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Trehalose Accumulation in the Tardigrade *Adorybiotus coronifer* During Anhydrobiosis

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ABSTRACT Changes in the trehalose level in the tardigrade *Adorybiotus coronifer* were observed during induction of, and arousal from, anhydrobiosis. A trehalose accumulation surpassing 1.6% dry weight (d.w.) in anhydrobiotic animals collected dry on their biotope was rapidly reduced on rehydration, reaching the level of active animals (0.1–0.2% d.w.) after 6 hours. Tardigrades dried while embedded in sand in the laboratory accumulated trehalose from 0.1% to 2.3% d.w. within 5–7 hours.

Induction of aerobic acidosis in arousing tardigrades by CO_2 perfusion reversibly arrested reactivation for at least 36 hours and induced a reduction in anabolic and catabolic activities, measured as a significant reduction in trehalose degradation, and a sevenfold reduction in the rate of protein synthesis.

These data support the hypotheis that trehalose generally serves a protective role in desiccation-tolerant Metazoa, but indicate that tardigrades require only a moderate level for efficient protection.

Trehalose accumulation

A positive correlation between the survival of the cryptobiotic state and the accumulation of the disaccharide trehalose has been demonstrated for a number of anhydrobiotic organisms, and concentrations as high as 13–18% dry weight have been reported in anhydrobiotic cysts of the crustacean *Artemia salina*[1] (Clegg, '64; '65) and in the nematode *Aphelenchus avenae* (Madin and Crowe, '75; Crowe et al., '77). Moreover, trehalose reduces damage, induced by freeze-drying, to isolated liposomes (Crowe et al., '86), microsomes from sarcoplasmatic reticulum (Crowe et al., '83), and the enzyme phosphofructokinase (Carpenter et al., '87; '88). Other data such as the physical properties of nearly dry *Artemia salina* cysts (Clegg et al., '82; Clegg, '86) and computerized modeling of molecular interactions between trehalose and a phospholipid bilayer (Gaber et al., '86) indicate that trehalose is a likely candidate for the polyhydroxy compound that, according to the water replacement hypothesis (Webb, '65; Crowe, '71), replaces the structural water of the cellular compounds during desiccation.

Anhydrobiosis is widespread in the phylum Tardigrada, but quantification of trehalose accumulation during the induction of anhydrobiosis has not yet been made in this phylum. Crowe ('75) reported that paper chromatography analysis of ethanol extracts of the tardigrade *Macrobiotus areolatus* showed more intense trehalose spots for anhydrobiotic animals than for active ones. The observations could not be quantified, and the author considered them tentative. In order to examine whether significant trehalose accumulation is a general feature of anhydrobiotic animals in different phyla, this paper is concerned with the changes (if any) of trehalose content during the induction of, and recovery from, anhydrobiosis in the tardigrade *Adorybiotus coronifer*.

pH_i-dependent regulation of metabolic status

The intracellular pH (pH_i) is known to play a regulatory role in transitions between different metabolic and developmental states in several biological systems (Busa and Nuccitelli, '84). An increase of pH_i is an important factor in the metabolic activation of the quiescent sea urchin eggs following fertilization (Shen and Steinhardt, '78), and even larger increases in pH_i are observed during germination of anhydrobiotic systems such as bacterial spores (Setlow and Setlow, '80) and probably yeast spores (Busa, '82; Barton et al., '80). In *A. salina* embryos, alkalization of the intracellular milieu up to 1.6 units has been observed during transition from an anaerobic, dormant state to aerobic development (Busa et al.,

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[1]The organism referred to in this work as *Artemia salina* has now been designated *Artemia franciscana* (Bowen et al., '80).

'82). Artificial inhibition of the increase in pH_i by addition of external CO_2 to the media (under aerobic conditions) reversibly prevents hatching of anhydrobiotic encysted embryos in this species (Busa and Crowe, '83) and suppresses the carbohydrate metabolism (Carpenter and Hand, '86). Trehalose is the major and perhaps only source of energy in newly hydrated *A. salina* cysts (Clegg, '64), and one mechanism by which pH_i regulates the metabolic status of the cysts has been elucidated by Hand and Carpenter ('86), who described a novel trehalase in extracts from newly hydrated cysts. The enzyme showed a significant decrease in catalytic activity when the pH was decreased from 7.9 to 6.3, an acidification comparable to the change in pH_i observed when the embryos enter anaerobic dormancy. However, Glasheen and Hand ('88) have recently shown that reversible reduction in the carbohydrate metabolism, observed during dehydration of *Artemia* cysts, is not dependent on intracellular acidification. At present, it appears that the reversible changes in metabolism may be triggered by both dehydration and acidification, but the pathways by which such changes are effected remains unresolved.

