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Hydrogen Peroxide Causes a Decrease in Aerobic Metabolic Rate and in Intracellular pH in the Shrimp *Crangon crangon*

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ABSTRACT. UV-dependent hydrogen peroxide formation can lead to high levels of hydrogen peroxide in tidepool waters. The effect of ambient hydrogen peroxide on the rate of oxygen consumption and on the levels of intracellular pH, lactate and high energy phosphates was studied in the shrimp, *Crangon crangon*. Exposure to elevated hydrogen peroxide levels caused a decrease in metabolic rate by an average value of 26% in intact animals and by up to 60% in isolated preparations of the tail muscle. Muscle intracellular pH decreased by 0.1–0.2 pH units in both isolated tissue and intact animal. The maintenance of lactate and ATP at control levels strongly suggests that the acidosis was not associated with anaerobic proton production and, as a corollary, must be explained through an effect of hydrogen peroxide on proton equivalent ion exchange mechanisms. Future studies will attempt to elucidate the nature of the effect and whether the associated acidosis leads to the observed decrease in aerobic metabolic rate. comp biochem physiol 117C;2:123–129, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Mudshrimp, hydrogen peroxide, oxygen consumption, intracellular pH

INTRODUCTION

UV-driven oxygen reduction causes the formation of hydrogen peroxide, a toxic oxygen species. Compared with oxygen radicals, hydrogen peroxide is a molecule of considerable stability, with a redox potential of +0.38 V (15). A half-life of 60 hr has been calculated for its decay in filtered seawater (0.2 μm), whereas the estimated half-life for superoxide anion radicals ($O_2{}^{*-}$) is 100 min (28).

Along the German Wadden Sea coast, large sandflat areas periodically emerge during low tides, leaving shallow intertidal pools exposed to irradiation, at times for more than 5 hr. In oxygenated tidepools, H_2O_2 photoproduction exceeds the biological and chemical decomposition, leading to a net-accumulation of micromolar concentrations of H_2O_2 (2). Thus, peroxide becomes an ecological factor, which affects redox processes, involving oxygen, in the sediment water boundary layer (24) and may also affect the biochemistry and the physiology of animals inhabiting intertidal pools and the sediments below.

The mudshrimp *Crangon crangon* is a regular inhabitant of these sandflats, to which it migrates during high tides. When the tide falls, most of the animals return to sublittoral waters (22). However, some shrimp are regularly trapped in tidepools during emersion periods, where they survive buried in the upper sediment layer. If that happens, they are subjected to strong changes in the abiotic conditions (e.g., temperature, salinity, pH and PO_2). Additionally, UV-driven hydrogen peroxide formation during summer (April–October) can yield maximum water concentrations between 3 and 5 μmol l^{-1} (6).

Although oxygen radical stress and antioxidant protection are coming increasingly into focus in marine invertebrate physiological research, only a few studies have considered the effects of elevated concentrations of reactive oxygen species (ROS) on invertebrate vital functions and metabolic rates. Studying the polychaete *Nereis diversicolor*, we found a depression of aerobic metabolic rates after exposure to 0.5 and 5 μmol l^{-1} H_2O_2 (4). Concomitantly, the worms ceased locomotory activities. Hydrogen peroxide concentrations of more than 4 μmol l^{-1} were found to induce a significant reduction of filtration rates of the intertidal bivalve *Cerastoderma edule* (3).

As hydrogen peroxide is an uncharged molecule, it easily passes through cell membranes by diffusion. Inside the cells, highly reactive hydroxyl radicals are liberated ($2\ H_2O_2 + e^- \rightarrow OH^* + OH^-$) in a reaction catalyzed by transition metals or other cellular reductants (34). At high concentrations, these radicals induce peroxidation of membrane lipids and proteins, which will probably disturb ion and proton transmembrane distribution and transport and may thereby also affect cellular energetics (i.e., ATP homeostasis).

