Bolletino di zoologia

ISSN: 0373-4137 (Print) (Online) Journal homepage: http://www.tandfonline.com/loi/tizo19

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To cite this article: Emilio Balletto , Attilio Arillo , Paola Mensi & Maria Adelaide Cherchi (1987) Zygaenid moths, cyanide and thanatosis, Bolletino di zoologia, 54:1, 59-63, DOI: 10.1080/11250008709355557

To link to this article: http://dx.doi.org/10.1080/11250008709355557

Boll. Zool. 54: 59-63 (1987)

Zygaenid moths, cyanide and thanatosis

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ABSTRACT

Sensitivity of burnet mitochondria to cyanide was studied and some detoxifying mechanisms were investigated.Results showed that burnet mitochondria are relatively sensitive to KCN and that the inhibitory effects of cyanide can be completely reversed by pyruvate. Additionally, some glycolytic intermediates were determined *in vivo* at differential times from the beginning of the crushing-induced thanatosis and at subsequent arousal.

On the basis of our results, a hypothesis is suggested to explain both the well-known resistance shown *in vivo* by burnets to hydrocyanic acid, and the biochemical mechanism at the root of thanatosis.

KEY WORDS: Zygaenids; Mitochondria; Cyanide; Thanatosis.

ACKNOWLEDGEMENTS

This work was financially supported by the Italian Ministry for Public Education (MPI) and by the Consiglio Nazionale delle Ricerche under «Gruppo nazionale di Biologia naturalistica».

(Accepted 10 February 1986)

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INTRODUCTION

Burnets (Genus *Zygaena*) constitute an aposematic Muellerian complex of unpalatable moths (Bullini *et al.*, 1969; Sbordoni & Bullini, 1971), whose main deterrent property is represented by their ability to release large amounts of hydrocyanic acid when crushed. This release was shown to occur both in their muscular tissues and haemolymph. The latter, in turn, may be emitted externally through specialized «bleeding areas» (Jones *et al.*, 1962). Cyanide production derives from the hydrolysis of the cyanoglucosides, linamarin and lotoaustralin, having either a dietary or a biosynthetic origin (Davis & Nahrstedt, 1979; 1982; Nahrstedt & Davis, 1981). Burnets, it is maintained, look perfectly dead when they are rejected by, say, an insectivorous bird who lets them immediately drop in the grass on perceiving the «bad taste» of their haemolymph. In fact, gulping the insect would probably be fatal, at least where some species are concerned (Davis & Nahrstedt, 1982). However, all lepidopterists know that even a severe crushing of the thorax seldom kills burnets. In fact, after a while they start walking again and attain complete recovery in a few minutes. This type of thanatosis, that may be defined as «severe thanatosis», is not to be confused with another behaviour. In the late evening, or in cool overcast weather, burnets perched on grass stems may let themselves drop to the ground when disturbed, instead of flying as they always do in warm, sunny conditions.

The latter behaviour («mild thanatosis») is probably linked to the difficulty, for these ectotherms, to produce high levels of metabolic activity at unfavourable temperatures. Though it may also have evolved as a consequence of the fact that in the shadow the bright red colour of their wings is far less visible than in the sun.

However, in this study we shall only refer to «severe thanatosis» and provide some suggestions that lead to the consideration that cyanide release and its subquent detoxification, are the prime biochemical mechanisms triggering this behaviour.

For this purpose, we tested *in vitro* the action of cyanide on the mitochondria from thorax muscles of *Zygaena fausta*. The variation of some glycolytic intermediates was studied *in vivo* during artificially induced thanatosis and subsequent arousal.

A mechanism for the cyclic removal of cyanide produced by crushing is also proposed.

MATERIALS AND METHODS

Mitochondrial respiration

Mitochondria from thorax muscles of specimens of *Zygaena fausta* (L.) were isolated as described by Van den Berg (1967). Oxygen consumption was determined polarographically with a Clark-type electrode (Gilson Instruments) at 25°C, using succinate as a substrate. The assay medium (pH 7.1) contained 10 mM Nacl, 25 mM TES, 16 μM albumin EFAF (SIGMA), 2 mM succinate, 1 μM

rotenone, 325 mM phosphate buffer, 5 mM $MgCl_2$, 1 mg mitochondrial protein. Proteins were determined by the method reported by Lowry *et al.* (1951). State 3 of oxidative phosphorylation was assessed by the addition to the incubation mixture (1.8 ml) of 200 nano-moles of ADP. The effect of cyanide was tested by following the variation in the respiration rate of phosphorylating mitochondria after the addition of 0.5-1.5 mM KCN to the incubation mixture.

