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STUDIES ON CYCLIC AMP MEDIATION OF HORMONALLY INDUCED CYTOLYSIS OF THE ALARY HYPODERMAL CELLS AND OF HORMONALLY CONTROLLED DOPA SYNTHESIS IN *LUCILIA CUPRINA*

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ABSTRACT

1. Cyclic AMP initiates cytolytic fragmentation of the hypodermal cells in the wings of newly emerged *Lucilia cuprina* (Australian sheep blowfly) adults and stimulates *in vivo* hydroxylation of tyrosine to DOPA.
2. 3 '5 '-Cyclic IMP is the only other nucleotide that mimics the action of the fragment disaggregating hormone (FDH). However, deamination of cyclic AMP is not a prerequisite for fragment dispersal.
3. The major cyclic nucleotide-dependent protein kinase obtained from homogenates of whole newly emerged flies is not concerned with cyclic AMP mediated, FDH-induced processes.
4. Cyclic AMP content of the flies increases significantly within 1 hour after adult ecdysis, but injection of hormonally active haemolymph into neck ligated, newly emerged flies does not induce an increased rate of cyclic AMP synthesis.

INTRODUCTION

The appearance of elongated cellular fragments in the haemolymph of imagines of *Lucilia cuprina* (the Australian sheep blowfly) is hormonally controlled. It appears likely that the proteinaceous tanning hormone bursicon (Fraenkel and Hsiao, 1965), and the hormone initiating dispersal of fragments are the same chemical entity (Seligman and Doy, 1972). The fragments are derived from the hypodermal cells of the wings and cytoplasmic strands joining the two integumental layers in the wing of the newly emerged adult fly. These strands resemble muscle insertions, having characteristic microtubules and filaments. The strands may occlude the wing during ecdysis or may simply represent an intermediate stage in the programmed cell death of the alary hypodermal cells (Seligman *et al.*, 1972).

3',5'-Cyclic-adenosine monophosphate (cyclic AMP) is a key regulatory agent in many tissues (Reviewed by Robison *et al.*, 1971). Only a few of the known hormonally stimulated processes in insects have been tested for sensitivity to cyclic nucleotides. Cyclic AMP mimiced the effects of serotonin on isolated salivary glands of *Calliphora erythrocephala* larvae. Serotonin stimulated an adenyl cyclase in the gland cells (Berridge, 1970; Berridge and Patel, 1968; Prince *et al.*, 1972). Cyclic AMP stimulated malpighian tubule secretion in locusts (Mordue, 1969), *Rhodnius* and *Carausius* (Maddrell *et al.*, 1971). Cockroach fatbody glycogen phosphorylase was

reported to be stimulated by cyclic AMP (Steele, 1964), but the phosphorylase from the fat body of some silk moths was unresponsive to cyclic AMP (Stevenson and Wyatt, 1964; Wiens and Gilbert, 1967).

Leenders *et al.* (1970) showed that dibutyryl cyclic AMP potentiated the effects of ecdysone on the puffing of polytene chromosomes in *Drosophila hydei.* Cyclic AMP synthesis was stimulated *in vivo* in the epidermis of chilled *Hyalophora gloveri* pupae injected with β-ecdysone. Stimulation was also obtained in pupal wings *in vitro* as well as in wing epidermal homogenates (Applebaum and Gilbert, 1972).

3′,5′-Cyclic-AMP and 3′,5′-cyclic-GMP have been found in whole homogenates of crickets (Ishikawa *et al.*, 1969) and in *Hyalophora cecropia* larval fatbody (Kuo *et al.*, 1972).

In order to investigate the mechanism of hormonal initiation of cytolysis of the wing hypodermal cells and initiation of hardening and darkening of the exocuticle, it was decided to test whether cyclic nucleotides are mediators of these hormonal responses. This investigation deals with the *in vivo* responses of newly emerged imagines to 3′,5′-cyclic-AMP, with identification of the nucleotide in homogenates of flies, and with measurement of changes in the amount of nucleotide soon after eclosion.