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The objective of the present study is to assess the effects of sublethal hydrogen peroxide concentrations on aerobic metabolic rates, energy status (i.e., conservation and turnover of high energy phosphates) and the intracellular acid-base regulation of the mudshrimp *C. crangon*. The aim was to see whether elevated hydrogen peroxide concentrations may substantially affect the vital metabolic functions of shrimp muscular tissues.

MATERIALS AND METHODS

Experimental Animals

Specimens of *C. crangon* were collected in shallow waters of the Wadden Sea, Lower Saxony. In the laboratory, the shrimp were held in natural seawater in large recirculated aquaria containing a 1- to 2-cm layer of sand. They were fed twice a week with pieces of polychaetes and fish. The animals were allowed to acclimatize for at least 2 weeks before experimentation. Females with eggs were not used in the experiments, and the animals were not fed 3 days before incubation. For experiments with living specimens, natural seawater with a salinity of 32‰ was filtered through a 0.4-μm and, subsequently, a 0.2-μm cellulose nitrate filter. Water temperature was kept constant throughout the experiment and was adjusted to 10°C (April) and 15°C (August). The water was kept oxygenated by constant aeration, and all experiments with living animals were conducted under low light conditions.

To obtain isolated muscle tissues, shrimp were quickly dissected and the abdominal muscle was separated from the exoskeleton. The muscle was cut up into slices, which were still connected, using scalpel and tweezer. Tissue preparation was carried out in an ice bath. In a preliminary experiment, the degree of slicing required to prevent muscle tissue from becoming ischemic over time was determined by monitoring intracellular pH. Isolated muscles were suspended in aerated Ringer solution, containing 440 mmol l^{-1} NaCl, 25 mmol l^{-1} Na_2SO_2, 10 mmol l^{-1} KCl, 10 mmol l^{-1} $CaCl_2$, 5 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} glucose and 5 mmol l^{-1} HEPES, pH 7.7.

Experimental Procedure

Oxygen uptake (MO_2) was measured in a flowthrough respirometer, described by Oeschger *et al.* (26). Measurements were carried out at 10 ± 0.1°C in 32‰ seawater filtered through 0.4-μm filters. For whole animals, respiration chambers (volume 40 ml) were perfused at known flow rates between 118 and 145 ml hr^{-1}. The oxygen partial pressures of in- and outflowing water were monitored continuously, and the rate of oxygen consumption was calculated by integrating the differential area between those recordings with a VIDS III-digitizing program. Oxygen consumption rates of individual shrimp were averaged for each hour during a measurement period of up to 12 hr. The normoxic oxygen consumption rate of each individual shrimp was determined before and after exposing them for 5 hr to 20 μmol l^{-1} H_2O_2 in a volume of 500 ml of aerated seawater without sediment. For a maximum effect, hydrogen peroxide levels were chosen higher than those found in the natural environment. H_2O_2 concentrations were monitored during exposure and readjusted to experimental levels when necessary. The first hour of recording was disregarded to exclude the effect of handling stress after placing the animal into the respiration chamber. After each measurement, the rate of oxygen consumption of empty chambers was monitored and the value subtracted from the respective value obtained for the animals. Additionally, control measurements were performed in shrimp kept in oxygenated seawater without the addition of hydrogen peroxide for 5 hr after the same protocol as described above (pseudo-treatment). The dry weight of whole animals was determined after 7 days of drying at 108°C.

Oxygen consumption of isolated muscles was measured in well-oxygenated Ringer solution using a 4.6-ml respiration chamber, perfused at a constant flow rate of 60 ml hr^{-1}. H_2O_2 incubations were carried out in the respirometer, by switching to a second water reserve, which contained peroxide at the indicated concentration. Initially, measurements were carried out to confirm the maintenance of constant rates of oxygen consumption and, thereby, the viability of isolated muscles in Ringer solution. For the determination of dry weight isolated muscles were heated to 60°C until weight constancy was reached.