Since a relation between pH_i and the regulation of the metabolic arrest has not yet been established for cryptobiotic tardigrades, this paper describes some basic experiments to investigate whether acidification with external CO_2 has any influence on the reactivation and metabolism during arousal from anhydrobiosis. Measurement of alterations in pH_i would be the natural way to start this study, but at present, we are not able to provide tardigrades in the quantities required for NMR determination of pH_i and are limited to indirect methods.

Experimental animal

The eutardigrade *Adorybiotus coronifer* (Richters 1903)[2] was selected for this work because it is a true anhydrobiote, able to survive both rapid desiccation and periods of at least a year in the dry state (R.M. Kristensen, personal communication). It thus seems plausible that metabolic features important for anhydrobiotic survival will be expressed clearly in *A. coronifer*. Furthermore, it is among the largest species in this phylum with a body length up to 1,000 μm (Maucci, '86), easily separated from other tardigrades by its characteristic yellow color and is locally abundant in mosses on carbonated bedrock. Together, these attributes make is suitable for biochemical analysis.

METHODS AND MATERIALS

Preparation of the tardigrades

Dry mosses containing anhydrobiotic *A. coronifer* were collected on Öland (Sweden) in August 1988 and kept dry in the laboratory until use (4–8 months). The tardigrades were gently washed from the moss using cold tap water and a sieve column with nets of decreasing mesh size (Endecotts Ltd.). To eliminate contamination of the samples, the animals were picked individually from the 125 μm fraction of the column with an Irwin loop and transferred to a few milliliters distilled water in a watch glass.

Trehalose anabolism

Eighteen samples, each containing 200 *A. coronifer*, were hydrated and kept for 24 hours in distilled water at 15°C. The animals were transferred to plastic tubes, the lower end bounded by a 30 μm nylon net, which were embedded 5mm into wet sand (0.4 g water/g dry sand). The sand was then dried under an air stream (20°C and 50% RH), and samples were simultaneously removed for trehalose analysis at intervals of 30–90 minutes. Water-loss from the sand was observed gravimetrically, and the water potential calculated according to Salisbury and Ross ('85). After a sample had been removed from the sand, the tardigrades were transferred to an Eppendorf tube containing 300 μl 40% ethanol, heated to 90°C for 2 minutes and sonicated 4 × 15 seconds in a 150 Watt MSE ultrasonic disintegrator. The viability of *A. coronifer* dried according to this procedure over 72 hours was also examined.

Trehalose catabolism

Eighteen samples of 100–200 tardigrades rehydrated for 12, 20, 35, 60, 120, 180, 240, and 360 minutes or 24 hours, respectively, were picked up from the 125 μm fraction and transferred to 40% ethanol, heated, and homogenized as above. To evaluate the extent of leakage of trehalose from the animals during rehydration, two groups of 200 animals dried on sand as above were transferred to 100 μl of distilled water. After 90 minutes hydration, the water was transferred to plastic tubes and the animals rinsed twice with 50 μl distilled water. The pooled water fractions were dried, silylated, and assayed for trehalose content as below.

[2]Synonym to *Rictercia coronifer* (Ricters, 1903) (see Pilato and Binda, '87).

Incorporation of ^{3}H-leucine

The rate of protein synthesis during the first 5 hours and after 24–27 hours following rehydration was examined by transferring 2 groups of 250 tardigrades to distilled water containing 1 mCi / ml L-(4,5-^{3}H) leucine (Amersham Lab., Buckinghamshire; specific activity 140 Ci/mmol). The first group was transferred to the radioactive medium 15 minutes after rehydration, and samples were removed for quantification of incorporated ^{3}H-leucine at hourly intervals. The other group was kept hydrated for 24 hours, transferred to the radioactive medium, and analyzed for ^{3}H-leucine incorporation over the following 3 hours. Each sample was fixed by removing the radioactive solution and substituting 120 µl 0.4% sodium deoxycholate in 0.1 M NaOH. The sample was sonicated for 15 seconds and separated into two 50 µl samples. The radioactivity of one of these was counted in a Tri Carb 2000 liquid scintillator, giving the total activity of the homogenate (activity incorporated in protein plus activity in the free amino acid). To the other tube was added 20 µl 50 mg/ml bovine serum albumin (to ensure quantitative protein precipitation) and 100 µl 20% TCA to precipitate the proteins. It was then centrifuged for 15 minutes at 11,000*g* and the activity of the supernatant determined by liquid scintillation, giving the activity *not* incorporated into protein. The activity incorporated into protein was then calculated as the difference between the two scintillations.