MATERIALS AND METHODS

Adenosine-8-^{3}H-3′,5′-cyclic phosphate, ammonium salt, 3 Ci/mmole; adenosine (base-^{14}C(U))-3′,5′-cyclic phosphate ammonium salt, ca. 100 mCi/mmole; adenosine-5-triphosphate- ^{32}P, ammonium salt; and tyrosine-^{14}C(U), 405 mCi/mmole were purchased from Radiochemical Centre, Amersham, Bucks. Adenosine 3′,5′-cyclic phosphate (cyclic AMP), N^{6},2′-O-dibutyryl adenosine 3′,5′-cyclic phosphate (dibutyryl cyclic AMP), tyrosine, dihydroxyphenylalanine (DOPA), imidazole, theophylline, calf thymus histone, alkaline phosphatase (calf intestine) acetone dry powder, and phosphoserine were purhcased from Calbiochem. Guanosine- and inosine- 3′,5′-cyclic phosphates; adenosine-, inosine-, and guanosine-2′,3′-cyclic phosphates were purchased from Sigma Chemical Co., St. Louis, Mo. Adenosine 5-triphosphate (ATP) and adenosine 5-monophosphate (AMP) were purchased from C.F. Boehringer and Soehne, GmbH, Mannheim. A sample of phosphothreonine was obtained from Dr. H. Rosenberg of the John Curtin School of Medical Research, Australian National University. Tyrosine phosphate was synthesized according to the method of Mitchell and Lunan (1964). The DOPA decarboxylase inhibitor, α-hydrazino-α-methyl-β-dihydroxyphenyl propionic acid (αMDH),, was a gift from Merck, Sharp and Dohme Research Laboratories, Rahway, N.J. Cellulose ester membrane filters (HA 0.45μ, 24mm) were purchased from Millipore Corp.

Definitions

(1) Active blood – haemolymph serum collected from flies 15 – 30 minutes after eclosion. The active principle of this preparation is called either bursicon or fragment disaggregating hormone (FDH), depending on whether the measured response was some aspect of cuticle differentiation or autolysis of wing epidermal cells.

(2) Inactive blood – haemolymph serum from flies that had just initiated ecdysis – i.e., their heads protruded from the puparium but they had not yet escaped.

(3) Bursicon and FDH bioassays – a ligature tied between head and thorax of the partially emerged fly (i.e., neck ligated) prevented release of hormone from the central nervous system. Thus, initiation of all hormone dependent process was inhibited. The bursicon and FDH assays were based on reversal of this inhibition by injection of the fly with active blood or some other test material.

Rearing and general bioassay procedures

Rearing of *Lucilia cuprina,* synchronization of emergence of flies from their puparia, procedures for collection of hormonally active and inactive haemolymph, manipulation of flies for bursicon and FDH assays, and the method used to estimate concentration of elongated cellular fragments in the haemolymph are described in detail elsewhere (Seligman and Doy, 1972). The bursicon assay is based on an examination of 6 – 12 neck ligated flies 1½ – 2 hrs. after they had been injected with the test material. The extent of darkening per fly was scored on a 3 point scale. A completely tanned fly scored 2, a partially tanned fly scored 1 and an untanned fly, 0. The FDH assay was based on an estimate of the concentration of elongated cellular fragments in the haemolymph 90 minutes after the flies were injected with the tested materials. Estimates of fragment concentration were made with a haemocytometer using phase optics.

In vivo metabolism of ^{14}C-tyrosine

Neck ligated flies (6 – 12 per replicate) were each injected with 0.005 μCi ^{14}C-tyrosine, 20 nanomoles αMDH to inhibit DOPA utilization and various substances as listed in the legend below Figure 4. Two hours later the flies were homogenized in 6% perchloric acid (2 flies/ml). The precipitated material was removed centrifugally and the supernatant neutralized with 2M KOH. After 10 minutes in ice the insoluble potassium perchlorate was compacted centrifugally and a portion of the supernatant (equivalent to 0.2 fly) dried under a stream of nitrogen at 45°C. The dried material was redissolved in 20 – 30 μl 0.1N HCl and streaked over 1" onto Whatman No. 1 chromatography paper. Chromatographic resolution of the metabolites of ^{14}C-tyrosine was achieved after 16 hours at room temperature in n-butanol/acetic acid/water (4:1:1, v/v). 4Pi geometric scans of radioactivity were made of the dried chromatographs. Only two metabolites were found, DOPA and tyrosine phosphate. DOPA was detected either with an ethylene diamine spray (Schneider and Gillis, 1965) or with a nitrite molybdate spray reagent based on the colourimetric determination of DOPA devised by Arnow (1937). The chromatographs were first sprayed with a freshly made 1:1 (v/v) mixture of 0.05N HCl and nitrite-molybdate reagent (10 g sodium nitrite and 10 g sodium molybdate in 100 ml distilled water) and then immediately thereafter with 1N NaOH. Trimethylsilyl derivatives of DOPA and tyrosine were synthesized and separated gas chromatographically (Atkinson *et al.*, 1971). Tyrosine phosphate was separated from threonine phosphate, serine phosphate and ethanolamine phosphate by ion exchange TLC (DEAE cellulose) in 1% formic acid according to the technique of Fahn *et al.* (1965). Further identification of this radioactive metabolite of ^{14}C-tyrosine as tyrosine phosphate was based on recovery of tyrosine after hydrolysis with alkaline

