PHYSIOLOGICAL CYCLIC AMP IN *DROSOPHILA*

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1. Introduction

Adenylate cyclase and phosphodiesterase have been identified in insect tissues and are actively investigated [1–4]. The physiological role of cyclic AMP has been studied by means of phosphodiesterase inhibitors [5,6] or exogenous cyclic AMP injected into the insects or added to isolated organs or cell cultures [7,8]. But, because of the small size of insect tissue samples and the small levels of cyclic AMP, the latter has never been accurately determined. A radioimmunoassay method [9] allowed us to measure these concentrations with great accuracy, both in pre-adult and adult stages of *Drosophila melanogaster*. We have found marked variations according to characteristic steps of the fly's development, formation of the puparium, nymphal and imaginal molts. Excepted these cyclic AMP crises, we observed much higher concentrations in imagos than in larvae and pupae.

2. Materials and methods

Drosophila melanogaster were reared at 28°C on a standard diet. The insects used for cyclic AMP assay were hatched from eggs laid within a one hour interval. They were sampled at critical phases of the postembryonic development, i.e. at the end of the third larval instar, during the puparium formation, during the pupal period, at the emergence of the imago, and at different times during their adult life. The formation of the puparium lasts only a few minutes, and this stage is used to select synchronous insects. Time zero represents puparium formation.

The cyclic AMP radioimmunoassay [9] was performed on the total extract of insects. Since the anti-cyclic AMP antibodies used were obtained against 2′*O*-succinyl cyclic AMP-albumin, the nucleotide to be measured was previously succinylated, giving a 100-fold more sensitive assay than usual [10].

Four to seven unanaesthetized insects were used for each extract sample and were quick-frozen in liquid nitrogen. They were then lyophilised and stored at −40°C until they were assayed. They were homogenised into 200 µl of 1 N $HClO_4$ by a micro Potter–Elvejhem apparatus. The homogenate was centrifuged 5 min at 15 000 rpm to eliminate proteins and chitin. 150 µl of the supernatant were neutralized with 15 µl of 6M K_2CO_3 and centrifuged 5 min at 15 000 rpm to discard the pellet of $KClO_4$. 100 µl of the supernatant were added to 5 mg of succinic anhydride and 10 µl of triethylamine. The incubation lasted 10 min at room temperature. The cyclic AMP contained in the sample was converted into 2′*O*-succinyl cyclic AMP.

After dilutions of 1/11 and 1/22, the unknown quantity of cyclic AMP was determined in duplicate samples by competition of radioactive analogue ([^{125}I]-succinyl cyclic AMP tyrosine methyl ester) and nonradioactive cyclic AMP, for the anti-3′,5′-cyclic AMP antibodies. Equilibrium dialysis was used to seperate free and bound nucleotides. Known quantities of succinyl cyclic AMP were used to construct a standard curve. 200 µl of 0.1 N NaOH were added to the pellet of the first centrifugation and stored overnight at room temperature. The residual insoluble material was then discarded by moderate centrifugation and the soluble

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proteins were assayed by Lowry's method.

The DNA assay was also performed in larvae, pupae and adults. About a hundred of lyophilised insects were homogenised in 1 ml of 1 N $HClO_4$ and centrifuged 5 min at 15 000 rpm. The supernatant was removed and the pellet resuspended in 1 ml $HClO_4$. After sonication of the sample, the DNA was hydrolysed 10 min at 90°C under agitation and the solution was centrifuged. The supernatant was collected and the pellet was resuspended in 1 ml $HClO_4$, then discarded. The supernatants were pooled and the DNA content of 500 μl aliquots determined in duplicate by diphenylamine coloration [10].

The results of the cyclic AMP assay, expressed as picomoles, were related to fresh and dry weight of tissues and to DNA and protein contents. We also considered the total cyclic AMP content of one insect at its different developmental stages.

3. Results

We observed that *Drosophila* contained from 0.03 to 1 pmole of cyclic AMP per mg fresh tissues. The lower values were observed in pre-adult stages (fig. 1). In the third instar larvae we noted 0.17 pmole/mg (0.29 pmole in a whole larva). This level is still lower in the pupae where it did not exceed 0.07 pmole/mg (0.09 pmole/individual). No change was seen during the pupaison, but it should be noted that the transition from the larva to the pupa, when the larva begins to form its puparium, was marked by a sharp increase of cyclic AMP concentration followed by an even more pronounced decrease.