Metabolism during CO_2 acidification

Two metabolic parameters (trehalose degradation and ^{3}H-leucine incorporation) were measured. Tardigrades were incubated in distilled water equilibrated with either a gas mixture of 63% N_2, 17% O_2, and 20% CO_2 (by volume) or with atmospheric air (78% N_2:21% O_2).

For measuring trehalose degradation, tardigrades were rinsed for 2–4 minutes with tap water in the sieve column and then transferred to water equilibrated with 20% CO_2. Groups of 100 tardigrades were removed, heated, and homogenized at intervals of 1 hour from 1 to 6 hours after rehydration.

To estimate ^{3}H-leucine incorporation, groups of 100 tardigrades hydrated for 24 hours were transferred to eight Eppendorf tubes containing 300 µl, 10 µCi/ml ^{3}H-leucine. Four of the tubes were equilibrated with the 20% CO_2 gas mixture, while the others were bubbled with atmospheric air. Samples were removed after 2–7 hours and analyzed for ^{3}H incorporation as mentioned before. All metabolic measurements were conducted at room temperature (20–22°C).

Dry weight determination

Eight groups of tardigrades (4 × 10 and 4 × 25) were initially dried in air (20°C and 50% RH) for 2 hours and weighed on a Cahn 23 automatic electrobalance. The animals were then placed for 90 minutes in an oven at 110°C and weighed again.

Trehalose analysis

The homogenates were centrifuged (10 minutes, 6,000*g*) and the supernatant transferred to a 300 µl tube. Precipitates were extracted three times with 100 µl boiling ethanol and once with 100 µl 20% ethanol. The pooled extracts and supernatants were placed in a heat block at 55°C and dried under a stream of nitrogen. They were then derivatized with 100 µl "Sigmasil A," a premixed reagent (Sigma, St. Louis, MO) containing trimethylchlorosilane and hexamethylsilazane dissolved in pyridine to form volatile trimethylsilyl carbohydrate derivatives suitable for gas chromatography (Sweeley et al., '63; Haverkamp et al., '71). Commercialy supplied trehalose (Sigma) was used for standards, and five standards were boiled and sonicated as above to test the stability of trehalose. Quantification was based on internal standardization with sorbitol (this polyalcohol could not be detected in crude extracts), and comparisons between chromatograms of samples and standards, made at three different temperatures and with two different columns, were used for qualitative identification of trehalose. All quantitative analysis was carried out on a Hewlett Packard 5840A gas chromatograph, equipped with a DB 1701 capillary column (30 m, 0.246 mm internal diameter), and an electronic integrator. Nitrogen was used as the carrier gas, and the oven temperature was 250°C. This method is comparable with the one described by Womersley ('81a).

RESULTS

Alterations in the trehalose level

Figure 1 shows a distinct accumulation of trehalose when *Adorybiotus coronifer* was exposed to decreasing water potentials. The trehalose level of animals hydrated for 24 hours (0 hours desiccation in Fig. 1) was approximately 0.1% of the d.w.,