Recovery was studied both as a result of spontaneous detoxification and after the addition of thiosulphate (50 mM), sodium pyruvate 0.5 mM), or aliquots of postmitochondrial fraction (PMF) (4-7 mg original tissue/ml assay mixture). PMF represents the supernatant obtained at 12 000 × g from 50 mg of thorax muscles homogenized in the same buffer used for the extraction of mitochondria.

A purified microsomal fraction, obtained from the thorax muscles of the same moths as suggested by Collins & Hooper (1984), was also employed of the same purpose.

Rodanese activity

The activity of this enzyme was assayed according to Sorbo (1955), both in the mitochondrial and in the postmitochondrial fractions.

Lactate, glycero-phosphate and pyruvate assay

The concentrations of these compounds were assayed enzymatically by following NAD^+ reduction in the presence of lactate dehydrogenase or glycerol-1-phosphate dehydrogenase, respectively for lactate and glycerophosphate (Hohorst, 1965a; b) and NADH oxidation for pyruvate (Buecher *et al.*, 1965).

RESULTS

Mitochondrial respiration

Mitochondrial oxygen consumption is inhibited (95%) by 0.5 mM KCN (Table I). Though, a 50% spontaneous recovery of respiration is observed after about one minute. No such recovery was detected with higher concentrations of cyanide (1.5 mM).

The cyanide-induced respiration block is unaffected either by the addition of 50 mM thiosulphate or by an equal amount of the microsomal fraction administered together with a NADPH regenerating system.

In contrast, the addition of a suspension with Na-pyruvate (pH 7.0) reinstates normal respiration after 4 minutes for the lower CN molarity (0.5 mM). At higher molarity (1.5 mM) the recovery after 5 minutes is only 55% of the initial respiration. Different results are also obtained by the addition to the mixture of various amounts of a crude postmitochondrial fraction (PMF). Higher amounts (7 mg original tissue/ml assay mixture) induce a practically immediate recovery of oxygen consumption, whereas smaller concentrations (4 mg/ml) only reinstate respiration by 65%.

Rodanese activity

The rodanese-like enzymatic activity is extremely scarce and only amounts to 1-2 nanomoles of thiocyanate formed per mg of protein in 5 minutes (data not shown).

Lactate, glycero-phospate, and pyruvate

Lactate undergoes a progressive increase, particularly towards the end of thanatosis and during arousal. Pyruvate concentration shows a sharp increase in advanced thanatosis and falls to values near those of the controls during arousal. Glycerophosphate remains constant throughout thanatosis, but doubles its concentration upon arousal (Fig. 1).

DISCUSSION

It is a well-known fact that burnets are comparatively more resistant to cyanid spontaneously hydrolyzed by air moisture than most other Lepidoptera (Rocci, 1914; Jordan, 1916). However, our results show that they are completely immobilized in about 15 secs when they are exposed to an HCN-saturated atmosphere.

The mitochondrial resistance to hydrocyanic acid of these moths is comparable to that known for mammalian organelles and little or no spontaneous recovery could be detected under the action of high concentrations of cyanide (1.5 mM).

In contrast, in the presence of 0.5 mM KCN, a significant spontaneous recovery occurred, so that about 50% of the original respiration rate was restored after 1 minute (Table I). It was possible to demonstrate that this recovery does not depend to any relevant degree on rodanese-like activities, that were shown to be very low in our mitochondrial preparations (see results). As a confirmation, the addition of thiosulphate to the polarographic assay mixture failed to activate the respiration of CN-inhibited mitochondria.

As o-type cytochromes, or similar, are not known to occur in the insects, it seems reasonable to exclude that the cyanide-resistant fraction of respiration may depend on alternative oxidase systems. In our opinion, the partial recovery shown by the zygaenid mitochondria after the addition of KCN, is probably dependent on a slight contamination with detoxifying cytoplasmic factors. In fact, some reactivation in the respiration rate could also be elicited by adding aliquots of crude postmitochondrial fraction to mitochondria previously treated with 0.5 mM cyanide (Table I). The level of restoration is proportional to the amount of PMF added.

