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SHORT COMMUNICATION

INTRACELLULAR pH DECREASES DURING ENTRY INTO ESTIVATION IN THE LAND SNAIL *OREOHELIX STRIGOSA*

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Given that respiratory acidosis occurs in land snails during entry into estivation (for a review, see Barnhart, 1989), it is of interest to know the extent of intracellular acidification during this transition. Efforts to measure intracellular pH (pHi) of estivating snails, however, have been complicated by the fact that manipulations inherent in some techniques (e.g. injection of radiolabeled tracers) may cause arousal of dormant snails (B. B. Rees, personal observations). In the present study, we report on the use of ^{31}P-n.m.r. as a non-invasive technique for measuring pHi in the land snail *Oreohelix strigosa* (Gould) during entry into estivation. This approach has allowed us to obtain a continuous record of pHi during this transition without disturbance to the animal. We have found that pHi declines rapidly and that over 3 days of estivation pHi drops by 0.4–0.5 units. Since the metabolism of a variety of organisms and cell types is sensitive to pHi (Busa and Nuccitelli, 1984), alterations of pHi during estivation may contribute to the overall metabolic suppression observed in this quiescent state.

O. strigosa were collected near Glenwood Springs, Colorado, in November 1990 and were maintained in the laboratory as previously described (Rees and Hand, 1990). The whole mass (soft tissue plus shell) of the snails ranged from 1.0 to 1.5 g. Following the n.m.r. experiments, species identification was confirmed electrophoretically (Rees, 1988).

n.m.r. studies were performed on a 7.05-T 10-cm bore cryomagnet with an AM-300 spectrometer (Bruker Instruments, Billerica, MA) using a home-built two-turn surface coil. The surface coil was made of insulated 2 mm copper wire and had a diameter of 1 cm. Fixed and variable capacitors (RS Laboratories, Oxford, UK) were placed for tuning and balanced matching. This coil had a loaded Q that exceeded 100.

Approximately 30 min prior to the start of an experiment, estivating snails were aroused by transferring them to dampened paper toweling. Snails were then

Key words: phosphorus nuclear magnetic resonance, acidosis, hypercapnia, dormancy, gastropod mollusc, *Oreohelix strigosa*.

individually secured to the coil with a circular band of elastic rubber. The coil was in direct contact with the dorsal aspect of the shell, encircling the first 3–4 whorls of the shell. It was tuned to the frequency of ^{31}P (121.5 MHz), and the snail and coil were placed in the magnet. The B_0 field was shimmed using the proton signal determined from the snail, as described by Ackerman *et al.* (1981). The proton linewidth was generally less than 120 Hz. Following shimming, ^{31}P-n.m.r. spectra were acquired serially using a pulse width of 6 μs (an apparent 45° tip at the center of the coil), a relaxation delay of 0.95 s, a sweep width of 10 kHz, data arrays of 1000, and 3600 transients. Each spectrum, therefore, represents 1 h of data acquisition. Free induction decays were zero-filled to 2000 before exponential multiplication with 35-Hz line broadening and Fourier transformation.

A typical ^{31}P-n.m.r. spectrum obtained with the above acquisition parameters from one *O. strigosa* is shown in Fig. 1. The major peaks correspond to inorganic phosphate (2.82 p.p.m.); arginine phosphate (−2.81 p.p.m.); and γ, α and β