The impact of elevated ambient hydrogen peroxide concentrations on the levels of high energy phosphates, lactate and pH_i in abdominal muscle tissue of *C. crangon* was studied in intact animals and in isolated, perforated muscle tissue. Living specimens and isolated tissues were exposed to 20 μmol l^{-1} H_2O_2. In the first experiment, one group of animals ($n = 7$) was exposed to 20 μmol l^{-1} H_2O_2 over 5 hr, whereas the control group ($n = 8$) was kept in peroxide-free seawater over the same time period. In a second experiment, changes in metabolite levels were recorded after exposure to 20 μmol l^{-1} H_2O_2 for 1 ($n = 3$), 3 ($n = 3$) and 5 ($n = 8$) hr. To reduce handling stress, this experiment was conducted in a flowthrough setup, where the H_2O_2 concentration was controlled in a second water reserve (500 ml), from which the water was pumped at a rate of 20 ml min^{-1} into the incubation aquarium (500 ml) containing the shrimp.

A third experiment was performed to test the effect of hydrogen peroxide on isolated muscle tissue after different time intervals. Muscles were incubated for 2 ($n = 8$) and 4 ($n = 8$) hr in 20 μmol l^{-1} H_2O_2, and controls, incubated in peroxide-free Ringer solution, were taken after 0 ($n = 3$) and 4 hr ($n = 6$). Hydrogen peroxide concentrations were controlled throughout the experiments and readjusted if necessary.

Analyses

H_2O_2 was determined fluorimetrically as described by Pamatmat (27) using scopoletin as a fluorescent dye. Intracel-

lular pH was determined according to Pörtner *et al.* (30) after grinding abdominal muscle of mudshrimp under liquid nitrogen, using mortar and pestle. Exposure of samples, tissue powder and liquid nitrogen to air was minimized to prevent CO_2 condensation. Aliquots of 30–80 mg of frozen tissue powder were immediately resuspended in up to 500 μl of ice-cold medium, which contained 160 mmol l^{-1} potassium fluoride and 1.0 mmol l^{-1} nitrilotriacetic acid at pH 7.3. After brief mixing on a Vortex mixer, the sample was centrifuged at 0°C and 13,000 ***g*** for 15 sec to remove insoluble tissue debris. Immediately after centrifugation, the pH in the supernatant was measured repeatedly at the experimental temperature in a capillary pH electrode (Radiometer, Copenhagen G297/G2) previously calibrated with precision phosphate buffers (Radiometer).

The concentrations of ATP, inorganic phosphate, phospho-L-arginine and arginine and lactate were determined enzymatically in neutralized perchloric acid extracts of abdominal muscle tissue (9). Phospho-L-arginine and arginine were determined after Grieshaber *et al.* (18), ATP and lactate according to Bergmeyer *et al.* (10) and inorganic phosphate according to Pörtner (29).

Catalase (E.C.1.11.1.6.) activity was determined after Aebi (7). Samples were homogenized in 50 mmol l^{-1} potassium phosphate buffer (pH 7.0) including one part of 1% Triton-X100 solution to 10 parts buffer. The catalase assay was conducted using potassium phosphate buffer (50 mmol l^{-1}, pH 7.0) and H_2O_2 as substrate at 240 nm. A catalase standard was purchased from Boehringer-Mannheim (2600 U/mg). Superoxidedismutase (SOD; E.C.1.15.1.) activity was determined according to Marklund and Marklund (23). A total of 0.05 mol l^{-1} Tris-succinate buffer (pH 8.2) was aerated over 1 hr before use. Pyrogallol solution (0.57 mg ml^{-1}) added to 950 μl buffer resulted in an absorbance increase of 0.020 abs min^{-1} at 420 nm. At 25°C and pH 8.2, one unit of commercially available SOD (Fluka) inhibited this increase by 50% (= 0.010 abs min^{-1}). Glutathione reductase (GR; E.C.1.6.4.2.) activity was measured following Abele-Oeschger *et al.* (4). The assay is based on the oxidation of NADPH during the reduction of oxidized (GSSG) to reduced glutathione (GSH). Deep-frozen tissue samples were ground in liquid nitrogen and extracted with 0.12 mol l^{-1} phosphate buffer (1:3, w/v, pH 7.2). GR activity in homogenates was assayed at 25°C and 340 nm in Tris buffer (0.1 mol l^{-1}, pH 8.2) to which EDTA (0.94 mmol l^{-1}), GSSG (4.6 mmol l^{-1}) and NADPH (0.16 mmol l^{-1}) were added. A GR standard was purchased from Boehringer-Mannheim (120 U mg^{-1}).

