Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 8435–8438, November 1988
Biochemistry

Acidic intracellular pH shift during *Caenorhabditis elegans* larval development

(growth control/nematode development/metabolism/dauer larva/nuclear magnetic resonance)

WILLIAM G. WADSWORTH AND DONALD L. RIDDLE

Division of Biological Sciences, 110 Tucker Hall, University of Missouri, Columbia, MO 65211

Communicated by W. B. Wood, August 10, 1988 (received for review February 2, 1988)

ABSTRACT **During recovery from the developmentally arrested, nonfeeding dauer stage of the nematode *Caenorhabditis elegans*, metabolic activation is accompanied by a decrease in intracellular pH (pH_i). Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) analyses of perchloric acid extracts show that inorganic phosphate predominates in dauer larvae, whereas ATP and other high-energy metabolites are abundant within 6 hr after dauer larvae have been placed in food to initiate development. Although metabolic activation has been associated with an alkaline pH_i shift in other organisms, *in vivo* ^{31}P NMR analysis of recovering dauer larvae shows a pH_i decrease from ≈7.3 to ≈6.3 within 3 hr after the animals encounter food. This shift occurs before feeding begins, and it coincides with, or soon follows, the developmental commitment to recover from the dauer stage, suggesting that control of pH_i may be important in the regulation of larval development in nematodes.**

Changes in intracellular pH (pH_i) often accompany the modulation of important cellular processes, including glycolysis, protein synthesis, and DNA synthesis (1, 2). It has been suggested that an alkaline pH_i shift is a highly conserved, obligatory signal for stimulation of eukaryotic cell metabolism and proliferation (3–6). A rise in pH_i has been shown to be important for initiating development in at least two instances: early development of sea urchin embryos (7) and the transition between dormancy and aerobic development in brine shrimp (8). By contrast, there is an acidic pH_i shift during initiation of development from the arrested dauer (German, "enduring") dispersal stage in the soil nematode *Caenorhabditis elegans*.

Under conditions of low population density and an abundant food supply, *C. elegans* development proceeds through four larval stages, L1–L4, to the millimeter-long adult ≈3 days after hatching. However, in response to overcrowding and limited food, development can be arrested at the second molt. An appropriately high ratio of a *Caenorhabditis*-specific, dauer-inducing pheromone to a bacterial food signal (9, 10) during the L1 stage induces formation of a predauer (L2d) larva that molts to the morphologically unique, third-stage dauer larva (11–13). The dauer larva is capable of rapid movement yet can survive for several months in the absence of food. It has been considered a nonaging form because the duration of the dauer stage does not alter postdauer life-span (14). When they encounter favorable growth conditions (reduced pheromone levels and increased food supply) dauer larvae begin to feed, then resume development and molt to the L4 stage after 10 hr. Recovery from the dauer stage is also affected by temperature (11) and by ion concentrations, but not by environmental pH of 6–8 (15). Under optimal conditions, developmental commitment to recovery occurs in about 50 min, although feeding does not begin for about 4 hr (11).

Dauer larva formation was initially chosen as an experimental model because of the advantages this developmental pathway offers for genetic and behavioral analysis (13, 16). However, little is known about the physiological mechanisms by which genetic and neuronal controls are implemented. Since dauer larvae are formed in response to limited nutrition, we examined energy metabolism both in the dauer stage itself and during the developmental transition to feeding and growth. Synchronous dauer larva recovery can be closely controlled in large laboratory populations, allowing this period of development to be studied with ^{31}P NMR spectroscopy, which requires relatively large samples (up to several million animals). The ^{31}P NMR spectrum reveals the relative concentrations of phosphorus-containing metabolites, and changes in the concentrations of these metabolites indicate changes in metabolic state. In the work described here, ^{31}P NMR was used to examine intact larvae as well as perchloric acid extracts. Profiles from the extracts indicate that there is stimulation of cellular metabolism during dauer larva recovery. Measurements of pH_i by *in vivo* ^{31}P NMR revealed that this process involves a large acidic pH_i shift.

METHODS

Sample Preparation for *in Vitro* ^{31}P NMR. Wild-type (N2) dauer larvae arrested in development were produced by placing synchronized L1 larvae (17) into 100-ml liquid cultures with exogenous dauer-inducing pheromone (9). The worms were harvested after 120 hr at 25°C. A mutant strain of genotype *daf-2(el370*ts) also was used to obtain cultures containing only dauer larvae. This temperature-sensitive dauer-constitutive strain (18) grows nearly normally at 20°C, but all progeny develop into dauer larvae at 25°C. Synchronized *daf-2* L1 larvae were placed on NG agar plates (19) with a lawn of *Escherichia coli* strain OP50 and were harvested after 44 hr at 25°C.

