrating PDE-II from *dunce*^{2} displays less of the high K_m component, consistent with the effects of proteolysis on the mobility and K_m of normal PDE-II. ▲——▲, *dunce*^{1} (*A*) or *dunce*^{2} (*B*) allele; ●——●, X chromosome carrying markers *sc* w^{bl} *ec cv*. *cA**, [^{3}H]cAMP; *cG*, cGMP.

ences arise from faulty post-translational modification of a normal polypeptide. Examination of activity in unpurified extracts of heterozygous flies tests for this possibility. If the structural gene for PDE-II from *dunce* stocks is identical to the normal version, then all of the PDE-II found in the heterozygote should behave uniformly. In fact, two classes of PDE-II were seen for both *dunce*^{1}/+ and *dunce*^{2}/+ (Figs. 6*B* and 5*B*, respectively). In each case, the two classes correspond closely to the pure mutant and pure normal PDE-II activities seen in homozygous flies. For each of the mutant phenotypes, the data obtained using heterozygous females parallel those obtained by mixing equal amounts of normal and mutant homogenates (data not shown).

The biphasic kinetics in *dunce*^{2}/+ heterozygotes (Fig. 5*B*) resemble those seen with purified *dunce*^{2} homozygotes (Fig. 7*B*). The mechanism producing the effect, however, is quite different. Under the conditions used for assay of the heterozygote, the effects of proteolysis are minimal. By contrast, results with the purified homozygote are strongly affected by unavoidable proteolysis.

Genetic mapping of PDE-II abnormalities. Both the high K_m of *dunce*^{2} PDE-II and the increased thermal lability of *dunce*^{1} PDE-II have been mapped to the *dunce* region of the X chromosome. The mapping experiment is important for excluding the possible action of modifier genes elsewhere in the genome as the cause of the measured phenotypes. For example, the fertility of *dunce*^{2} females is quite sensitive to genetic background; two independent modifiers of this phenotype have been tentatively mapped to chromosome 2 (M. A. Crosby, personal communication). The enzyme defects in *dunce*^{1} and *dunce*^{2} were mapped by recombination, which eliminates possible ambiguities arising from a nearby chromosomal breakpoint or from aneuploidy effects. Since gene dosage experiments have already localized the presumptive PDE-II gene to salivary band 3D4, a small number of recombinants showing the expected linkages of PDE-II defects with flanking markers suffices to exclude the possibility that modifier genes distributed throughout the genome are the source of the defect. As seen in Table IV, the *dunce* enzyme defects segregated along with the mutant-derived band 3D4 for all of the recombinants between outside flanking markers. For recombinant classes in which the distribution of the mutant-derived 3D4 cannot be determined by inspection of the flanking markers, only some of the recombinants showed the *dunce* defects, the expected result.

TABLE IV

Genetic mapping of intrinsic defects in PDE-II

Mutant *dunce*^{1} or *dunce*^{2} males were mated to virgin females homozygous for the marked X chromosome *sc* w^{bl} *ec cv*. The F_1 progeny thus contained males of the *sc* w^{bl} *ec cv* genotype and heterozygous females. These males and females were mated and F_2 male progeny were selected which showed a recombination of the visible markers. These males were mated to *y f* $\overline{XX}$ females to establish a balanced stock which produces multiple male progeny of the same X chromosome genotype as the originally selected recombinant fathers. For the first recombinant class, line 1, females were assayed and showed fully normal PDE-II, establishing that the defects are linked to the X chromosome. For the recombinant males, the data show the fraction of each recombinant genotype showing the enzyme defect characteristic of the *dunce* allele in question. The results show that both enzyme defects map between w^{bl} and *ec*, just as for the learning defect and the total PDE-II level-controlling effect of chromosomal region 3D4. Lines 1 and 2 map the defects to the right of *sc*, lines 3 and 4 map them between w^{bl} and *ec*, and lines 5 and 6 map them to the left of *cv*. Assays were performed as described in the legends to Figures 5 and 6.