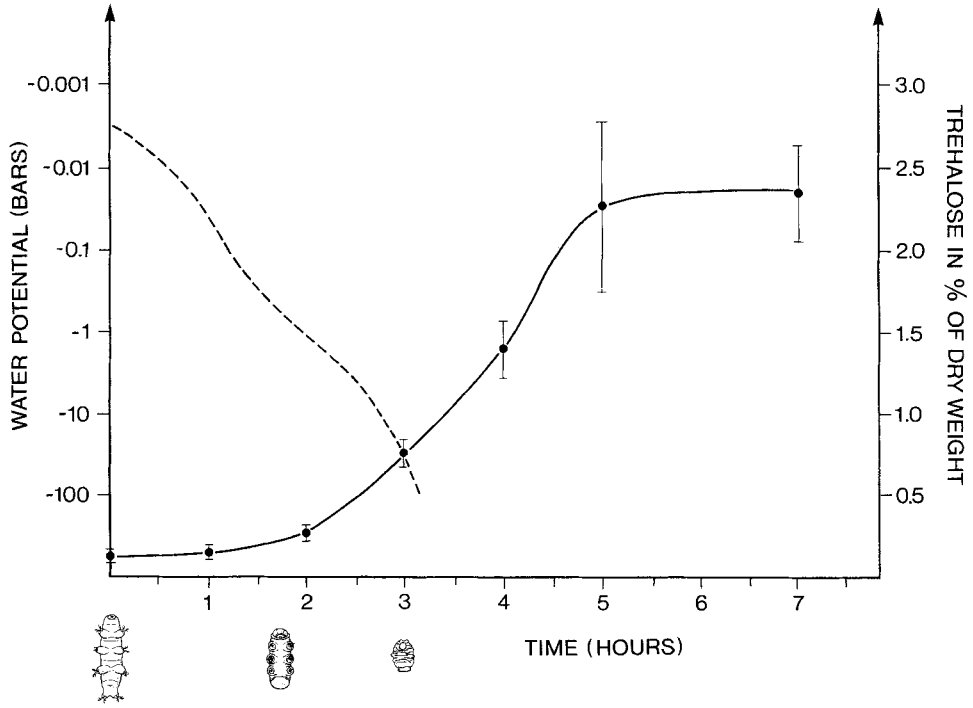

Fig. 1. Trehalose content in *Adorybiotus coronifer* dried embedded in sand. 0 hours represents active animals (hydrated 24 hours). (-----) Sand water potential (data omitted). (●——●) Trehalose level (vertical bars represent SEM of three to four measurements). Drawings below time axis refer to the morphological stage of the animals. Typical tun states are observed after 3 hours. The sand water potential is specified by the osmotic pressure (in bars) (Salisbury and Ross, '85).

whereas the level after 7 hours drying rose to 2.3% d.w. Since the tardigrades were embedded into the sand, the water potential (μ_w) they were exposed to must have been close to μ_w of the sand (dashed line in Fig. 1), and trehalose accumulation is thus observed even at reductions in μ_w of a few bars. After 3 hours, the sand was dry, and the animals, now exposed to μ_w of the air stream (approx. −900 bars), had all assumed a typical tun state characteristic of anhydrobiotic tardigrades (Baumann, '22). The trehalose concentration reached a stable level after 5–7 hours. The viability of *A. coronifer* for this desiccation procedure followed by rehydration in water was 92 ± 4% (SE) (after 72 hours desiccation).

Figure 2 illustrates the degradation of trehalose in *A. coronifer* when specimens dried on their biotope are rehydrated in distilled water. The intercept between the curve and the ordinate axis represents the trehalose content of anhydrobiotic animals. Since the method used demanded a minimum of 12 minutes for processing a sample, it is difficult to estimate the initial trehalose level (see Fig. 2), but this value is above 1.6% d.w. The trehalose concentration declined to less than half of the initial value within the first hour of hydration and approached the level of active animals (0.1–0.2% d.w.) after 6 hours. The viability after this hydration procedure was 94 ± 5% (SE).

The assay for leakage of trehalose to the surroundings during rehydration showed detectable amounts of leakage. The amount of trehalose leaked, however, was below the limit of quantification. Since the method used easily detects trehalose levels of 0.1 μg trehalose/sample, the leaked trehalose constitutes less than 1% of the initial trehalose content of the animals.

Protein synthesis

Incorporation of ^{3}H-leucine could not be detected in the first hour following rehydration. The incorporation from 1 to 4 hours was 9.8×10^3 DPM/animal/hour (calculated as the slope of a line fitted to the curve in Figure 2 from 1 to 4 hours), whereas the rate 24 hours after hydration had risen to 2.1×10^4 DMP/animal/hour. This indicates that protein synthesis, in contrast to trehalose degradation, is resumed relatively slowly after rehydration.

