Carbon dioxide — more rapidly impairing the glycolytic energy production than nitrogen?

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Abstract

To compare the effects of different controlled atmospheres on anaerobic energy production of insects, wheat grains infested with pupae of the granary weevil *Sitophilus granarius* (L.) were exposed to pure nitrogen or pure carbon dioxide for times between 2 hours and 21 days at 20 ± 1 °C, 76% r.h. Under anoxic conditions, 10–12 pupae (approx. 50 mg) were removed from each grain sample, weighed and their lactate content was determined using a standardised enzyme test kit.

It could be demonstrated that granary weevil pupae produce lactate under anoxic conditions. The contents of lactate increased most strongly within the first 24 hours of anoxia. Later the lactate production decreased to almost zero, suggesting an inhibition of this metabolic pathway. The lactate levels in pupae fumigated with CO_2 were only about one third of those found in N_2-fumigated pupae. This may be attributed to an acidification caused by carbonic acid and lactic acid which in N_2-fumigated pupae is attained by the accumulation of greater amounts of lactic acid alone. A higher concentration of hydrogen ions could inhibit the production of phosphoenol-pyruvate out of glycerol-3-phosphate by causing a shortage of free NAD^+ which is needed in this glycolytic reaction. Compared with nitrogen, the inhibition of glycolysis at lower lactate levels, corresponding to a lower energy yield, could be a reason for the more rapid lethal action of CO_2-rich atmospheres.

Introduction

Energy production from nutrients is always connected to a partial or complete oxidation of the substrate with electrons or hydrogen ions being transferred to an acceptor, or down a chain of acceptors. In this process, the primary or intermediate acceptors are reoxidised while the terminal acceptor accumulates in the reduced state. Oxygen is an ideal terminal acceptor because water can be utilised, accumulated and excreted without damaging the organism (Urich 1990). Eucaryotic cells first utilised oxygen for respiration about 1.4 billion years ago. This oxygen had previously been produced by the photosynthesis of prokaryotes, themselves using other terminal acceptors in an originally anoxic atmosphere (Wegener 1988).

Even animals that live in habitats extremely poor in oxygen, such as parasites of the intestinal tract, depend at least once in their ontogenesis on the availability of oxygen (Wegener 1988). As Wegener states, there are several good reasons for the uniformity in which oxygen is used as a terminal acceptor:

1. Different nutritive compounds, such as lipids, proteins and carbohydrates can be utilised, whereas without oxygen only glycolysis and, in a few cases, the breakdown of certain amino acids can be utilised for the production of the energy-conserving adenosine triphosphate (ATP).
2. Only in the presence of oxygen can substrates be metabolised with a maximum energy yield. In contrast, the energy yield of anoxic metabolism is rather low.
3. The main foodstuffs, carbohydrates and lipids, can be broken down completely to the harmless and readily excretable end-products carbon dioxide and water. Anaerobic metabolism, on the other hand, usually gives rise to acidic compounds and protons that may interfere with cellular functions and that are excreted or accumulated with much more difficulty.

If an organism is frequently confronted with a lack of oxygen in its habitat it can react in one or several of the following ways.

Migration

Migration to sources of oxygen can be observed in many aquatic organisms that actively move into water layers with a high oxygen content. Extreme examples are some fish species (e.g. of the genus *Gambusia*) that can survive in eutrophic, oxygen-free water by swallowing air.

Energy conservation

Carrying out microcalorimetric studies with the desert locust *Locusta migratoria* L. and the tobacco hornworm *Manduca sexta* (Joh.), Moratzky et al. (1992) could prove that these insects reduce their heat production to less than 5% of the normal values after 4 or 5.5 hours under anoxia, respectively. This enormous conservation of energy can therefore be seen as a major factor rendering insects much more tolerant to the lack of oxygen than vertebrates, for example (for mechanisms of energy conservation see Gäde 1985).

Anoxic energy production

Glycolysis does not require oxygen. Therefore, this reaction can be utilised under anoxic conditions. To balance the redox state, the resulting pyruvate can be reduced to lactate by a reaction with NADH, in this way replacing oxygen as a terminal acceptor. This is known to happen in the muscles of terrestrial animals during periods of high activity and insufficient oxygen supply (described for the saltatoric muscle in the hind leg of *L. migratoria* in Gäde 1985). The production of lactate will supply an organism with 3 mol ATP per glycosylic unit while a total of 36 mol ATP is produced if pyruvate can be transformed into malate and broken down in the Krebs cycle under consumption of oxygen.

Many aquatic animals are able to produce excretable end-products of anaerobic metabolism like propionate (via succinate) while at the same time achieving a higher energy

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yield (7 mol ATP) (Fig. 1). The production of propionate, however, has not yet been described for insects.