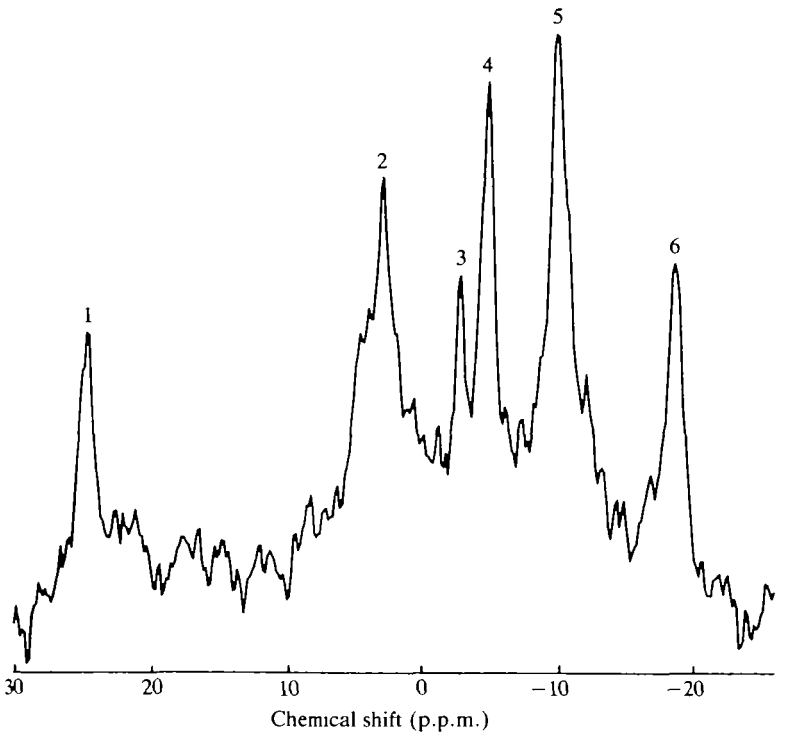

Fig. 1. A ^{31}P-n.m.r. spectrum obtained from 3600 scans of *Oreohelix strigosa*. The abscissa is chemical shift in p.p.m. (1 p.p.m. = 121.5 Hz) relative to an external standard of 85 % phosphoric acid. The major peaks correspond to (1) unknown phosphonate; (2) inorganic phosphate; (3) arginine phosphate; (4, 5, 6) γ, α and β resonances, respectively, of ATP. Peak assignments for 2–6 were verified by spiking a neutralized perchloric acid extract with known amounts of inorganic phosphate, arginine phosphate and ATP.

resonances of ATP (−4.90, −10.04 and −18.40 p.p.m., respectively). In all snails studied, a large peak was observed far downfield (24.69 p.p.m.; Fig. 1), and in some cases an additional peak was noted in this portion of the spectrum (approximately 22.5 p.p.m.). Resonances in this region are typical of phosphonate compounds, and we have assigned these peaks as unknown phosphonates. Similar unidentified resonances have been observed in the freshwater snail *Biomphalaria glabrata* (Thompson and Lee, 1985). The chemical shifts of these unknown peaks indicate that neither resonance is due to 2-aminoethylphosphonic acid, which is predominant in the egg masses of the snail *Helisoma* (Miceli *et al.* 1980). Preliminary analyses have shown that these peaks are in neither the shell nor the food of the snails, and are at least partially recovered in perchloric acid extracts of whole *O. strigosa*. Furthermore, the resonances are not observed in the perchloric-acid-insoluble material or in chloroform:methanol extracts of snails, suggesting that the unknown phosphonates are not associated with protein or lipid.

Intracellular pH was determined based on the difference between the chemical shifts of inorganic phosphate and arginine phosphate (cf. Ellington, 1983). With the surface coil oriented as described above, the digestive gland occupied a large portion of the sensitive volume of the coil, although the spectra are probably derived from several tissues. Hence, the measured pH reflects an average of several compartments, and the absolute value of pHi of any particular tissue is uncertain. This point is not critical to this study as the relative changes in pHi were of prime interest. It is appropriate to note that the contribution of extracellular phosphate to the measured pH values should be minimal as the concentration of phosphate in the hemolymph of land snails is extremely low (Burton, 1983).

In order to determine pHi from the chemical shifts of inorganic phosphate and arginine phosphate, we generated a titration curve with solutions containing (in $mmol\,l^{-1}$): potassium acetate, 40; K_2HPO_4, 10; NaCl, 13; glycine, 8; alanine, 6.5; glutamate, 4.5; aspartate, 1; arginine phosphate, 3; and EDTA, 5 (Meincke, 1975; Wieser and Schuster, 1975). The titration curve ranged from pH 5 to pH 8, in increments of approximately 0.2 units. Spectra at each value of pH were obtained with a commercial broad-band probe (Bruker Instruments) and the following acquisition parameters: pulse width of 25 μs (90° tip); relaxation delay of 10 s; sweep width of 3.3 kHz; 2000 data arrays; four transients. The data were fitted to the equation:

$$pH = pK' + \log_{10}[(d_{obs} - d_a)/(d_b - d_{obs})],$$

where d_{obs} is the observed difference in chemical shift of inorganic phosphate relative to arginine phosphate, and d_a and d_b are the chemical shift differences of fully protonated and fully dissociated inorganic phosphate, respectively (Shapiro, 1990). The values for the constants and their 95 % confidence intervals were: $pK'=6.88\pm0.03$; $d_a=3.74\pm0.03$ p.p.m. and $d_b=6.12\pm0.02$ p.p.m. The limit of resolution of this technique was 0.08 p.p.m. with our acquisition parameters, corresponding to approximately 0.1 pH units.