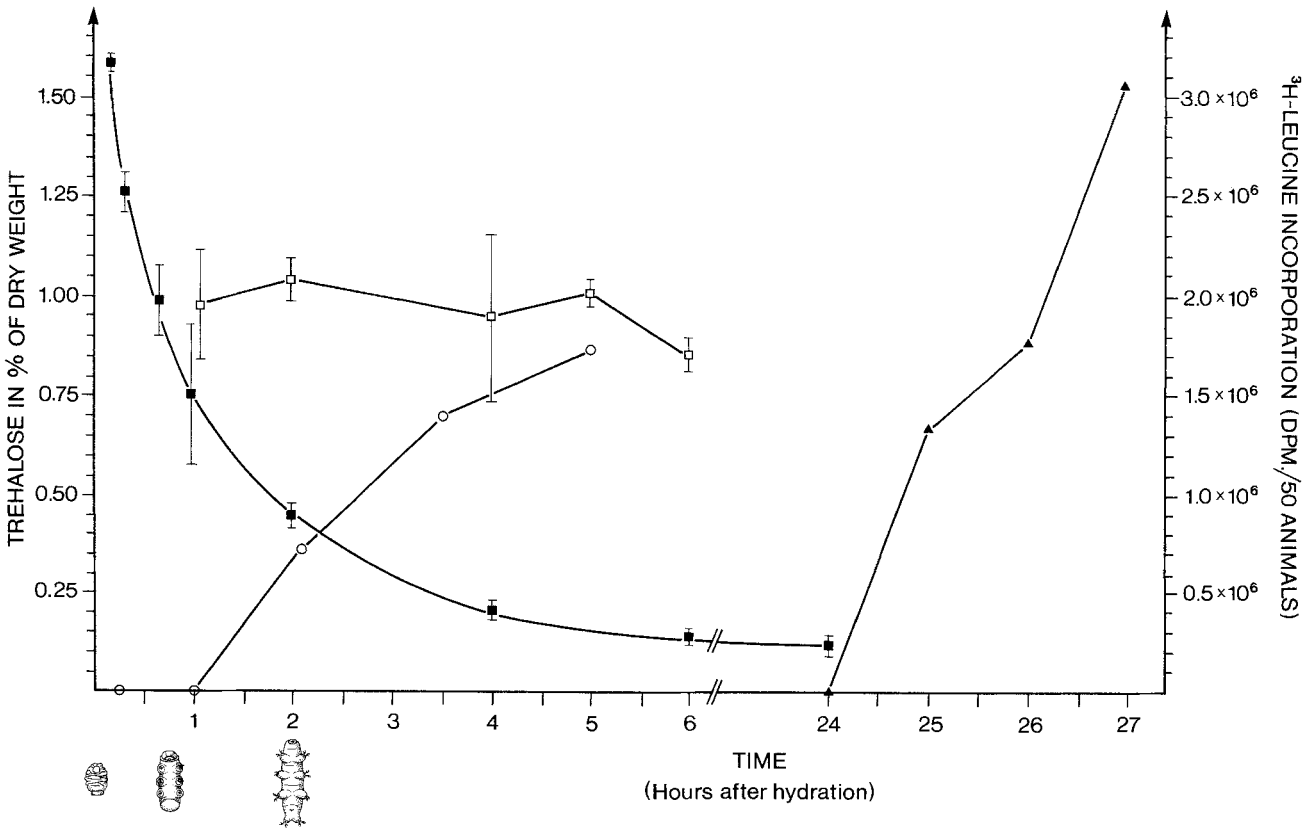

Fig. 2. Trehalose content and ^{3}H-leucine incorporation in rehydrating *Adorybiotus coronifer* collected dry on the biotope. (■——■) Trehalose level when rehydrated in distilled water under normal gassing conditions, i.e., without CO_2 (vertical bars represent SEM of two to three measurements). (□——□) Trehalose level when rehydrated in CO_2-perfused medium (vertical bars represent SEM of two to three measurements). (○——○) ^{3}H-leucine incorporation under normal gassing conditions (^{3}H-leucine added 15 minutes after rehydration). (▲——▲) ^{3}H-leucine incorporation under normal gassing conditions (^{3}H-leucine added 24 hours after rehydration). Drawings below time axis refer to the morphological state of the animals. Locomotion can be observed after 45 minutes, and normal activity is resumed after approximately 2 hours.