The mud-inhabiting larvae of *Chironomus riparius* (formerly *C. thummi thummi*) are known to excrete ethanol (Zebe 1977). But generally, the production of lactate seems to be the main metabolic pathway of anoxic energy production in insects.

Adult cerambycid beetles *Rhagium inquisitor* L. surviving arctic winters enclosed in ice were found to have high lactate levels after being kept in pure nitrogen gas at 5°C for a month (Zachariassen and Pasche 1976). Also, soil-inhabiting larvae of the fly *Callitroga macellaria* F. were found to accumulate lactate under experimental anoxia (Gäde 1985).

Research findings on the respiratory physiology of stored-product insects have so far been published mainly by scientists from the Volcani Center, Bet Dagan, Israel. Navarro and Friedlander (1975) exposed pupae of *Ephestia cautella* for 24 hours to 10 % O_2 and different concentration of CO_2 (20–89%). Proportional to the increase of CO_2 in the experimental atmosphere, they could find a rising level of lactate in the haemolymph. In atmospheres of nitrogen and small amounts of oxygen, the lactate levels rose sharply if the oxygen content was reduced below 3%. In another survey, the same authors found a significant reduction in glucose levels under hypoxic conditions, whereas under hypercarbia these levels remained constant (Friedlander and Navarro 1979). The amounts of citrate were found to be reduced under both hypoxic and hypercarbic atmospheres which could be a consequence of disrupting the Krebs cycle.

Till now, no studies have been published on the physiological effects of controlled atmospheres on the granary weevil, *Sitophilus granarius*, though this insect is known to be quite tolerant to hypoxia and hypercarbia. The pupa of this species was chosen for the following experiments because pupae are the most tolerant developmental stage and because they combine both high body volume and a low degree of sclerotisation.

The levels of lactate in pupae fumigated with pure nitrogen or pure carbon dioxide were determined by high performance liquid chromatography (HPLC) and by a standardised enzymatic test. The HPLC study was carried out only with pupae fumigated for 3 weeks to detect the presence of lactate and to receive a first estimate of the maximum amount of lactate to be expected after a treatment with controlled atmospheres.

The results of this study aided calculation of the appropriate dilution factors for the enzymatic analysis. The enzymatic test, more accurate in quatitative terms, was carried out with pupae being exposed to the respective atmospheres for various periods. This was done in order to determine the influence of exposure time and atmosphere on the lactate production.

Materials and Methods

Wheat grains infested with 28–31-day-old developmental stages (mainly pupae) from a culture kept at 25 ± 1°C, 75 ± 5 % r.h., were placed in wire mesh cages. Three cages were placed into a 500 mL Dressel flask that was then purged with pure nitrogen or pure CO_2 (flow rate 1000 mL/minute). Before use, the gases had been humidified to 76% r.h. by passing them through a saturated sodium chloride solution (Winston and Bates 1960). When the Drechsel flask contained an atmosphere with 0.1% O_2 or less, the taps of its gas inlet and gas outlet tubes were closed. The flasks were then stored at 201°C for various periods. The following exposure times were chosen:

N_2-fumigation: 2 hours, 4 hours, 1 day, 4 days, 7 days, 14 days and 21 days;

CO_2-fumigation: 1 day, 4 days, 7 days, 14 days and 21 days.

At the end of the exposure period, the flasks were checked for their oxygen content. In a glove box continously flushed with pure N_2, the bottles were opened and the pupae were removed from the grain. About 50 mg of intact whitish pupae were weighed within the glove box using a CAHN model 4400 electrobalance and were then blended after addition of 200 µL of 3 N perchloric acid. This helped to stop all enzymatic processes immediately. Untreated control samples were weighed and blended in ambient air following the same procedure.

