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0014-4754/83/020161-03$1.50+0.20/0


## About the photostimulation of ATP production in the integument of insects

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*Summary.* The photostimulation of ATP production in the integument of *Pieris brassicae* has been reexamined, using 2 different methods for ATP estimation. As could be demonstrated with the luciferin-luciferase assay, the so-called photostimulation is an artefact which is due to inappropriate utilization of the Boehringer kit assay.

Vuillaume et al.[2,3] described a photostimulated synthesis of ATP in integument homogenates and in cell-free extracts of the same tegument in the caterpillar of *Pieris brassicae*.

Because of the great potential importance of the newly described system, which would give to the insects a very unusual and surprising position, near to all the chlorophyll-containing plants or bacteria and certain microorganisms like *Halobacterium halobium*[4] which solar energy for phosphorylation, we started a program aimed at the identification of the reactive molecule (or molecules) involved. To increase the sensitivity and specificity of ATP estimation, we adopted the luciferin-luciferase assay[5].

Only very small amounts of ATP were found. None of the qualitative and quantitative results described in the published papers could be reproduced. To understand the origin of such a discrepancy, we felt is necessary to consider again the method used by Vuillaume et al.[2,3] for measuring ATP. In their work, they use an assay kit based on the decrease of NADH, which might be nonspecific in a complex medium.

In fact, depending of the method used, 2 opposite results are obtained. As already described, using the Boehringer kit for ATP assay, an important decrease in NADH is observed with extracts of integuments from *Pieris brassicae*, which can be enhanced under illumination. It is this decrease in NADH which is translated into ATP. But, with the specific luciferin-luciferase assay, only a very low amount of ATP is found and it is rapidly degraded, whether under illumination or in darkness.

*Materials and methods.* The 5th instar larvae of *Pieris brassicae* were taken under the same conditions as described by previous authors[2,3]: at the end of the instar (mean fresh weight 500 mg), bred from hatching in white light, photoperiod 16/8 h, temperature 20 °C. The homogenate of the integument was prepared as described in darkness with a mechanical Potter homogenizer and the same cold buffer pH 7.2 (0.05 M Tris-HCl, 0.25 M sucrose, 2.5 mM $CaCl_2$). Examination of the extracted integuments under a binocular microscope showed total epidermis extraction.

The Boehringer kit for ATP assay (B.M. assay) from Boehringer-Mannheim based on the following sequence of reactions:

$$\text{glycerate-3-P} + \text{ATP} \xrightarrow{\text{PGK}} \text{glycerate-1,3-P}_2 + \text{ADP}$$

$$\text{glycerate-1,3-P}_2 + \text{NADH} + \text{H}^+ \xrightarrow{\text{GAPDH}} \text{GAP} + \text{P}_i + \text{NAD}^+$$

$$\text{GAP} \xrightarrow{\text{TIM}} \text{DAP}$$

$$\text{DAP} + \text{NADH} + \text{H}^+ \xrightarrow{\text{GDH}} \text{glycerol-1-P} + \text{NAD}^+$$

Abbreviations used: PGK, phosphoglycerate kinase; GAPDH, glyceraldehyde phosphate dehydrogenase; TIM, triose phosphate isomerase; GDH, glycerol phosphate dehydrogenase.

Luciferin-luciferase, Firefly lantern extract (L.L. assay) and ATP were from Sigma. All other chemicals were of the purest quality available from Prolabo or Merck.

Incubation of the extracts under light was as described by previous authors[2,3].

*Results.* One example of quantitative determinations by both types of assay and different treatments is presented in the table. With the luciferin-luciferase assay, ATP concentration is about 100–1000 times smaller than with the Boehringer assay, and there is no increase after incubation. If a known amount of ATP is added, it is recovered by either of the methods. After 1-h incubation most of this added ATP is hydrolyzed: about 70% with the Boehringer

Amount of ATP detected by 2 different assays

| Boehringer assay | ATP mg/g | Luciferin-luciferase assay | ATP µg/g |
|---|---|---|---|
| T=0 | 0.25 | T=0 | 17.8 |
| T=0+ATP | 0.71 | T=0+ATP | 64.7 |
| T=1 L | 1.02 | T=1 L | 1.1 |
| T=1 L+ATP | 1.15 | T=1 D | 9.7 |
| T=2 L | 1.41 | | |

The results are the average of duplicate assays and are expressed in mg or µg of ATP in the total extract obtained from 1 g of original wet integument. T=0, ATP measured without incubation, T=1 L and T=2 L, after incubation, respectively for 1 h or 2 h under illumination; T=1 D after incubation in darkness. All incubations were performed at 20 °C. For controls, ATP was added: 0.5 mg/g for the B.M. assay und 50 µg for the L.L. assay. The caterpillars were bred under white light, with photoperiod 16 h illumination, 8 h darkness during their larval life.

assay; with the luciferin-luciferase assay more than 90% of the added ATP is hydrolyzed within 1 h. If the samples are incubated after perchloric acid deproteinization, this ATP is stable and is measured in both types of assay (results not shown).