Metabolism and reactivation during exposure to external CO_2

When anhydrobiotic animals were hydrated in 20% CO_2:17% O_2 equilibrated water, they remained motionless and assumed an outstretched appearance in contrast to the "rugose" look of the active state. The motionless state could easily be reverted to normal activity within 30–50 minutes by changing the perfusion gas to atmospheric air. Two groups of 100 tardigrades, kept for 36 hours in CO_2-equilibrated media, did not show exaggerated mortality (survival 88 ± 6% SE) when external CO_2 supply ceased.

Trehalose degradation is markedly reduced by CO_2-perfusion, and no significant decline in the level is observed within the first 6 hours following rehydration. The mean level of this curve (0.95% d.w.) does not correspond to the trehalose concentration of anhydrobiotic animals, and possible reasons for this are considered in the Discussion. Incorporation of ^{3}H-leucine (Fig. 3) is also suppressed by exposure to CO_2. The slopes of the curves in Figure 3 (9.7×10^3 and 1.3×10^3 DPM/animal/hour, respectively) indicate an 85% reduction in protein synthesis. Thus, perfusion with external CO_2 induces a general inhibition of activity, as well as at least some catabolic and anabolic reactions.

Standards and dry weight

Standards heated and sonicated before analysis in the chromatograph showed no signs of trehalose hydrolysis. The average mass of *A. coronifer* in this population was 2.9 ± 0.3 μg/animal when

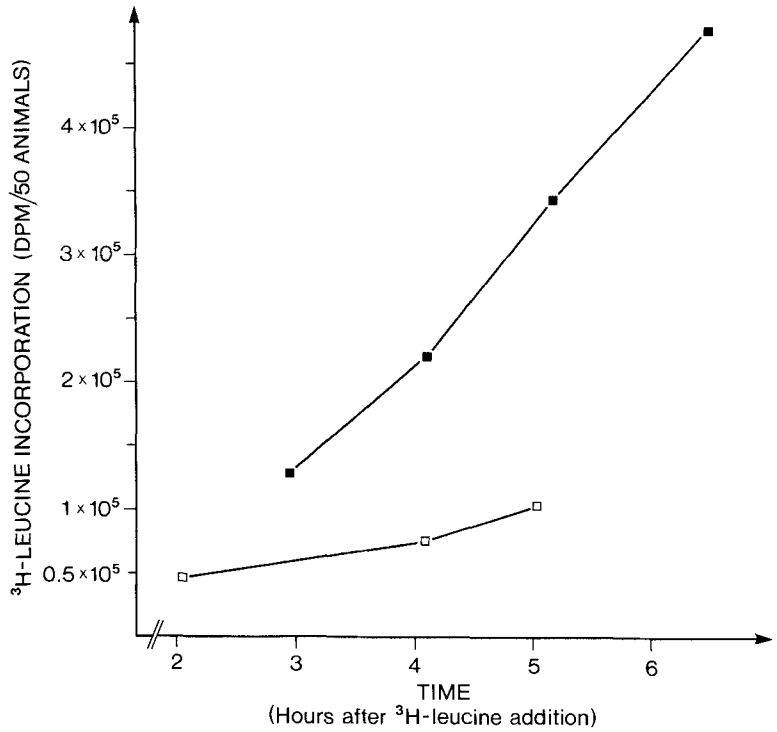

Fig. 3. Effect of CO_2 perfusion on the incorporation of ^{3}H-leucine in *Adorybiotus coronifer* rehydrated with a medium containing ^{3}H-leucine. (□——□) Medium equilibrated with 20% CO_2. (■——■) Medium equilibrated with atmospheric air. The slope of the curves reflect the rate of protein synthesis.

equilibrated with air at 20°C, 50% RH. Ninety minutes desiccation at 110°C did not reduce the mass (now 2.9 ± 0.1 μg/animal), indicating that the residual amount of "bound" water is small. This mass (2.9 μg/animal) is thus considered a reasonable estimate for the average dry weight of this population and used in the concentration calculations.

DISCUSSION

Alterations in the trehalose level

The results shows that trehalose accumulated by *A. coronifer* during drying on the biotope is degraded rapidly following rehydration (Fig. 2). Since the animals are incubated in distilled water, one could argue that the radical decrease in the trehalose level is simply a consequence of starvation or degradation of sugar absorbed from moss in the intestine. We find this explanation unlikely since tardigrades starved for 24 hours followed by desiccation in the laboratory accumulate trehalose in quantities at least as high as the ones dried on their biotope with nutrition available (Fig. 1), and we therefore suggest that a strict correlation exists between trehalose accumulation and induction of anhydrobiosis in *A. coronifer*.