Glutathione peroxidase (GPOX) was assayed in a coupled enzyme test in which GSH is oxidized by GPOX using added H_2O_2 as a substrate, following Günzler and Flohé (20). The resulting GSSG was re-reduced by GR involving the oxidation of the co-substrate NADPH at 340 nm. Tissues were extracted with a Tris–HCl buffer (20 mmol l^{-1}, pH 7.6), containing EDTA (1 mmol l^{-1}) and dithiothreitol (1 mmol l^{-1}). Tissue extraction was carried out on ice in a pre-cooled glass potter, the tissue buffer relation being 1:3. The assay was carried out in a KPi buffer (100 mmol l^{-1}, pH 7.0) with EDTA (1 mmol l^{-1}). To 600 μl KPi buffer, 100 μl tissue extract, 10 μl NaCN (100 mmol l^{-1}), 1U GR and 100 μl of GSH (100 10 mmol l^{-1}) were added. NaCN was used as a specific inhibitor of catalase, competing for the H_2O_2 substrate. This mix was incubated for 10 min at room temperature followed by the addition of 100 μl NADPH (1.5 mmol l^{-1}, ϵ_{340}nm: 6.2 mmol l^{-1} cm^{-1}). The resulting peroxide independent NADPH oxidation (ΔE_1) was measured for 3 min, whereafter 100 μl H_2O_2 (3 mmol l^{-1}) was added, resulting in increased NADPH oxidation (ΔE_2) due to the H_2O_2 driven reaction of GPOX. One unit GPOX is defined as the amount of enzyme, oxidizing 1 μmol of GSH per min at 25°C and pH 7.0. The assay was checked using a GPOX standard (Fluka, 100 U mg^{-1}).

The total amount of GSH and GSSG was determined spectrophotometrically using 5,5-dithio-2-nitrobenzoic acid as described in Buchner and Abele-Oeschger (14).

Changes over time were tested for significance at the 5% level by using one-way ANOVA and by performing the Bonferroni/Dunn post-hoc test for group comparisons using SuperAnova, Abacus Concepts. Values are presented as means ± SD throughout. Comparisons of means for treated and pseudo-treated groups were performed using Student's *t*-tests.

RESULTS

Oxygen uptake of three animals exposed to hydrogen peroxide was significantly reduced, on average by 25.7% (Table 1). Control animals exhibited no significant drop in oxygen consumption after pseudo-treatment. On a dry weight basis, the oxygen uptake rate of isolated abdominal muscle tissues was found to be similar to the rates found in whole animals and, moreover, showed the same reaction to hydrogen peroxide. The preliminary results, however, do not reflect a dose-response relationship, probably owing to the high concentrations tested. Therefore, results were grouped to test for the presence of a response to hydrogen peroxide *per se* (Table 1).