phosphatase (as described by Seligman *et al.*, 1969b). Amino acid phosphates were stained with ninhydrin (Blackburn, 1968). DOPA and tyrosine phosphate were separated from each other and from their parent compound by high voltage electrophoresis at pH 1.9 according to the technique of Katz *et al.* (1959).

Cyclic AMP activated protein kinases and cyclic AMP binding proteins

The assay for endogenous cyclic AMP was based on the association of nucleotide with a binding protein. The protein binder was obtained from a homogenate of newly emerged imaginal flies processed through the first steps of the purification procedure described by Miyamoto *et al.* (1969) for cyclic nucleotide-dependent protein kinases.

Preparation of protein kinase and cyclic AMP binding protein: Flies were homogenized in 3 volumes cold, neutral 4 mM EDTA in a Virtis homogenizer and centrifuged at 27,000g for 30 minutes. The supernatant was adjusted to pH 4.8 by gradual addition of 1N acetic acid and the resultant precipitate was discarded. The pH of the supernatant was adjusted to pH 6.5 with 1M potassium phosphate buffer pH 7.2 containing 2 mM EDTA. Kinase and binding proteins were precipitated by the addition of ammonium sulphate (32.5 g per 100 ml.). The precipitate was redissolved in a minimal amount of potassium phosphate buffer (5 mM, pH 7.0) containing 2 mM EDTA and then dialysed against an excess of buffer with at least 2 changes over a 14 hour period. The precipitate formed during dialysis was discarded. The supernatant was dispensed (½ ml. aliquots) into pyrex tubes, quick frozen in liquid nitrogen and stored at -20^0C. Although kinase activity had declined appreciably after 5 – 6 weeks storage, binding activity remained high for several months.

Kinase assay: The assay mixture consisted of 10 μ moles sodium phosphate buffer, pH 6.0; 0.6 mg histone; 1.06 nanomoles γ–^{32}P-ATP, 1 x 10^6 dpm; 2 μ moles magnesium acetate; 2 μ moles sodium fluoride; 0.06 μ moles EDTA; 10^{-9} moles cyclic nucleotide and enzyme (200 μg) in a total volume of 0.2 ml. The mixture was incubated for the indicated times at 30^0C. The reaction was stopped with 4 ml ice cold 5% trichloracetic acid and 0.2 ml 0.6% bovine serum albumen was added to the mixture. The mixture was kept on ice for 5 minutes. After centrifugation, the protein pellet was redissolved in 0.1 ml 1N NaOH and the proteins reprecipitated with 2 ml 5% trichloracetic acid. The washing procedure was repeated twice. The proteins were finally dissolved in 0.1 ml 1M Hyamine hydroxide in methanol (Packard) and washed into scintillation vials with scintillation fluid (Bray, 1960).

Cyclic AMP assay: The assay mixture consisted of 12.5 μ moles potassium phosphate buffer (pH 6.5), 5.3 x 10^{-8}M cyclic-(^{3}H)-AMP (31 mμ Ci.), 0 to 100 picomoles cyclic AMP (for standards or from flies) and approximately 200 μg enzyme in a total volume of 0.25 ml. The mixture was incubated for ½ hour at 30^0C and then cooled in an ice bath. The free and protein bound cyclic AMP were separated by filtration through cellulose membrane filters as described by Walton and Garren (1970).

Extraction of cyclic AMP from flies: Flies (20 per replicate) were quick frozen in liquid nitrogen at specified times after eclosion. They were homogenized in 5 ml 0.6% perchloric acid with tritiated cyclic AMP added to monitor recovery. After centrifugation the supernatant was neutralized to pH 6 – 7 with 2M KOH and stood on ice for 10 minutes. The insoluble potassium perchlorate was compacted