Cultures enriched for N2 dauer larvae also were prepared by allowing worms to grow and reproduce at 25°C in liquid S medium (20) containing 5% (wt/wt) *E. coli* strain X1666. After 3 weeks the food supply was depleted, with essentially only arrested dauer larvae remaining. Recovering animals were obtained by placing these dauer larvae on a lawn of *E. coli* at 25°C for 6 hr before harvesting. L4 larvae that had not passed through the dauer stage were produced by placing synchronized L1 larvae on a lawn of *E. coli* at 25°C and harvesting 31 hr later.

Between 4 and 8×10^5 wild-type larvae were used for each experiment. The animals were collected by low-speed centrifugation, thoroughly washed in H_2O to remove the bacteria and sonically disrupted in 8% perchloric acid. After the debris was removed by centrifugation, the extracts were

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Abbreviation: pH_i, intracellular pH.

adjusted to pH 7.2 with cold 1.0 M KOH, and the $KClO_4$ precipitate was removed by centrifugation. After lyophilization to a powder, the extracts were dissolved in ^2H_2O (Aldrich).

Sample Preparation for *in Vivo* ^{31}P NMR. To prepare N2 dauer larvae or recovering dauer larvae, animals from starved liquid cultures (see above) were used directly or were placed into a fresh liquid culture of 5% *E. coli* at 25°C for a specified recovery time. Mutant *daf-2* animals were initially grown in liquid medium at 20°C, placed in a fresh liquid culture at 25°C for 2 weeks to form dauer larvae, and shifted to 15°C for recovery (21).

Worms were collected by centrifugation, washed in M9 buffer (19) to remove bacteria, and suspended in 40–50 ml of 20 mM Tris buffer (pH 7.2). The worms were allowed to settle by gravity and transferred to the NMR tube. In this manner, 8–10 ml of worms were harvested within 15 min. Control populations were grown in the same way but were extracted with perchloric acid (as above) for *in vitro* NMR analysis.

NMR Analysis. Samples were analyzed with a Nicolet NT-300 spectrometer in the Fourier transform mode. Signal assignments were made by the addition of known compounds to the extracts. The identity of the signal in the phosphodiester region of the spectrum was tested by the addition of acetyl phosphate, glycerophosphate, glycerophosphocholine, glycerophosphoethanolamine, phosphocreatine, phosphoarginine, phosphocholine, and phospho*enol*pyruvate (all from Sigma).

The *in vivo* NMR spectra were recorded by using a 20-mm tube with a coaxial reference tube containing 100 mM phosphocreatine in 20 mM Tris buffer (pH 7.0). The inorganic phosphate (P_i) signal was identified by the chemical shift relative to the external standard and by comparison to the spectra of extracts. The pH_i was determined by comparing the chemical shift of P_i to a standard curve (22), which had been prepared by adjusting the pH of dauer larva extracts to values between pH 5 and pH 8 and plotting these values against the chemical shift of P_i.

RESULTS AND DISCUSSION

Exit from the dauer stage involves a transition from an apparently low-energy state to one that is metabolically more active. P_i was the major phosphorus compound in perchloric acid extracts of dauer larvae (Fig. 1 *A* and *B*). However, when dauer larvae were placed on Petri plates containing food (*E. coli*) and were allowed to resume development for 6 hr prior to harvest and extraction with perchloric acid, signals corresponding to ATP, ADP, AMP, and sugar phosphates were seen in the spectra (Fig. 1*C*). Such spectra resemble those obtained from L4 larvae that have not been through the dauer stage (Fig. 1*D*), with the exception that they contain relatively more P_i.

Spectra from L4 larvae (Fig. 1*D*) resemble those from the nematode *Ascaris* (23), the fluke *Fasciola* (23, 24), and the tapeworm *Hymenolepis* (25). Neither phosphocreatine nor phosphoarginine was detected, but another signal was present in the phosphodiester region of the spectrum. In previously reported nematode spectra, a similar signal was assigned to glycerophosphocholine (23), but addition of this compound to *C. elegans* extracts produced a distinct signal, demonstrating that it was different from any component of the extract. Similarly, other metabolites known to produce signals in the phosphodiester region were also shown to be distinct (see *Methods*). The unidentified *C. elegans* metabolite has been purified from extracts, but its structure has not been determined.