Values of intracellular pH, lactate levels and high-energy phosphates in muscle *in vivo* are summarized in Table 2. With the exception of a decrease in pH_i, no significant changes in individual metabolic parameters were found upon peroxide exposure. ATP was present at "resting" levels in both control and incubated animals. The ratio of phospho-L-arginine over the sum of phospho-L-arginine and L-arginine concentrations was below 0.5 in this and all consecutive experiments, which indicates that burst activity had occurred when samples were taken. This is a common phenomenon often reported in the literature when tissues are sampled invasively from non-narcotized animals. Lactate levels showed a minor, but insignificant, increase between the unexposed and the exposed group (Table 2). However, lactate concentrations in both groups were clearly

TABLE 1. Oxygen consumption of living sand crab (*Crangon crangon*) specimens and isolated tail muscle before and after exposure to H_2O_2

	Before treatment	After treatment	% Change
		μmol $O_2 h^{-1}$ g^{-1} dry mass	
Control animals	9.8 ± 2.3	9.9 ± 3.1	−0.14 ± 13.2 (5)
Animals exposed to H_2O_2 (5 hr)	13.1 ± 2.9	9.6 ± 2.5	−25.7 ± 14.2 (3)*
Muscle exposed to H_2O_2 (2 hr)	14.2 ± 4.1	6.3 ± 3.9	−57.7 ± 14.8 (4)*

Whole animals: 5 hr to 20 μmol l^{-1} H_2O_2, isolated tail muscle: 2 hr to 10–20 μmol l^{-1} H_2O_2. Control animals were pseudo-treated in peroxide free seawater, using the same protocol as for the exposed specimens. Asterisk indicates a significant difference ($P < 0.05$) compared with control levels.

close to resting values, indicating that anaerobic ATP production was minimal during normoxic peroxide exposure.

Intracellular pH in isolated muscle tissues showed the same reaction to hydrogen peroxide as the pH_i in muscle tissue collected from whole animals. pH_i decreased from 7.31 ± 0.03 in the control group to 7.12 ± 0.08 (2 hr) and to 7.19 ± 0.08 after 4 hr of exposure, whereas pH_i remained constant in the control group (4 hr, Fig. 1). Again, lactate levels were not affected by H_2O_2 but remained constant at an even lower level (0.1–0.2 μmol g^{-1} wet weight) than observed *in vivo* (Table 3b).

A second incubation experiment with intact animals was performed monitoring the time course of changes during 5 hr of exposure to 20 μmol l^{-1} hydrogen peroxide. Peroxide incubation caused pH_i to decrease from 7.46 ± 0.02 to 7.34 ± 0.07 after 5 hr of exposure, whereas pH_i in pseudo-treated animals (7.45 ± 0.07) did not differ from the control group (Fig. 1). Lactate levels were higher in this than in the previous experiment (Table 2), probably owing to seasonal differences and the associated higher metabolic rates in summer animals.

Results of the antioxidant enzyme measurements in mudshrimp abdominal muscle and haemolymph are depicted in Table 4. The major H_2O_2 metabolizing enzyme catalase was of extremely low activity in abdominal muscle and below detection limit in the hemolymph. Moreover, we failed to induce catalase activity upon experimental H_2O_2 exposure (unpublished data). SOD was also below detection limit in the hemolymph but showed activities comparable with other invertebrates in muscle tissue. In the absence of catalase, the glutathione system, consisting of GR, GPOX and the substrate glutathione, may constitute a major proportion of the hydrogen peroxide metabolizing antioxidant system of *C. crangon*, as well in muscle tissue as in the respiratory fluid.