centrifugally and 0.2 ml 5% $ZnSO_4$ was added to 3 ml of the supernatant, the mixture was then neutralized with 0.3N $Ba(OH)_2$ and kept in ice for 10 minutes. After further centrifugation 1.5 ml of the supernatant was applied to a 7 x 80 mm Dowex 50W-X8 (100 – 200 mesh) column in the H^+ form. Cyclic AMP was eluted with water in the 5 – 8 ml fraction. This fraction was dried in a rotorary evaporator at 45°C. The residue was taken up in 0.5 ml .05M potassium phosphate buffer (pH 6.5). Duplicate assays were done with 0.2 ml of this solution and 50 µl was used to monitor recovery. In order to check the validity of the extraction procedure, ^{14}C-cyclic AMP was added to the original homogenate and estimates of specific activity (cpm per picomole) were made at each step during the purification procedure (Table 1). The increase in specific activity effected by chromatography on Dowex 50 was probably due to removal of trace amounts of cations that inhibited the binding assay. These cations were introduced into the sytem during the purification procedure. Subsequent paper chromatography did not alter the specific activity of the cyclic AMP thus indicating that the purification procedure through to the Dowex 50 chromatographic step was sufficient for the determination of *in vivo* cyclic AMP levels. Whatman No. 4 paper was first washed with the chromatography solvent (isopropanol/ammonia/water – 7:1:4, v/v) and then dried. After chromatography, the cyclic AMP was eluted from the paper with distilled water.

RESULTS AND DISCUSSION

The minimal requirements necessary to establish that cyclic AMP mediates the transmission of a hormonal signal are discussed by Robison *et al.* (1971). The criteria are based on pharmacological and biochemical properties of the enzymes controlling the levels of cyclic AMP in the target tissue. Pharmacological evidence includes measurements of responses of the tissue (or organism) to application of exogenous cyclic AMP and its analogues, and use of diagnostic, pharmacologically active compounds that specifically activate or inhibit the enzymes synthesising or degrading cyclic AMP. The biochemical evidence required are demonstration of hormonal induction of cyclic AMP synthesis and identification of a membrane bound adenyl cyclase whose activity is modified by the hormone. It is also possible that the hormone controls cyclic AMP levels indirectly, or that some process other than cyclic AMP synthesis– e.g., cyclic AMP hydrolysis, transport or binding– is regulated by the hormone.

Five nanomoles cyclic AMP injected into a neck ligated fly mimiced FDH. The response was indistinguishable from that obtained with hormone (Figure 1). Theophylline – an inhibitor of the phosphodiesterase that hydrolyses cyclic AMP – potentiated the action of FDH (Figure 2). FDH activity in "hormonally active blood" diluted 10 fold was maximal in the presence of 50 nanomoles theophylline. FDH activity was inhibited by a high dose of imidazole (250 nanomoles) – a purported phosphodiesterase activator (Figure 3). These pharmacological data suggest that FDH activity is, indeed, mediated by a cyclic nucleotide.

Only 3′,5′-cyclic AMP and 3′,5′-cycic IMP initiated fragment dispersal. 5′-AMP; ATP; 2-deoxy-3′,5′-cyclic AMP; 2′,3′-cyclic AMP; N^6,2′-0-dibutyryl-3′,5′-cyclic AMP and 3′,5′-cyclic GMP were all inactive (Table 2). The fragment dispersing response thus appears to be specific for 3′,5′-cyclic AMP and its deaminated derivative, 3′,5′-cyclic

Table 1. Purification of ^{14}C-cyclic AMP added to a homogenate of newly emerged flies to constant specific activity*

	^{14}C per unit vol.	pico moles per unit vol.	Specific activity
Perchloric acid extract neutralized with KOH	144	19	7.2
Precipitation of interfering substances with Ba^{++} and Zn^{++}	90	8.7	10.2
Cyclic AMP fraction from Dowex 50 column	145	5.2	28
Paper chromatography (Isopropanol/NH_3 /H_2O– 7:1:2, v/v)	46	1.8	26

*Details of the procedure are described in the text.

Table 2. Effects of various nucleotides related to cyclic AMP on fragment dispersal*

Nucleotide (5 nanomoles/fly)	Fragments per ml x 10^{-4}
ATP	0.25
AMP	9.5
2',3'-cyclic AMP	3.8
3',5'-dibutyryl-cyclic AMP	48
3',5'-cyclic GMP	7.5
3',5'-cyclic IMP	1907±370
3',5'-cyclic AMP	1613±256

*Estimates of fragment concentration were made as described in the legend of Figure 1.