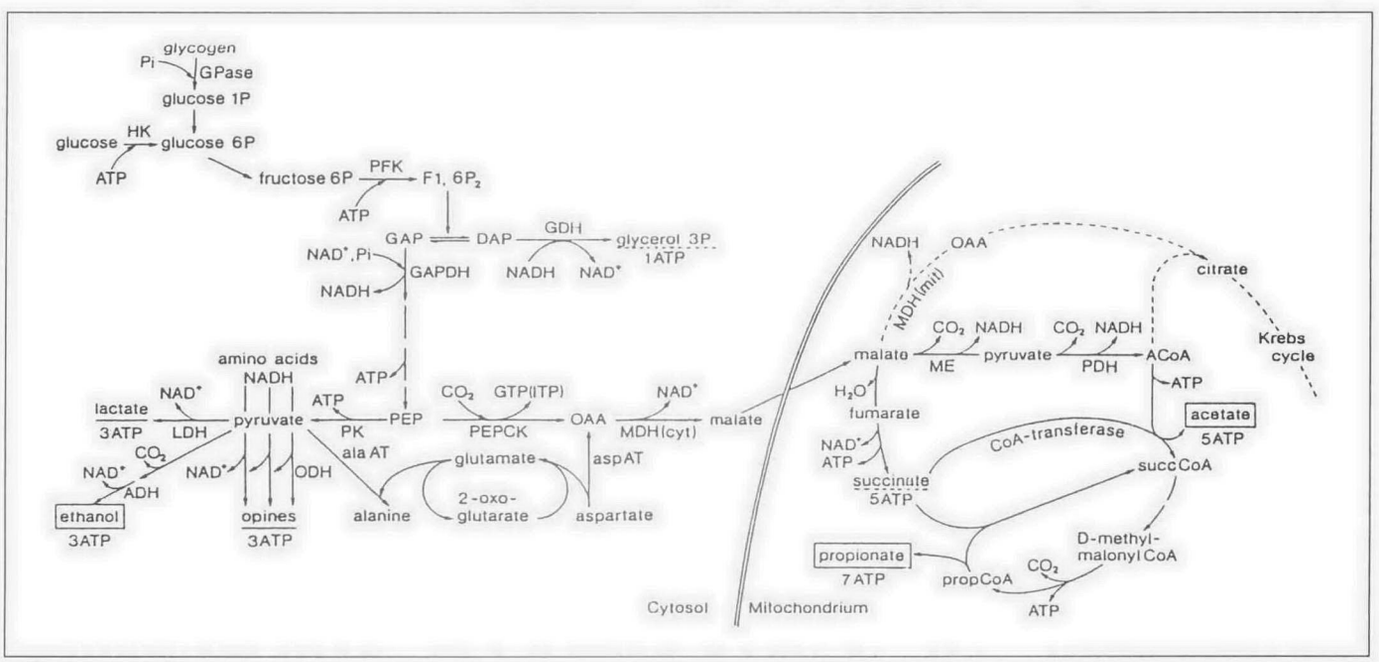

Fig. 1. Scheme of anaerobic energy metabolism (from Wegener 1988)

The homogenate, together with 200 µL of twice distilled water (to rinse the blender), was transferred into an Eppendorf vessel and neutralised by 200 µL 2.55-m potassium hydroxide solution. All probes were then centrifuged (2 times 5 minutes at 12 000 rpm and 4°C) to remove all particles (for details see Adler 1993). Samples were taken from the liquid fraction for both the HPLC-determination and the standardised enzymatic l-lactate (+) test (test kit from Boehringer Mannheim). In this test lactate is determined through the addition of NAD resulting in:

L-lactate (+) + NAD pyruvate + NADH.

This reaction takes place in the presence of lactate anhydrogenase. The resulting NADH causes a change in absorption of light (at = 340 nm) and thus allows an indirect determination of lactate contents by photometry (Bergmeyer and Graßl 1983).

Results

It was demonstrated that granary weevil pupae produce lactate under anoxic conditions. In the HPLC determination, a distinctive peak was recorded after the appropriate retention time (*rt* = 12.93). Lactate levels for pupae exposed for 21 days to pure nitrogen or pure carbon dioxide were 195 ± 5 mg/100 mL and 65 ± 5 mg/100 mL, respectively.

Further peaks were recorded after retention times close to those of -ketoglutarate (*rt* = 8.27 instead of 8.18), succinate (*rt* = 11.84 instead of 12.26) and fumarate (*rt* 15.28 instead of 15.24).

Lactate contents as determined by the enzymatic analysis are presented in Figure 2.

Discussion

The two corresponding values from HPLC determination are considerably lower than those attained in the enzymatic survey which may have been caused by filtering the samples with a molecular sieve prior to injection into the HPLC column. The accuracy of the enzymatic determination was secured by a lactate sample with known concentration (real concentration 19.9 mg/100 mL, determined concentration: 20.2 mg/100 mL).

The lactate levels measured by Navarro and Friedlander (1975) in the haemolymph of *Ephestia cautella* pupae after 24 hours exposure to N_2 with less than 3% O_2 (287 mL/100 mL) are a little higher than those found in the present study (200 mg/100 mL after 24 hours exposure to pure N_2). It has to be considered, however, that the amounts of lactate may be higher in haemolymph than in the whole body tissue. Moreover, the residual oxygen in their controlled atmosphere (<3 %) could be responsible for this difference as well as different rates of anoxic energy production in the pupae of two different insect orders.