DISCUSSION

To our knowledge, this is the first study to report that hydrogen peroxide causes a decrease in intracellular pH in the musculature of an animal that may very well be exposed to high ambient peroxide levels in its natural environment. We have investigated the influence of 20 μmol l^{-1} H_2O_2 on aerobic metabolic rates, the energy metabolism and intracellular acid-base regulation of *C. crangon*. The concentrations used in this study were 4-fold higher than the maximum values so far observed in the field (6). These values were chosen in an attempt to not only elucidate the mode of action of hydrogen peroxide on invertebrate vital functions but also to investigate the maximum effect. Levels comparable with those used experimentally have been reported for the body fluids of benthic bivalves (*Astarte borealis*) and polychaetes (*Arenicola marina*) where under hypoxic conditions hemolymph H_2O_2 increased to up to 21 μmol l^{-1} (1). However, exposure to H_2O_2 levels of 4 μmol l^{-1}, which were sufficient to reduce oxygen consumption in the polychaete *Nereis diversicolor*, failed to show an effect on mudshrimp metabolic rates. Possibly the exoskeleton provides a diffusion barrier, sufficient to exclude hydrogen peroxide from shrimp tissues at environmental concentrations. This could be crucial for the shrimp, which in contrast to the polychaetes do not dig into the sediment but stay in surface pools, directly exposed to the accumulating hydrogen peroxide.

The antioxidant enzyme activities developed by the *Crangon* range at a surprisingly low level, as compared with previously investigated polychaetes. This applies mainly to the major H_2O_2 metabolizing enzyme catalase that was scarcely detectable in the investigated shrimp. Although SOD is in the same range as in other non-hemoglobin invertebrates (5), the glutathione system is again several orders of magnitude below the levels found in the polychaete *Arenicola marina* (14). However, there are hints for the exis-

TABLE 2. Effect of 20 μmol l^{-1} hydrogen peroxide exposure on pH_i and the levels of lactate, ATP, phosphagen (Arg-P) and arginine (μmol g^{-1} wet weight) in abdominal muscle tissue of whole animals

	Controls	5 hr H_2O_2
pH_i	7.32 ± 0.04	7.22 ± 0.05*
[Lactate]	0.55 ± 0.54	1.14 ± 1.04
[ATP]	4.66 ± 0.94	5.05 ± 0.95
[Arg-P]	16.1 ± 4.5	14.6 ± 7.1
[Aginine]	19.2 ± 6.1	22.3 ± 9.9
[Arg-P]/[Arg-P] + [Arg]	0.40 ± 0.15	0.35 ± 0.17

Data from April 1995, $n = 6–10$. Asterisk indicates a significant difference ($P < 0.05$) compared with control levels.