IMP. The lack of response to the dibutyryl derivative of cyclic AMP was probably due to the absence of an esterase in the fly able to hydrolyse the aliphatic side chains in the molecule. Different responses of a tissue to cyclic AMP and dibutyryl cyclic AMP are not unusual. Berridge (1970) showed that dibutyryl cyclic AMP induced a slower rate of water secretion by the salivary glands of *Calliphora erythrocephala* than cyclic AMP. In isolated rat fat cells cyclic AMP stimulated production of CO_2 from lipids and glucose, whereas its dibutyryl derivative inhibited this conversion. On the other hand, dibutyryl cyclic AMP augmented the lipolytic response induced by ACTH, whereas cyclic AMP inhibited this lipolysis (Solomon *et al.*, 1971). No radioactive cyclic IMP could be demonstrated in flies injected with ^{14}C-cyclic AMP. Thus, deamination of cyclic-AMP to cyclic IMP does not appear to be necessary prerequisite for FDH action.

Hardening and darkening of the exocuticle is less sensitive to cyclic nucleotides than is initiation of alary hypodermal cell destruction (unpublished data). However, *in vivo* hydroxylation of ^{14}C-tyrosine to DOPA was clearly stimulated in neck ligated flies injected with 20 nanomoles *a* MDH and active blood or 5 nanomoles cyclic AMP. the metabolites of ^{14}C-tyrosine were separated as described in the method section. The distribution of radioactivity on the paper chromatographs and the relative amount of radioactivity in each peak was calculated (Figure 4). Twenty nanomoles *a* MDH inhibited tanning of the exocuticle but did not stop or retard autolysis of the alary hypodermal cells. More DOPA was formed in the presence of hormone than in the presence of 5 nanomoles cyclic AMP, a dose sufficient to induce a normal fragment disaggregating response. This correlates well with the lower efficiacy of cyclic AMP in stimulating a complete tanning response than that observed with bursicon. *In vivo* formation of tyrosine phosphate was unaffected by both bursicon and cyclic AMP (Figure 4). Thus, more attention should be paid to the integrity of the adenyl cyclase system when studying *in vitro* activation of tyrosine hydroxylation by bursicon. Indentification of DOPA and tyrosine phosphate eluted from the chromatographic paper is discussed in the methods section.

As newly emerged flies were responsive to cyclic AMP it was of interest to determine if flies at this stage have a protein kinase activated by cyclic nucleotides. A preparation of protein kinase was made according to the procedure of Miyamoto *et al.* (1969) and the kinase and cyclic AMP binding properties of this preparation was tested. Cyclic IMP and cyclic GMP stimulated the kinase equally, but to a lesser extent than cyclic AMP (Figure 5). Thus, the major protein kinase in the preparation was not concerned with degeneration of the alary hypodermal cells. The difference between the two cyclic nucleotide activated systems was that cyclic IMP induced degerneration of the alary hypodermal cells as effectively as cyclic AMP, unlike cyclic GMP which was entirely inactive; whereas activation of the protein kinase by cyclic IMP and cyclic GMP had similar kinetics. The apparent Ka for cyclic AMP and for cyclic GMP were 0.14μM and o.36 μM respectively (Figure 6). Kuo et al. (1971) surveyed the protein kinases from several arthropod species. Their preparation of cyclic nucleotide dependent protein kinase from whole *Drosophila melanogaster* adults resembled our preparations from *Lucilia cuprina.* The preponderance of the cyclic AMP activated kinase appears to be a characteristic of enzymes derived from insect flight muscle.

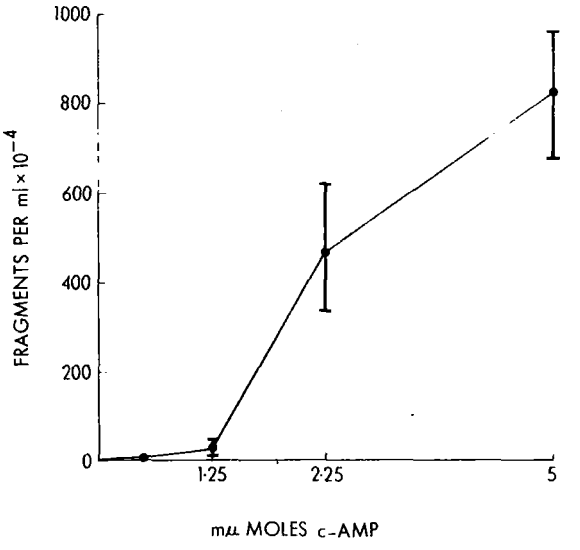

Fig. 1: Fragment dispersal induced by cyclic AMP. Neck ligated flies were injected with an aqueous solution of cyclic AMP (1 μl) and the injection wound covered with bees wax. Ninety minutes later the flies were bled and estimates of fragment concentration were made on a haemocytometer using phase optics. Six to twelve flies were used per replicate and each determination was repeated 4 to 8 times. The I shaped bars denote S.D.