With higher concentrations of cyanide (1.5 mM), the effect of the PMF is still perceptible, albeit very scarce (data not shown). However microsomal metabolism does not participate in the detoxifying process, as the addition of a microsomal fraction to the polarographic mixture had no demonstrable effect on the respiration rate (Table I). Therefore it seems more likely that contaminant cytoplasmic keto-acids (or keto-acid producing enzymatic systems) may represent the principal factor responsible for the apparently cyanide-insensitive part of respiration. In fact, it has been known for a longtime that cyanide is ineffective as an inhibitor of respiration in the presence of such compounds owing

TABLE I – *Respiration of the mitochondria from the thorax muscles of* Zygaena fausta *(L.) under several experimental conditions. Assay mixtures, all containing 1 mg/ml mitochondrial proteins, plus the addition of the compounds shown. Respiration rate was assayed before (1) and after (2) the addition of the compounds tested. Time required by the added compounds to produce the indicated level of recovery (3) is reported in brackets. Level of recovery is also expressed as percent value of the initial respiration. PMF = post mitochondrial fraction (supernatant recovered after 12 000 × g).*

Compounds added	O_2 consumption (n atoms mol/ min/mg prot. at state 3 of respiration) initial respiration (1)	residual respiration (2)	reactivation of respiration (3)	approximate of initial respiration
none	49.5 ± 0.45			
KCN (0.5 nM)	50.1 ± 0.38	2.47 ± 0.22	24.1 ± 2.7 (1 min)	50%
KCN (0.5 mM) + Na-pyruvate (0.5 mM)	44.4 ± 0.42	2.40 ± 0.15	43.2 ± 5.01 (4 min)	100%
KCN (0.5 mM) + PMF (7 mg/ml)	50.3 ± 0.57	47.8 ± 0.25	47.8 ± 0.25 (0 min)	95%
KCN (0.5 mM) + PMF (4 mg/ml)	48.6 ± 0.50	3.1 ± 0.25	31.6 ± 3.42 (4 min)	65%
KCN (0.5 mM) + thiosulphate (50 mM)	50.3 ± 0.48	2.31 ± 0.15	25.2 ± 3.15 (1 min)	50%
KCN (0.5 mM) + microsomal fraction (1 mg protein/ml)	47.9 ± 0.45	2.39 ± 0.20	22.9 ± 2.68 (1 min)	48%
KCN (1.5 mM)	47.5 ± 0.40	0.54 ± 0.03	9.98 ± 1.02 (2 min)	20%
KCN (1.5 mM) + Na-pyruvate (1.5 mM)	48.3 ± 0.35	0.62 ± 0.04	27.1 ± 2.91 (5 min)	55%

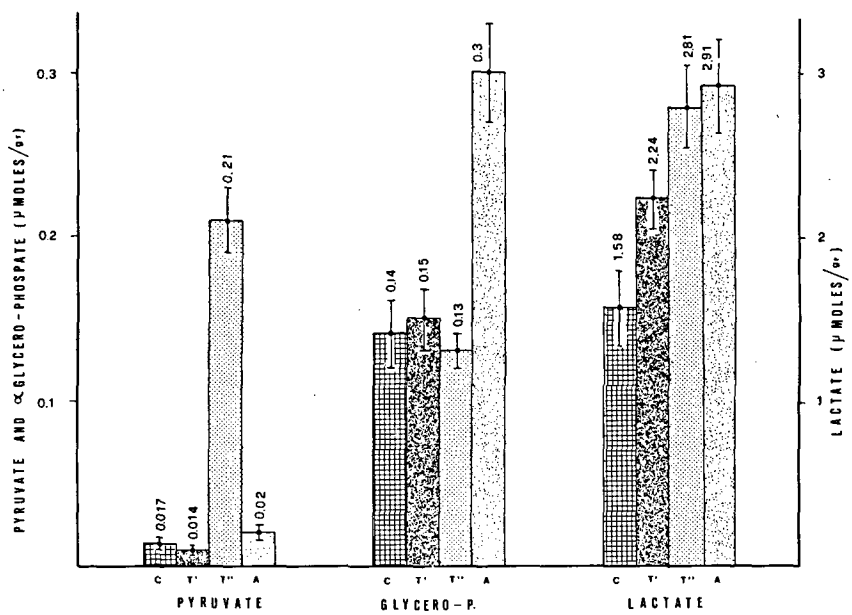

Fig. 1 - Pyruvate, glycero-phosphate and lactate concentrations detected before thanatosis, during thanatosis and on arousal. C = before crushing of the thorax (controls); T' = on immobilization (beginning of thanatosis); T" = one minute after immobilization; A = 15 seconds after T" (arousal). Data represent the means of 8 samples ± SE (vertical bars).

to its removal by cyanhydrin formation (Slater, 1967). Accordingly, the addition of pyruvate can reinstate normal respiration in mitochondria treated with 0.5 mM KCN within 4 minutes. In contrast, pyruvic acid can induce only a slow and partial improvement in the respiration rate of organelles treated with 1.5 mM KCN.