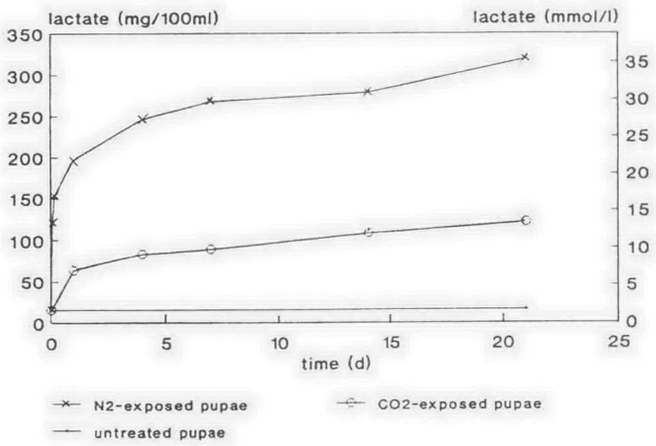

Fig. 2. Lactate contents in *S. granarius* pupae exposed to pure N_2 or pure CO_2 gas. Data from enzymatic analysis (100 mL liquid fraction corresponds to about 100 g fresh weight).

Another interesting point in the findings of Navarro and Friedlander is that lactate levels shot up only when the O_2 contents were reduced below 3%. If this value is exceeded, lethal exposure periods are extremely prolonged in several stored-product species (Lindgren and Vincent 1970). Therefore, this seems to correspond to the 'critical partial pressure' of oxygen where the energy metabolism is converted from aerobic to anaerobic (discussed in Grieshaber et al. 1988).

In both the enzymatic and the HPLC type of lactate determination, lactate levels in pupae exposed to CO_2 were much lower than those of pupae exposed to N_2 for the same time span. As the results of the enzymatic test show, after 24 hours exposure, the level of accumulated lactate in CO_2-treated individuals amounted to only one third of the value detected in N_2-exposed pupae. This relation remained constant through all exposure periods tested (see Fig. 2). Another remarkable fact is that, in both controlled atmospheres, the greatest increase in lactate took place within the first 24 hours whereas the production was reduced later, resulting in a more or less hyperbolic function of lactate generation (Fig. 2). These findings immediately give rise to two questions:

1. Why is so much less lactate produced in an atmosphere of pure carbon dioxide?

2. Why does the production of lactate decrease in both atmospheres after the first 24 hours?

Both questions may be answered by the following hypothesis: CO_2, readily dissolving in all body liquids forms carbonic acid and causes an acidification on the cellular level. Together with lactate, itself being an acidic compound, the excessive amount of hydrogen ions present could directly or indirectly inhibit glycolysis. In vertebrates, a pathologically high accumulation of lactate in body tissues causes a severe disturbance in the redox balance known as lactic acidosis.

In his extensive studies on the induced tolerance of *Tribolium castaneum* Herbst to anoxia and hypercarbia, Donahaye (1991) found that the population selected for tolerance against hypercarbia had a significantly higher body mass than unselected individuals of the same laboratory strain. This result is supported by a comparative fumigation of 10 different strains of *S. granarius*, where Adler (1993) found that the average adult body weight of strains more tolerant to a treatment with 95% CO_2 (rest air) was significantly higher than that of susceptible strains. A higher body mass correlates with more body liquids which could allow a longer exposure to CO_2 before the same level of acidification is reached.

Studies on the selection of *Sitophilus* weevils for tolerance against atmospheres high in CO_2-contents were carried out by Bond and Buckland (1979) and Navarro et al. (1985). Donahaye (1991) selected *T. castaneum* for tolerance not only against hypercarbia but in a second study also against hypoxic atmospheres. In all these experiments the selection was successful under laboratory conditions with extreme moisture contents (≥95% r.h.) and all authors mention the susceptibility of fumigated individuals to desiccation. These findings stress the importance of body water and fit into the picture of acidification being caused by hypercarbia or anoxia, respectively.

In pupae exposed to nitrogen, glycolysis take place until a much higher level of lactate alone has caused a similar acidification as carbonic and lactic acid cause in CO_2-treated pupae.

A step in glycolysis that could be directly affected by acidification is the reaction from glycerolaldehyde 3-phosphate to phosphoenol pyruvate where hydrogen ions are released (Fig. 1). Nicotin adenosyl diphosphate (NAD^+) needed as an acceptor in this reaction would not be available in an acidic environment.

Donahaye (1991) attributed the mortality of treated individuals to desiccation and the exhaustion of triglycerides as energy reserves. This exhaustion theory would, however, not explain the more rapid lethal action of hypercarbia.

It remains debatable what the ultimate cause of insect mortality is, but compared with hypoxia, the rapid inhibition of the glycolytic energy production by hypercarbia could be a reason for the faster lethal action of atmospheres high in CO_2.

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