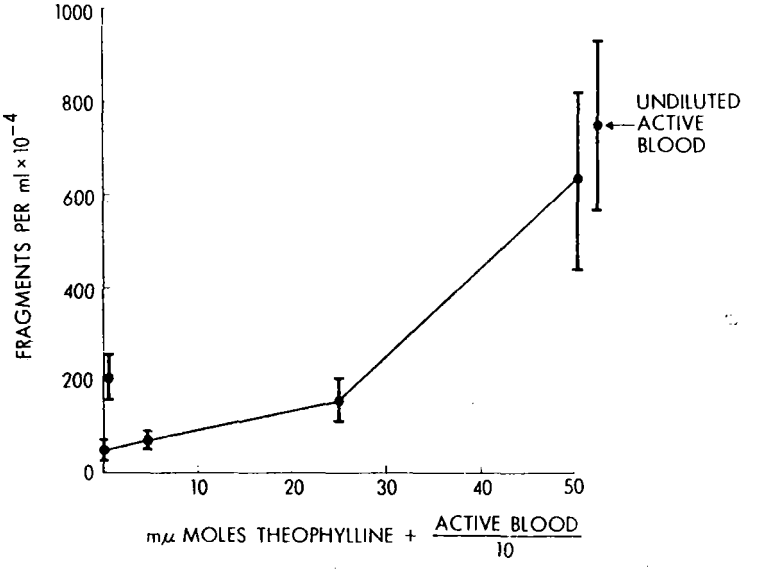

Fig. 2: Potentiation of the FDH activity with theophylline. Ten times diluted active blood and an aqueous solution of theophylline (1:1, v/v) were mixed and 1 μl of the mixture injected into each fly. The active blood was diluted with Ephrussi-Beadle ringer solution. Estimates of fragment concentration were made as described in the legend of Figure 1.

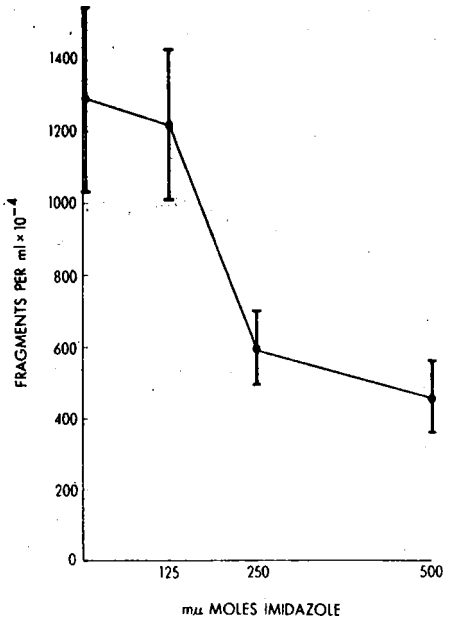

Fig. 3: Inhibition of FDH activity with imidazole. Active blood and an aqueous solution of imidazole (1:1, v/v) were mixed and 1 μl of the mixture injected into each fly. Estimates of fragment concentration were made as described in the legend of Figure 1.

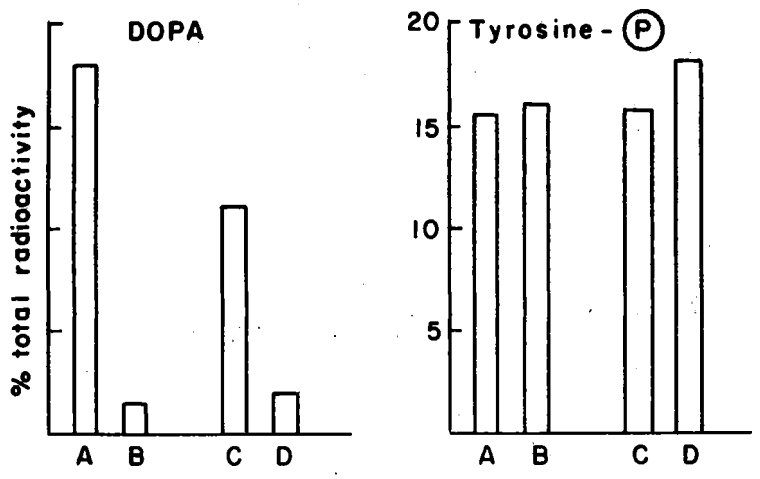

Fig. 4: Effects of active blood, inactive blood, cyclic AMP, and 5 AMP on the *in vivo* hydroxylation of tyrosine in the presence of αMDH. Experimental and chromatographic procedures are described in the text. A = 1 μl active blood; B = 1 μl inactive blood; C = 5 nonomoles cyclic AMP; D = 5 nanomoles 5 AMP.