The trehalose degradation is induced within 12 minutes following rehydration, while animals are still in the tun state (see drawings in Fig. 2), indistinguishable from unhydrated animals under a binocular. Incorporation of ^{3}H-leucine was not detected within the first hour following rehydration, and the rate of incorporation from 1 to 4 hours was less than half of the rate after 24 hours. These data indicate that while trehalose catabolism is an early metabolic event in rehydrating tardigrades, resumption of the protein synthesis is a much slower process.

It has previously been reported that anhydrobiotic nematodes leak low molecular weight metabolites such as primary amines, inorganic ions, and glycerol during rehydration (Crowe et al., '79; Crowe et al., '77; Womersley, '81b). The present results indicate that only a minor fraction of the trehalose accumulated in *A. coronifer* is lost by leakage during rehydration.

Accumulation of trehalose has been observed in several anhydrobiotic organisms, and especially two systems, *Artemia* cysts and the nematode

Aphelencus avenae, have been extensively investigated. Anhydrobiotic *Artemia* cysts have a trehalose level of 15–18% of the dry weight, whereas less than 0.3% d.w. is found in newly emerged nauplii (Clegg, '62, '65). The decay of accumulated trehalose is relatively slow: Clegg ('64) found that the trehalose concentration reached half of the initial level approximately 8 hours after hydration, whereas Carpenter and Hand ('86) reported that 10–12 hours was needed for the first 50% reduction (incubation was made in 0.25 M NaCl in both cases). The soil-dwelling *A. avenae* raises the level from 0.2% d.w., in the active state, to 12–13% d.w. after 72 hours exposure to air at 97% relative humidity (Madin and Crowe, '75). In this case, the trehalose level decreases rapidly (initial half-time 30 minutes) when the anhydrobiotic nematodes are transferred to water (Crowe et al., '77). Finally, three species of plant-parasitic and free-living nematodes have been studied for trehalose accumulation during the induction of anhydrobiosis, and the concentrations found ranged from 4.6 to 15% d.w. (Womersley and Smith, '81; Womersley, '81c, '88).

Alterations in the trehalose level induced by hydration and dehydration in the other anhydrobiotes examined are thus generally slower, but of a much larger amplitude than the variations observed in *A. coronifer* (except for the case of degradation in *A. avenae,* where the kinetics resemble those observed in *A. coronifer*).

Tardigrades used in this study were collected from mosses growing on limestone in an area with only scattered vegetation. We have recorded remarkably rapid alterations in temperature and humidity on this biotope (unpublished results), compared to other biotopes such as soil mosses or epiphytes on trees (for a review see Ramazzotti and Maucci, '83; Kristensen, '86). *A. avenae,* on the other hand, is a soil-dwelling nematode never exposed to fast dehydration; Womersley ('81c) even stresses that it is still to be ascertained whether it is a true anhydrobiote. We consider the ability of *A. coronifer* to accumulate the necessary concentration of trehalose approximately ten times faster and survive anhydrobiosis with a level at least five times lower than that reported for *A. avenae* (Madin and Crowe, '75) as an adaptation of *A. coronifer* to the rapid cycles of hydration and dehydration occurring in their biotope.

The water permeability in the cuticle of at least some tardigrade species decreases when the animals lose water (Crowe, '72; Wright, '89 a,b). Both authors mention that the permeability decrease might play an important physiological role prolonging the time available for biochemical preparation for the dry state. From Figure 1 it appears that the sand is dry after 3 hours and that *A. coronifer* accumulates trehalose for a further 2–4 hours. The animals are in tun states during this period and exposed to the low water potential (−900 bars) of the air. Since fully hydrated tardigrades lose water very rapidly when exposed to 80% relative humidity (Wright, '89a), accumulation of trehalose for several hours under the above-mentioned conditions seems to be in accordance with the existence of a hydration-dependent water-retentive mechanism.

In conclusion, the presented data support the hypothesis that accumulation of trehalose is an important factor in the desiccation-tolerance of anhydrobiotes from different metazoan phyla. High viability, combined with a low trehalose accumulation, indicates that *A. coronifer* either requires only a moderate level for optimal protection or possesses an ability to allocate the disaccharide selectively to drought-sensitive structures.