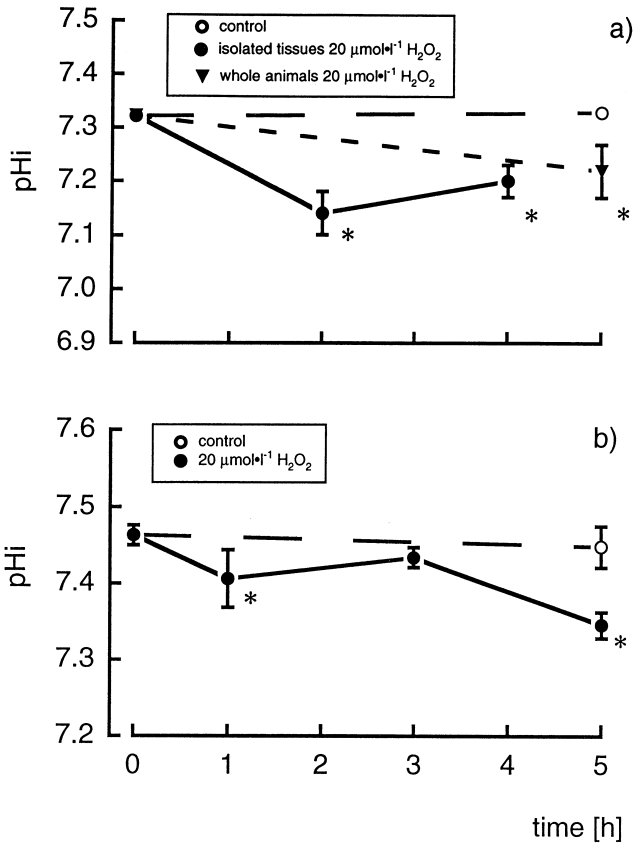

FIG. 1. (a) Effect of 20 μmol l^{-1} H_2O_2 exposure on pH_i in isolated abdominal muscle tissues and in whole animals. Data from April 1995, n = 6–10. Controls were pseudo-incubated in peroxide free seawater (whole animals) or saline (isolated tissues) under the same conditions as the peroxide incubation experiments. Asterisk indicates a significant difference ($P < 0.05$) compared with control levels. (b) Effect of 20 μmol l^{-1} H_2O_2 exposure on pH_i in abdominal muscle tissues of whole animals. Data from August 1995, n = 6–10. Asterisk indicates a significant difference ($P < 0.05$) compared with control levels.

tence of very effective antioxidants in crustacean muscular tissue, as shown for Antarctic krill, *Euphausia superba*, by Seher and Löschner (33). These authors characterized the antioxidant fraction in krill tissue to consist of tocopherol and a variety of free amino acids. It seems noteworthy that catalase is also completely absent in krill abdominal muscle (Abele-Oeschger, unpublished data). It will have to be tested whether a comparable set of chemical antioxidants can be determined in muscles of mudshrimp.

In our study, the peroxide-induced decrease in oxygen consumption was more pronounced in isolated tissues than in whole animals (Table 1). The lower degree of change observed in whole animals might be caused by the maintenance of a concentration gradient for hydrogen peroxide between external water and the body fluids. However, it has to be kept in mind that in isolated tissues, a considerable depression of aerobic metabolic rates was already induced at 10 μmol l^{-1}. In other invertebrates, a reduction in pH_i is discussed to be a main factor in causing a decrease in metabolic rate [e.g., (21)]. It is controversial, however, whether this is a general phenomenon (13,31). Therefore, it has to be emphasized that it is unknown for marine crustaceans whether a decrease in pH_i as observed in the present study causes metabolic depression. Although the reduction in aerobic metabolism was more pronounced in isolated muscle than in whole animals, the decrease in pH_i was similar in both experiments. The reason may be that the decrease in metabolic rate *in vivo* is largely caused by metabolic depression in the musculature with possibly less effect of H_2O_2 on the other tissues.

The intracellular acidification was not caused by anaerobic metabolism because lactate production and accumulation did not occur in either whole animals or in isolated tissues. ATP levels also remained unchanged; thus the observed intracellular acidosis could not be attributed to protons released during ATP hydrolysis. The decrease in pH_i must, therefore, be the consequence of a change in proton

TABLE 3. (a) Effect of 20 μmol l^{-1} hydrogen peroxide exposure on pHi and the levels of lactate, ATP, phosphagen (Arg-P) and arginine (μmol g^{-1} wet weight) as well as the ratio of PLA over the sum of PLA + L-Arg concentrations in abdominal muscle tissue of whole animals (data from August 1995) and (b) effect of 20 μmol l^{-1} hydrogen peroxide on pHi and lactate (μmol g^{-1} fresh mass) concentrations in isolated abdominal muscles of *C. crangon* (data from June 1995, n = 8)

	Controls			H_2O_2	
(a)	**0 hr**	**5 hr**	**1 hr**	**3 hr**	**5 hr**
pH_i	7.46 ± 0.02	7.45 ± 0.07	7.41 ± 0.07	7.43 ± 0.02	7.34 ± 0.03
[Lactate]	2.17 ± 0.47	0.94 ± 0.40	1.42 ± 0.47	2.24 ± 0.29	2.15 ± 1.30
[ATP]	5.06 ± 1.10	6.73 ± 1.22	6.25 ± 0.32	6.02 ± 1.36	7.09 ± 2.81
[Pi]	23.44 ± 11.45	26.31 ± 8.57	24.39 ± 1.81	34.31 ± 9.05	30.80 ± 12.44
[Arg-P]	17.38 ± 8.96	24.97 ± 12.37	25.52 ± 10.77	30.06 ± 5.78	30.94 ± 7.91
[Arginine]	25.08 ± 8.03	20.72 ± 12.61	27.12 ± 1.66	37.63 ± 11.72	31.43 ± 7.77
[Arg-P]/[Arg-P] + [Arginine]	0.41 ± 0.17	0.55 ± 0.26	0.48 ± 0.11	0.44 ± 0.11	0.42 ± 0.07