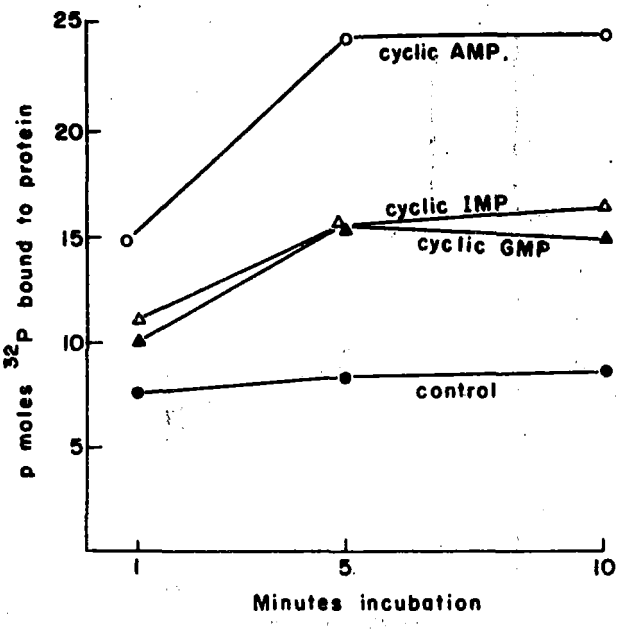

Fig. 5: Time course of protein kinase activity in the presence of various 3',5'-cyclic nucleotides. Details of the assay are given in the text. O——O cyclic AMP; +——+ cyclic GMP; Δ——Δ cyclic IMP.

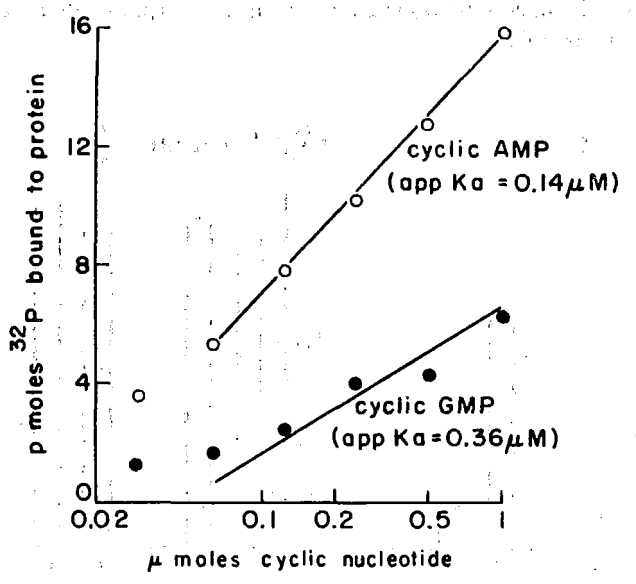

Fig. 6: Effect of varying cyclic AMP and cyclic GMP concentration on the activity of the protein kinase preparation. Incubation conditions were as described in the text, except for variation in amount of cyclic nucleotide as indicated. Values have been corrected for protein phosphorylation occurring in the absence of added cyclic nucleotide (7.3 pmoles). Incubation time = 5 minutes. The regression lines were estimated by the method of least squares. Each point represents the mean of duplicate assays.