As a consequence, detoxifying mechanisms seem to be effective only under relatively low cyanide concentrations, at least *in vitro*.

The possibility that the increased respiration rate produced by pyruvate may depend on the presence of an additional electron donor more effective than succinate, can be excluded for the following reasons: 1. all polarographic assays were carried out in the presence of rotenone; 2. in our mitochondrial preparations the rate of pyruvate oxidation was particularly low; 3. pyruvate does not increase oxygen consumption when added to succinate oxidizing mitochondria, either in the absence of cyanide or in the presence of rotenone.

Therefore the evidence suggests that pyruvate can produce its detoxifying action by cyanhydrin formation, as it has been known for a longtime to occur in mammalian mitochondria (Cittadini *et al.*, 1972).

Results obtained for specimens where thanatosis was artificially induced (Fig. 1) demonstrate that the level of CN-release produced by the crushing of the thorax is enough to block cellular respiration *in vivo* as it does *in vitro*. In fact, the concentrations of lactate, α-glycerophosphate and pyruvate found during thanatosis and early phases of arousal are consistent with those known to occur in insects under anaerobic conditions (Hochachka & Somero, 1973), thus demonstrating that anaerobic glycolisis is stimulated during thanatosis.

Nevertheless, it should be observed that while lactic acid accumulates progressively with thanatosis, pyruvate and glycero-phosphate show a sharp increase at differential times. This means that pyruvate cannot accumulate during the early phases of thanatosis, as it is cyclically combined with cyanide in the cyanhydrin-producing reactions. Only later on, and after the cyanide is completely removed, the turnover of the glycolytic compounds will revert to values that are typical of normal anaerobiosis, and lead to the increased concentration of (unmetabolized) pyruvate and glycerophosphate found at the end of thanatosis. The fact that arousal takes place immediately after the rise of pyruvate and α-glycerophosphate is in accordance with the previous hypothesis, as the oxidative metabolism can only be reset after the cyanide is completely removed, allowing normal mitochondrial functioning. In this framework it can finally be recalled that respiration is greatly facilitated by the stimulating effect of α-glycerophosphate, that represents a major substrate for insect muscular function.

A tentative reconstruction of the biochemical mechanisms involved in the thanatosis of burnets and their subsequent arousal is summarized in Figure 2.

If our hypothesis is correct, cyanide is to be regarded as the primary agent responsible for thanatosis in burnets. In this connection, as reported above, it is relevant to note that submitting these burnets to an HCN-saturated atmosphere for 15 secs, produces a complete, but reversible immobilization of the insect, mimicking in a surprising manner natural thanatosis. The immobilizing action of cyanide may be exerted both directly, through cellular metabolism, and by the mediation of some neurotransmitter. Though the latter aspect so far remains un-investigated.

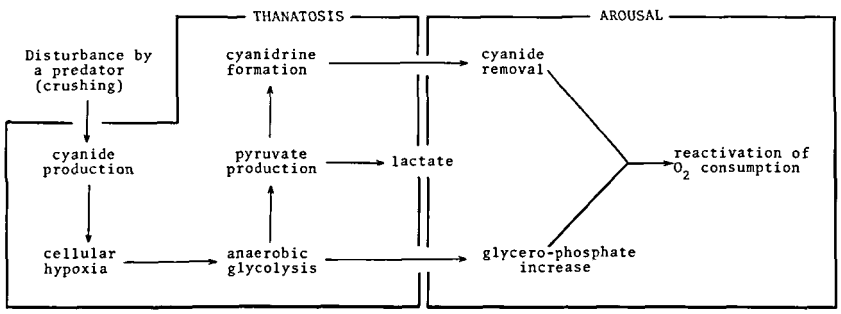

Fig. 2 - Biochemical mechanisms which may be involved in «severe» (cyanide triggered) thanatosis.

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