Another substantial and quick variation of cyclic AMP content occured at the emergence of the imago from the puparium and in adults the level was not much less than 1 pmole/mg of wet tissues. This corresponds to a micromolar concentration in homogenised tissues. If we consider one fly in its entity, we can see that the total body content is 0.9 pmole in males and 1.8 in females. This difference is due to the large size of the female since the cyclic AMP content per mg wet tissues of both males and females were not significantly different. Similarly, when the cyclic AMP content of females in different stages of their life cycle was determined no significant difference was found between flies at the emergence of the puparium and during active reproduction. We observed then no further

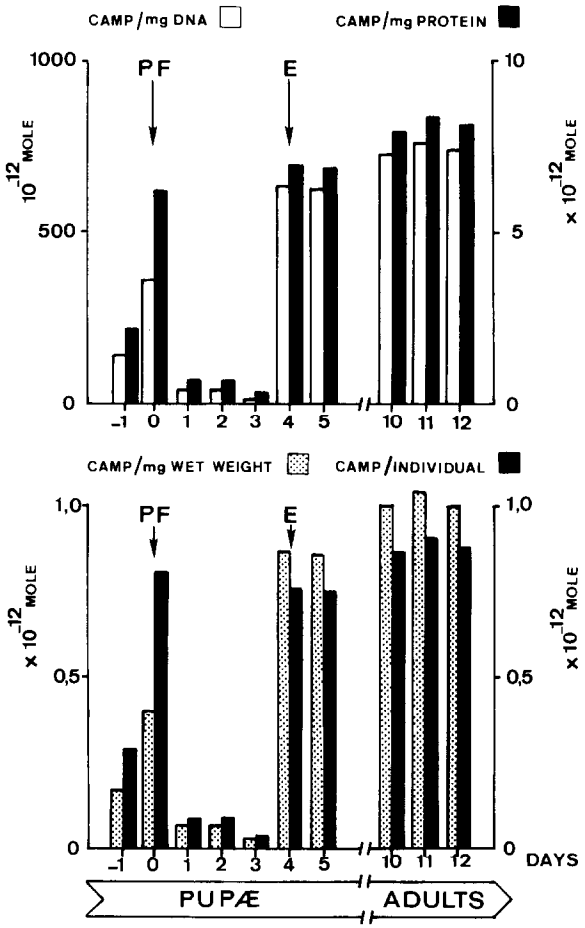

Fig. 1. Cyclic AMP was expressed at pmoles vs.: mg DNA; mg protein: mg wet weight; individual. All the flies were males. PF: puparium formation. E: emergence.

noticeable variation in cyclic AMP level throughout the adult life, even in the last surviving individuals, 24 days after emergence.

A DNA concentration of 0.047 mg per mg dry tissues was found in adults and no significant variation from this value was observed in larvae and in pupae. It follows that the ratio of total number of cells per mg dry weight is remarkably constant throughout the *Drosophila* life. Moreover, the protein content of the insect was determined as 30% of the dry tissues weight in larvae and in pupae and as 45% in adults. It follows, as shown in the figure, that the described variations of cyclic AMP concentration in *Drosophila* developmental stages and in

adults remains valid whether DNA or protein was used as reference system.

4. Discussion and conclusions

The observed enhancement of cyclic AMP levels at the emergence of the insect from its puparium agrees in part with the results of Castillon et al. [1]. They noted an increase of adenylate cyclase activity during the last half of the nymphal period of the fly *Ceratitis capitata*. This observation, however, does not necessarily imply that there is a simultaneous increment of cyclic AMP concentrations which is determined both by adenyl cyclase and phosphodiesterase activity.

The physiological significance of our observations is unclear at present. In mammals, it is known that cyclic AMP acts as a second hormonal messenger [12], but this role for cyclic AMP in insects has received much attention only very recently. Circumstantial evidence has been provided that this cyclic nucleotide is probably implicated in the process of activation of the prothoracic glands by the brain hormone in *Manduca sexta* [3]. In fact, the peak of cyclic AMP concentrations that we observed in *Drosophila* during the formation of the puparium may be related to the hormonal events that control the transformation of the larvae. We like to draw special attention to the ten fold increase in cyclic AMP concentrations observed at the emergence from the puparium and to its persistent high value during adult life. This high cyclic AMP level, as compared to the low level in larvae and pupae may be due to endocrine activities of the imago. Work is now in progress to determine the influence of endocrine activity on cyclic AMP levels in adult *Drosophila*.

The radioimmunoassay method used in our experiments allows us to determine easily and accurately endogenous levels of cyclic AMP. It should prove to be an excellent tool for further studies aimed to clarify the physiological role of cyclic AMP in insects.

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