P_i was the predominant phosphorus compound in the spectra of extracts made from dauer larvae, whether they were formed in liquid or on agar (Fig. 1 *A* and *B*). Two mutant

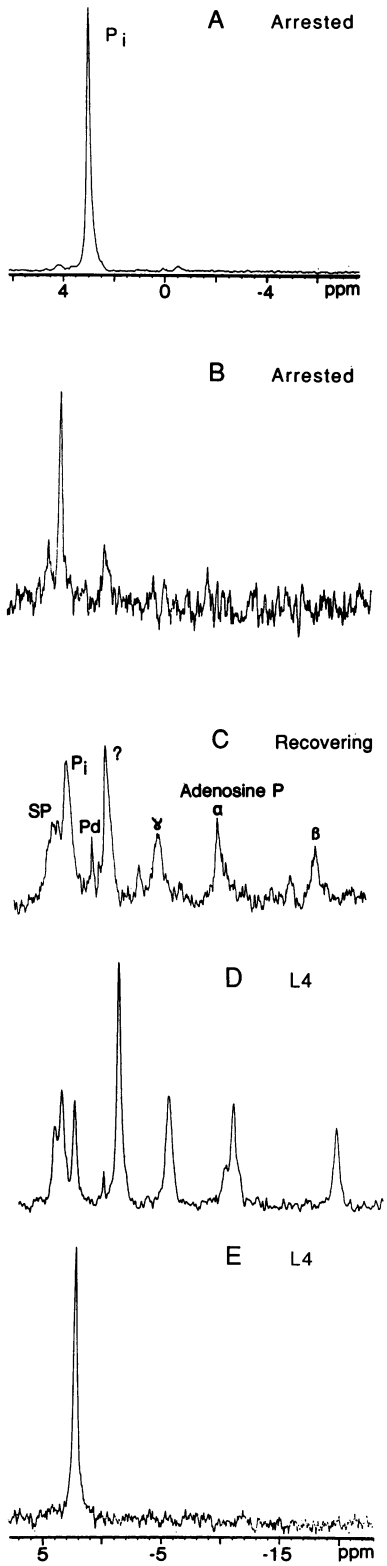

FIG. 1. ^{31}P NMR spectra of perchloric acid extracts from larvae grown at 25°C. (*A*) Developmentally arrested N2 dauer larvae produced in liquid culture by treatment with dauer-inducing pheromone. A portion of the total spectrum is enlarged to emphasize the detectable signals. (*B*) Mutant *daf-2* dauer larvae formed constitutively on a lawn of *E. coli*. (*C*) N2 dauer larvae after 6 hr on a bacterial lawn. (*D*) N2 L4 larvae that had not passed through the dauer stage and were grown on a bacterial lawn. (*E*) N2 L4 larvae developed from dauer larvae incubated in a liquid bacterial suspension for 16 hr. The same scale is used for *B–E*. Signal assignments: SP, sugar phosphates and AMP; P_i, inorganic phosphate; Pd, a phosphodiester, probably glycerophosphocholine or glycerophosphoethanolamine; ?, unidentified; Adenosine P, α, β, and γ phosphates of ATP and ADP.

strains (*daf-2* and *daf-7*) that enter the dauer stage constitutively at 25°C were used to follow the metabolic transition into the dauer stage in synchronous populations grown on Petri dishes with abundant food. Analysis of extracts showed that the levels of high-energy phosphates decreased during the L2d (predauer) stage, whereas the relative concentration of P_i increased (unpublished data). A spectrum from *daf-2* dauer larvae extracted soon after they were formed constitutively on a lawn of *E. coli* at 25°C (Fig. 1*B*) showed relatively small amounts of AMP and the unknown metabolite relative to P_i. Only P_i was detected in a spectrum from wild-type dauer larvae extracted ≈4 days after they were formed in response to addition of dauer-inducing pheromone to a liquid culture (Fig. 1*A*).

In contrast to dauer larvae, other stages produced quite different spectra depending on whether they were grown in liquid or on agar. Unlike the spectra from populations grown on agar (Fig. 1 *C* and *D*), spectra from recovering dauer larvae extracted after 6 hr in liquid culture (data not shown) or even after 16 hr (Fig. 1*E*), when the worms had become L4 larvae, showed that the predominant soluble phosphorus compound in the animals was P_i. The reduced high-energy phosphate pools in animals grown in liquid relative to animals of the same stage grown on a lawn of *E. coli* may be the result of rapid energy consumption by active swimming. Also, feeding may be less efficient because the concentration of bacterial cells in liquid suspension is less than that on an agar surface.