Then, instead of the awaited increase, there is an absolute decrease in ATP concentration even with the Boehringer assay, since only 1.15 mg/ATP was found where 1.52 (1.02 + 0.50) was expected.

Very similar results were obtained with larvae reared under red light or in darkness in the same described conditions[2,3]. Between 3 independent sets of experiments, ATP concentration in fresh integument extracts varied considerably (up to 100%), but within each series of incubations, the results are comparable to those of the table.

*Discussion.* Comparing the data obtained with the specific luciferin-luciferase assay with those based on an extensive sequence of reactions, it is obvious that the 'light-dependent ATP production system (LAPS)' in *Pieris brassicae*[2,3] is an artefact due to the inappropriate utilization of the Boehringer kit, prepared for ATP estimation in human blood. With the luciferin-luciferase assay, there is no measurable LAPS. Not only is ATP not synthesized under illumination, but instead, upon incubation it is used up, probably hydrolyzed. To explain the high ATP concentration found by Vuillaume et al.[2,3], one must suppose that, in the integument extracts, there must be some molecule which is estimated as ATP (like other nucleosides triphosphate) or more likely is involved in some reaction, induced by the kit enzymes, which utilizes NADH as a cosubstrate. The amount of this molecule is changed under illumination. The published values for ATP concentration in integument extracts were rather high, one of the highest concentration known for ATP reaching 4–5 mg/g integument, when rat or rabbit muscle[6] has only about 2 mg/g and *E. coli*[7] about 2 mg/g dry wt.

We must also remark that the so-called ATP synthesis was, for these authors, related to the presence of the pigment Pterobiline in the epidermis where it would act as a photoreceptor, absorbing in the red part of the spectrum and therefore preventing diapause[8-11]. Consequently, all explanations in which diapause is related to pterobiline and LAPS must be viewed with caution.

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0014-4754/83/020163-02$1.50 + 0.20/0


## Effects of cysteine and N-acetyl cysteine on GSH content of brain of adult rats

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*Summary.* Intraperitoneal injections of cysteine or N-acetyl cysteine induce a depletion of reduced glutathione (GSH) in rat brain. The doses required to promote GSH depletion are lower than those reported to cause a disseminate neurodegenerative syndrome. Since physiological GSH concentrations are required to maintain cell membranes, we suggest that consideration of the cysteine-induced GSH depletion is important in attempts to understand the mechanism of cysteine-induced cytotoxicity in brain.

We reported earlier[1,2] that cysteine induces a depletion of reduced glutathione (GSH) in isolated hepatocytes and that i.p. injections of cysteine, or its derivative N-acetyl cysteine, also promote a depletion of hepatic GSH[3]. Olney and Ho[4] have reported that very high doses of cysteine result in the death of infant rats. However, sublethal doses of cysteine induce a widely disseminated neurodegenerative syndrome[5]. Here we report that cysteine induces a depletion of GSH in the brain of adult rats.

*Materials and methods.* L-Cysteine or N-acetyl cysteine were injected i.p. to adult Wistar rats fed with a standard laboratory chow (from Prasa, Vara de Quart, Valencia, Spain).

Solutions of the amino acids were made in water immediately before use and care was taken that the pH was 7.0 ± 0.1. The animals were killed 2 h after the injection, by decapitation. The brain was immediately removed, weighed and homogenized in 10 vol. of a solution of 2% perchloric acid in ice-cold physiological saline. The time that elapsed between killing the rat and obtaining the acid homogenate was always less than 2 min.

GSH was determined under the conditions described by Viña et al.[1]. All values are means ± SD for the number of observations in parentheses.

The concentration of GSH in the brain of untreated fed rats was 3.25 ± 0.38 μmoles/g fresh weight.

*Results and discussion.* The effect of various doses of cysteine on GSH concentration in rat brain is shown in table 1. It is important to notice that injections of 0.25 g/kg b.wt, of cysteine are sufficient to induce a depletion of GSH to values of 60% of the controls. Morphological alterations were observed when the doses of cysteine used were 0.8 g/kg[5]. However, when N-acetyl cysteine was used, a higher dose (1 g/kg) was needed to induce a depletion of GSH to values of 60% of controls (table 2). N-acetyl cysteine is rapidly deacetylated in liver to yield free cysteine[6] which may, in turn, induce a depletion of GSH in brain.