Parental genotypes	*sc* +	w^{bl} +	+ *dunce*	*ec* +	*cv* +
Salivary band location	1B3	3C2	3D4	3F1	4F9
Recombination map position	0.0	1.5	~4	5.5	13.7

Recombinant Genotype	Enzyme Defect Present	
	Cross of *dunce*^{1}	Cross of *dunce*^{2}
1. *sc* + + +	5/5	3/3
2. + w^{bl} *ec cv*	0/3	0/3
3. *sc* w^{bl} + +	2/6	1/5
4. + + *ec cv*	2/3	1/4
5. *sc* w^{bl} *ec* +	0/5	0/4
6. + + + *cv*	5/5	4/4

Discussion

Byers et al. (1981) showed by genetic mapping that the same *Drosophila* gene, *dunce*, had been identified independently twice, once via point mutations leading to poor learning (Dudai et al., 1976) and once as a chromosomal region having major effects on cAMP phosphodiesterase (PDE) activity (Kiger and Golanty, 1977). Subsequent work by Kiger and colleagues (Kiger and Golanty, 1979; Davis and Kiger, 1980, 1981) showed that *Drosophila* appeared to have two cyclic nucleotide phosphodiesterases, only one of which was affected by *dunce*, the form here designated PDE-II (previously denoted as heat labile or form II).

For several well characterized structural genes in *Drosophila*, it has been found that enzyme activity is proportional to gene dosage (O'Brien and MacIntyre, 1978). By extrapolation, gene dosage dependence is a suggestive guide to locating structural genes for other enzymes. In 1972, Lindsley, Sandler and 14 collaborators isolated a set of *Drosophila* stocks which allows creation of duplication and deficiency aneuploids for regions of chromosomes averaging about 5% of the genome. Using these stocks, Kiger and Golanty (1977) found a number of chromosomal regions giving elevated total cAMP phosphodiesterase activity when duplicated. In spite of the complications arising from the presence of two independent enzymes active on cAMP, the *dunce* region was identified as having major effects on total cAMP phosphodiesterase activity in duplication and deficiency aneuploids. Finer analysis reduced the chromosomal region involved to approximately one salivary gland chromosome band, band 3D4, providing suggestive evidence that it contains the structural gene for one form of PDE. Shotwell (1981) has repeated this result using substrate specificity to define the different PDE forms and has provided confirmatory data on additional intermediate gene dosages.

In the present report, the two major PDE enzymes of *Drosophila* have been sufficiently well characterized to allow a purification to near homogeneity with the suggestion that each is a monomer. Two different parameters reflecting intrinsic properties of enzymes have been shown to be abnormal in PDE-II from *dunce* mutant stocks: the K_m of PDE-II from $dunce^2$ is increased 10-fold and the thermal stability of PDE-II from $dunce^1$ is decreased substantially. These defects have been mapped to the *dunce* site by genetic recombination experiments. Both normal and abnormal PDE-II activities have been demonstrated in heterozygous flies, eliminating abnormal post-translational modification of a normal enzyme as the cause of the *dunce* defects. For both $dunce^1$ and $dunce^2$, the defects present in crude homogenates are also apparent after extensive purification of PDE-II, thus establishing that the defects are due to alterations of the enzymes themselves and not to an abnormal milieu. Variability in PDE activity as a function of assay procedures has been linked to partial proteolysis since the effects can be mimicked by limited trypsin digestion. These variations do not account for the abnormalities in $dunce^1$ and $dunce^2$. The accumulated evidence, although short of a direct demonstration of a change in amino acid sequence, argues quite strongly that *dunce* is the structural gene for PDE-II.

In mammals, cyclic nucleotide phosphodiesterase activity also has been shown to occur in multiple forms (for recent reviews, see Appleman et al., 1982; Thompson et al., 1979b; Wells and Hardman, 1977; Amer and Kreighbaum, 1975). Although a number of groups have reported purification of a PDE (Ho et al., 1977; Sharma et al., 1980; Morrill et al., 1979; Klee et al., 1979; Thompson et al., 1979a; Marchmont et al., 1981), the relationship of the multiple forms to each other is not firmly established. The physiological roles of the different forms is also unclear, partly due to the lack of drugs which specifically inhibit only one form (Weiss and Hait, 1977).

Flies totally lacking PDE-II, either by a chromosomal deletion or a null activity point mutation, are viable and appear largely normal in morphology and general behavior. This suggests that the poor performance of *dunce* flies in the conditioning paradigms is not a distant consequence of nonspecific disruption of many processes irrelevant to the learning process itself. Dudai (1979) has presented evidence that *dunce* flies are able to learn but forget very rapidly. Recently, B. Tempel and W. Quinn (personal communication) have used an olfactory conditioning paradigm involving reward instead of punishment to show normal conditioning but accelerated memory decay in *dunce*. The clear establishment of the biochemical defect in *dunce* makes it useful as a genetic analog to a highly specific drug. Further exploration of physiological and biochemical aspects of cAMP action, for example, in controlling protein phosphorylation, should be facilitated by comparison of *dunce* and normal flies.

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