In spite of the different energy status, *C. elegans* development in liquid cultures proceeds normally. Developmental time from egg to adult is not increased. Recovery from the dauer stage also proceeds normally, although less synchronously, showing that the necessary metabolic transition to growth takes place. Dauer larvae placed in fresh liquid medium with *E. coli* began to feed after 4 hr, just as they would on a bacterial lawn.

The predominance of P_i in extracts from developmentally arrested dauer larvae led us to examine intracellular pH, based on the idea that pH_i may be more acidic in arrested dauer larvae than in other stages. *In vivo* ^{31}P NMR spectra were used to measure pH_i both in dauer larvae and in synchronously recovering dauer larvae. The chemical shift of P_i is pH-dependent within the physiological range and can be used to measure the pH_i by standard techniques (22). Spectral data, collected in blocks of 10 min each, showed that for up to 30 min in the NMR spectrometer there were no significant changes in the spectra. This eliminated the possibility that spectral changes might occur because of stress induced by settling the animals in the NMR tube. An exception was a spectrum from postdauer L4 larvae (not shown), in which several different P_i resonances became apparent after 20 min. Nevertheless, spectra could be acquired before changes due to anoxia or other possible stress occurred, thus avoiding the need for a perfusion system within the instrument.

For *in vivo* spectra, liquid cultures were employed to obtain a sufficient number of animals and to minimize the time required for transfer from the culture to the NMR spectrometer (about 15 min). *In vivo* spectra of arrested dauer larvae from liquid cultures in which the bacteria had been depleted (Fig. 2*A*) closely resembled spectra from extracts of arrested dauer larvae produced with exogenously added dauer-inducing pheromone (Fig. 1*A*). Also, the *in vitro* spectrum from animals recovering from the dauer stage in liquid culture (not shown) was essentially the same as the *in vivo* spectrum of such animals (Fig. 2*B*), demonstrating that the brief preparation of living animals for *in vivo* NMR analysis did not alter the phosphate pools in a detectable way.

In the *in vivo* spectra from recovering dauer larvae, two peaks (Fig. 2*C*, P_ix and P_iy) were present, with the major signal shifted relative to an external standard and to the P_i signal in dauer larvae. In a control experiment, an extract of

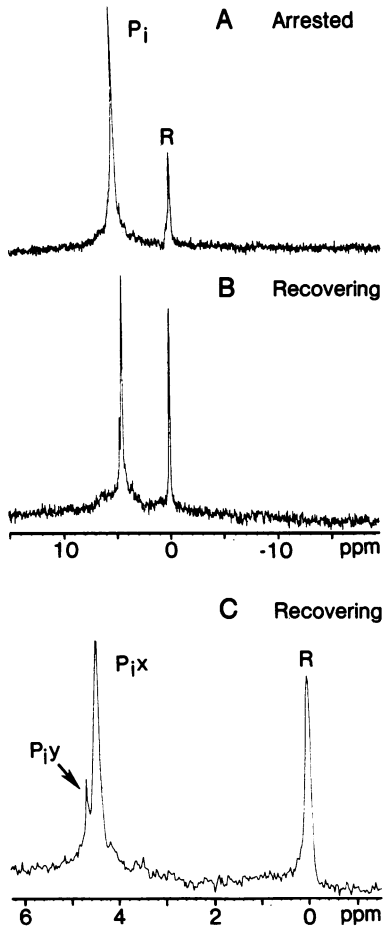

FIG. 2. *In vivo* ^{31}P NMR spectra of arrested (*A*) and recovering (*B* and *C*) N2 dauer larvae prepared from liquid cultures. The region corresponding to the P_i signals in *B* is shown enlarged in *C*. Worms used for *B* were collected 3 hr after they had been placed in food. The peak labeled R represents the reference signal, phosphocreatine at pH 7.0.

recovering dauer larvae was spiked to identify the single resonance peak as P_i. Hence, peak P_ix is not a signal from some novel phosphorus-containing metabolite that was synthesized from the P_i pool. The existence of the two signals indicates that P_i is present in at least two compartments with different pH values. The pH remained constant in the minor compartment during dauer larva recovery. This compartmentalized pH_i may be subcellular (e.g., mitochondrial vs. cytoplasmic) or the result of differences between tissues. As shown by electron micrographs of sections through larvae (16), virtually all of their internal volume is intracellular (in dauer larvae even the intestinal lumen is constricted). Therefore, the major P_i signal (P_ix) represents the pH_i of most cells within the animal.