Regulation of the trehalose metabolism

Some of the regulatory mechanisms involved in accumulation of cryoprotectants in cold-hardy insects are brought about by simple temperature effects (Storey and Storey, '88; Haykawa, '85), and restriction of cellular water might have a direct regulatory effect by preferentially influencing the activities of specific enzymes in some anhydrobiotes (Glasheen and Hand, '88; Loomis et al., '80). A mechanism analogous to these could be responsible for the trehalose build-up observed in *A. coronifer.* Thus, the catabolic pathways could be more drought-sensitive than the anabolic ones leading to trehalose accumulation under water stress. This would be consistent with the fact that the desiccation-stressed anhydrobiote *A. coronifer* accumulates smaller quantities of trehalose than other examined anhydrobiotes, simply because less time is available between the onset of water stress and the entry to the ametabolic domain. Still, other regulatory mechanisms are indicated by the significant trehalose accumulation observed at water potentials as high as −5 to −2 bars (Fig. 1), where appreciable water stress on enzyme function seems improbable. Furthermore, this model for regulation of the trehalose level could hardly account for the rapid degradation when animals are rehydrated in water.

Influence of pH_i on resumption of metabolism

Intracellular pH is considered an important factor influencing transitions between active and hypo- or ametabolic states in many organisms (Busa and Nuccitelli, '84). Cysts of different *Artemia* species (*A. salina* and *A. fransiscana*) showed an intracellular acidification of more than 0.7 units when the medium was perfused with 20% CO_2 (Busa and Crowe, '83; Drinkwater and Crowe, '87). Assuming that 20% CO_2 perfusion also gives rise to intracellular acidification in *A. coronifer,* we have compared earlier reports on the effect of aerobic acidosis in arousing *Artemia* cysts with the effect of newly hydrated *A. coronifer.*

Artemia. Aerobic acidosis induced by perfusion with 60% CO_2:40% O_2 leads to a 70% reduction in oxygen consumption (whereas 20% CO_2 reduces O_2 uptake by 55%) (Busa and Crowe, '83) and a significant reduction in trehalose degradation (Carpenter and Hand, '86). Development of *Artemia* is reversibly arrested by perfusion with CO_2 even at low concentrations (11%), and when the perfusion gas is changed from 60% CO_2:40% O_2 to 60% N_2:40% O_2, hatching resumes at the normal frequency and with normal kinetics (Busa and Crowe, '83). The activity of trehalase extracted from *Artemia* is strongly dependent on pH (Hand and Carpenter, '86). At pH 7.8, the activity is maximal, whereas pH 6.3 induced polymerization of the enzyme and markedly reduced activity. Activation of the polymerized enzyme could be completed within a few minutes, while inactivation (polymerization) of monomers, by transfer from pH 8.6 to pH 6.3, required more than 1 hour (Hand and Carpenter, '86).

In *A. coronifer*, newly hydrated tardigrades show reduced trehalose degradation during aerobic acidosis (Fig. 2). The trehalose level is almost constant (0.95% d.w.) 1–6 hours after hydration. They also show a seven-fold reduction in leucine incorporation, indicating an 85% reduction in protein synthesis (Fig. 3). Reactivation of *A. coronifer* is reversibly arrested by perfusion with 20% CO_2, and the time needed for the animals to resume locomotion after removal of CO_2 from the perfusion gas (30–50 minutes) is similar to the time required for anhydrobiotic tun states to commence movement after rehydration (Wright, 89a). The mean level of trehalose in the animals under CO_2 perfusion (0.95% d.w.) corresponds to the concentration in tardigrades hydrated for 40–50 minutes, and not, as one might expect, to the level in anhydrobiotic animals. A possible explanation for this is a regulation of the catabolic enzyme activity similar to that reported for *Artemia* trehalase. The catabolic pathway could thus be activated during the initial 2–4 minute rinse in tapwater and subsequently suppressed gradually within the first hour following transfer to CO_2 media.

The presented data constitute only preliminary observations of the regulatory effect performed by alterations in the intracellular pH during the resumption of metabolism in tardigrades. The difference in oxygen concentration in the CO_2-perfused and the control groups (17% and 21%, respectively) and general suppressing or toxic effects of CO_2 may substantially influence the results. Still, we consider the similar effects of CO_2 acidification on reactivation and metabolism of *Artemia* cysts and *A. coronifer* as indicating that pH_i in these two species might play an analogous regulatory role during resumption of metabolism.

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