(b)	**Controls**	**2 hr H_2O_2**	**4 hr H_2O_2**	**4 hr controls**
pH_i	7.31 ± 0.03	7.12 ± 0.08*	7.19 ± 0.08*	7.33 ± 0.01
[Lactate]	0.11 ± 0.12	0.21 ± 0.09	0.11 ± 0.14	<0.05

TABLE 4. Antioxidant enzyme activities and glutathione concentrations in *Crangon crangon* abdominal muscle and hemolymph

	Catalase	SOD	Glutathione reductase	Glutathione peroxidase	Glutathione (GSH + GSSG)
Abdominal muscle	1.8 ± 0.56 (n = 3)	4.37 ± 1.07 (n = 8)	0.00026 ± 0.00038 (n = 8)	0.0137 ± 0.0028 (n = 8)	165.25 ± 29.87 (n = 8)
Hemolymph	b.d.l. (n = 3)	b.d.l. (n = 8)	0.0322 ± 0.045 (n = 8)	n.d.	0.031 ± 0.016 (n = 6)

Enzyme activities are in U mg^{-1} muscle protein and in U ml^{-1} hemolymph. Glutathione concentration is in nmol g^{-1} fresh weight for abdominal muscle and in nmol ml^{-1} for hemolymph. Data from April 1996. b.d.l., below detection limit; n.d.; not determined.

equivalent ion exchange mechanisms. In animal tissues, pH_i is not in equilibrium across the plasma membrane because the equilibrium pH would be close to pH 6.0, whereas the actual intracellular pH is usually found above 7.0. Transmembrane ion transport mechanisms play a central role in maintaining pH_i above equilibrium values (32). For example, an electroneutral, amiloride-sensitive Na/H exchanger has been found in crayfish neuron (25) and muscle (17), whereas a 4,4-diisothiocyano-2,2′-stilbene-disulfonicacid sensitive Na/HCO_3 exchanger has been identified in barnacle muscle (12) as well as in crayfish neuron and muscle (19,25). It is presently unknown whether and which of these mechanisms is affected by elevated hydrogen peroxide levels.

Detailed studies of the sublethal effects of ROS on basic cellular functions have been carried out using isolated vertebrate cells. Canine myocyte cells, treated with 2 μmol l^{-1} hydrogen peroxide or higher concentrations for 9–27 min, became depolarized and inexcitable (8), an observation that the authors attributed to the alteration of specific currents contributing to the myocyte action potential. Hydrogen peroxide has been found to decrease the activity of membrane Ca^{2+}- and Na^+-K^+-ATPases in human lens cells, leading to major disturbances of the intracellular ion concentrations in the lenses (11). If membrane function in *C. crangon* is also impaired by peroxide, this might explain the development of the intracellular acidosis. However, the effect of hydrogen peroxide might also be associated with a reduction in energy turnover by membrane transport and may therefore contribute to the reduction of metabolic rate during exposure to. H_2O_2. Obviously, these energy savings are not associated with any significant effect of hydrogen peroxide on the levels of high energy phosphates.

As a corollary, hydrogen peroxide, the concentration of which fluctuates in the natural environment of *C. crangon*, significantly influences the physiology of this inter- and subtidal species. An increase in UV-B radiation expected and already described during periods of atmospheric ozone depletion will lead to increased levels of peroxide in marine surface waters in general (16,28) and especially in the intertidal pools (2). Future work must show to what extent the physiological effects of ambient peroxide will affect survival and composition of the benthic community in those UV-exposed environments. This research must also address the mechanisms by which the action of hydrogen peroxide affects cellular functions.

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