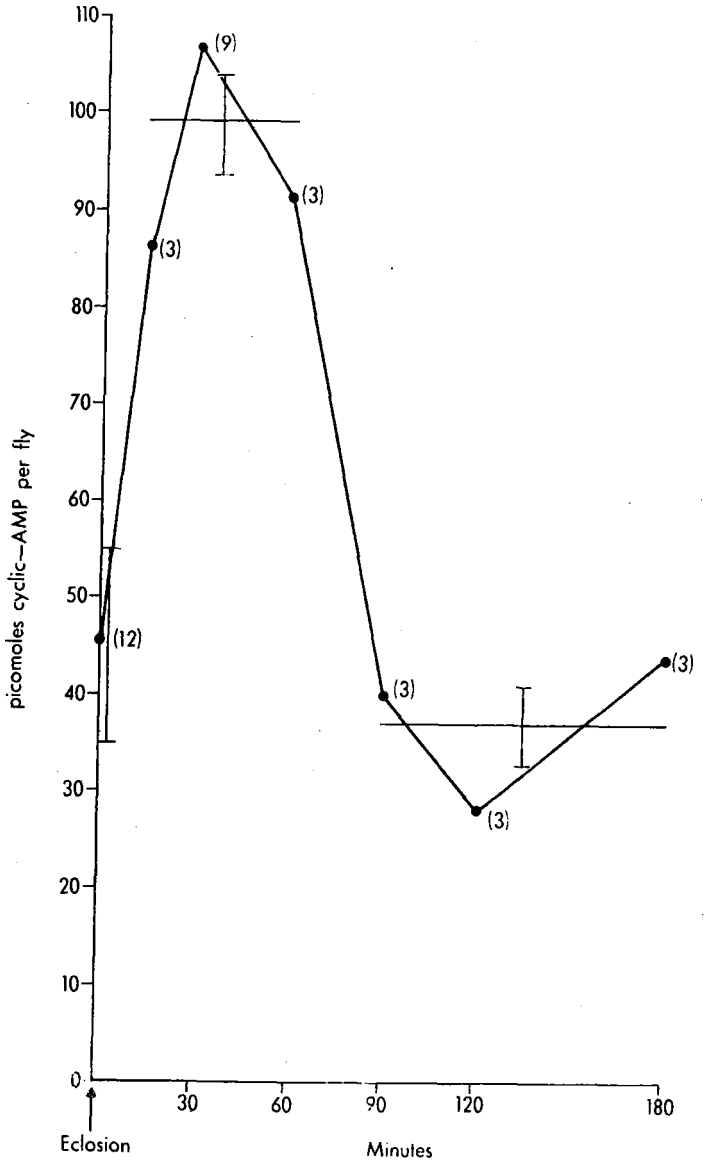

Fig. 7: Flux of cyclic AMP in whole flies after eclosion. Ten to twenty flies were used per replicate and the number in parathenses at each point signifies the number of replicates. Horizontal lines represent the mean of all values within the indicated time interval. The I shaped vertical bars represent S.E. Details of the procedure are described in the text.

Cyclic AMP binding by the *Lucilia cuprina* preparation was considerable and was used routinely for the assay of endogenous cyclic AMP in whole flies. Levels of cyclic AMP increased significantly within 1 hour after eclosion ($p<0.01$) and then subsided again to the level found in newly emerged flies (Figure 6). However, an increase in cyclic AMP in whole flies was not induced by injection of active blood irrespective of the addition of theophylline. We could not demonstrate *in vivo* biosynthesis of cyclic AMP in neck ligated animals coinjected with ^{14}C-adenosine and active blood, and we were unable to find an adenyl cyclase in homogenates of whole animals activated by "active blood". Thus, the increased levels of cyclic AMP soon after eclosion might not be a direct consequence of the release of a hormone from the central nervous system of the fly. However, since these experiments were done on whole animals the effects we sought might have been masked by the presence of a large amount of unresponsive tissue. Endogenously produced hormone(s) greatly exceeded the amount injected into neck ligated test animals. The normal haemolymph volume of a newly emerged fly is 5 – 10 µl and only 0.5 µl active blood was used to test for *in vivo* stimulation of cyclic AMP production.

The metabolic fate of cyclic AMP is an important and possibly critical factor that has not been controlled adequately in these experiments. Uda *et al.* (1969), using usual chemical procedures, found that the catabolic products of RNA in *Drosophila melanogaster* were the 2′,3′-cyclic nucleoside monophosphates of guanosine, uracil, cytosine and inosine. They showed that replacement of 2′,3′-cyclic AMP by 2′,3′-cyclic IMP was due to the presence of an adenine deaminase. Thus, *in vivo* measurements of cyclic AMP alone might be an insufficient indicator of cyclase activity as the intracellular hormonal mediators might be deaminated rapidly in the tissues. Interpretation of experiments on the metabolism of ^{14}C-cyclic AMP injected into flies are equivocal as the quantitatively important transformation probably occurred outside the target tissue. These experiments need to be repeated on isolated target tissues. If the hypodermal cells of the wings are indeed a target tissue of FDH (Seligman *et al.*, 1972) then this is the tissue of choice for further work on the mode of action of this hormone.

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