Table 1 summarizes the pH_i of dauer larvae, recovering dauer larvae, and postdauer L4 larvae. The experiments were performed with both the N2 strain of *C. elegans* and strain CB1370, *daf-2(el370ts)*, which carries a temperature-sensitive mutation allowing 100% of the population to form dauer larvae at 25°C, even in the presence of abundant food (18). When shifted to 15°C, these dauer larvae recover normally (21). Neither this mutation nor the temperature of recovery affected the transition in pH_i values. Arrested dauer larvae had a pH_i value of 7.1–7.5, but within 3 hr after the larvae were placed in food, the pH_i decreased by ≈1.0 unit, to pH ≈6.3. This decrease occurred before feeding began, and the lower pH_i continued through the recovery period into the L4 stage.

Table 1. Chemical shifts and the corresponding pH_i values for arrested and recovering dauer larvae

Larvae	Recovery time, hr	Chemical shift, ppm	pH_i	ΔpH_i*
N2 (wild type)				
Arrested dauer	—	5.3, 5.2	7.5, 7.3	—
Recovering dauer	3	4.4	6.3	−1.1
	4	3.9	5.7	−1.7
	5	4.4	6.3	−1.1
Postdauer L4	22	4.5	6.4	−1.0
daf-2 (mutant)				
Arrested dauer	—	5.1	7.1	—
Recovering dauer	4	4.1	6.0	−1.1
	11	4.0	5.8	−1.3

Recovery time for N2 was the time elapsed from being placed in food, and for *daf-2* was the time elapsed from the temperature downshift. All chemical shifts are in reference to phosphocreatine at pH 7.0.

*An average of pH 7.4 was used for arrested N2 dauer larvae to calculate ΔpH_i in recovering N2 larvae; pH 7.1 was used to calculate ΔpH_i in recovering *daf-2* larvae.

Although the worms were washed with buffer to remove bacteria, a trace might have remained. However, no signals were contributed to the spectra by bacteria because of the low sensitivity of ^{31}P NMR. This was demonstrated by the fact that spectra from wild-type dauer larvae formed by starvation were identical to those from *daf-2* dauer larvae formed in the presence of abundant food. The nematode spectra (Fig. 1 *C* and *D*), including the predominant signal in the phosphodiester region, are different from published *E. coli* spectra (26). Hence, spectral changes are a result of the physiological state of the nematodes and not the result of external culture constituents.

We conclude that the early phase of dauer larva recovery, prior to the initiation of feeding, involves a change in pH_i. Unlike the changes during metabolic activation so far reported, pH_i decreases, and the magnitude of the change is much greater than the 0.3- to 0.5-unit changes observed during activation of most cells. It approaches the largest reported thus far, a change from pH 6.3 to 7.9 during the transition between anaerobic dormancy and aerobic development in the brine shrimp *Artemia* (27). The large decrease in pH_i upon initiation of dauer larva recovery presumably affects many cellular activities important to executing the developmental transition. Therefore, the control of pH_i in *C. elegans* may be important both in the maintenance of the dauer state and in the reinitiation of development.

The *C. elegans* dauer larva may be a useful system for studying the mechanisms employed for metabolic and developmental transitions common to a variety of organisms. Data from our laboratory indicate that dauer larvae possess cyclic AMP-dependent and -independent protein kinase activities and that certain proteins become dephosphorylated within 1 hr after dauer larvae are placed in food (A. Ray and D.L.R., unpublished data). Regulation of pH_i may be a key event involving growth factor-associated protein phosphorylation in mammals (6). Recent studies with third-stage "infective juveniles" of the parasitic nematode *Haemonchus contortus* suggest that the host agents H_2CO_3 and HCl may initiate Ca^{2+}-dependent development by inducing changes in pH_i (28). Since third-stage infective larvae of many parasitic nematodes are developmentally analogous to dauer larvae (29), an understanding of the cellular physiology of the *C. elegans* dauer larva might be applicable to parasite control.

We thank Dr. Hanna Gracz and the University of Missouri–Columbia NMR Facility for assistance in obtaining NMR spectra and Dr. Christopher Womersley for the suggestion to examine intracellular pH. The Nicolet NT-300 spectrometer was purchased in part by a National Science Foundation grant. This work was supported by Department of Health and Human Services Grant HD11239 to D.L.R.; W.G.W. was a predoctoral trainee supported by GM07494.

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