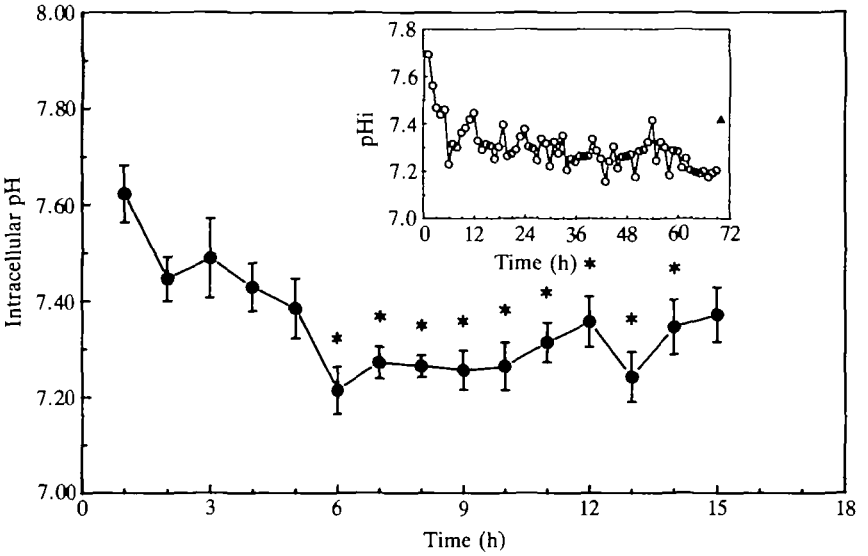

Fig. 2. Intracellular pH (pHi) of *Oreohelix strigosa* during estivation. The main panel shows the time course of intracellular acidification during entry into estivation. Asterisks indicate values significantly different from the first hour value (95% confidence intervals do not overlap). Symbols represent the mean hourly pHi values of nine experiments, except for hour 1 when there are data from 13 experiments, with six individuals, and bars are 1 S.E.M. The inset shows pHi during 3 days of estivation and the recovery of pHi during arousal. During 66 h of estivation, 54 of the 1-h intervals were significantly lower than the value for non-estivating snails (hour 1). At the end of the experiments, humidity was elevated for 1 h and pHi was not significantly different from the non-estivating pHi value (solid triangle). Symbols represent mean values from three experiments with three individual snails (except for hours 18–20 and 70, when data were collected from two snails). Standard errors were generally similar to those in the main panel, and error bars have been omitted for clarity. The temperature was 24±1°C throughout.

^{31}P-n.m.r. spectra were collected from *O. strigosa* continuously as snails were induced to estivate by lowering the ambient relative humidity (Fig. 2). Experiments were begun with active snails and, over the first 1-h sampling interval, the pHi was 7.62±0.060 (S.E.M., N=13). The extracellular pH (pHe) of non-estivating *Oreohelix* spp. is 8.03 (Rees and Hand, 1990), reflecting a difference between pHi and pHe of 0.41 units. The relative humidity was approximately 20% and, at this humidity, snails withdraw into their shells and begin estivation within a few hours (B. B. Rees, personal observations). Over 6 h, the pHi of these snails declined to a value of 7.21±0.049 (N=9). The pHi appeared to recover partially from this acidosis, as values fluctuated between 7.25 and 7.35 over the next 9 h. The extent of intracellular acidification during entry into estivation corresponds well with the drop in pHe measured during this transition in *O. strigosa* (Rees and Hand, 1990).

To follow pHi over several days of estivation, and to assess the reversibility of the intracellular acidification, a perfusion system was employed (inset to Fig. 2).

These experiments were begun with active snails perfused with humid air [approx. 500 ml min^{-1}, relative humidity (RH)$\geq$80 %]. The pHi over the first 1-h interval was 7.69±0.011 (N=3). After 3 h of high humidity, snails were perfused with dry air (RH$\leq$20 %) for the next 66 h. The majority of the decline in pHi occurred within the first 6–12 h, and over the next 3 days the pHi oscillated around 7.3. The oscillation of pHi may reflect periodic bursts of ventilation and fluctuations of extracellular acid–base variables (cf. Barnhart and McMahon, 1987). At 66 h of estivation, the pHi was 7.20±0.039 (N=3), compared to a pHe of 7.52 at 72 h (Rees and Hand, 1990). These data show that over this period of estivation the difference between pHi and pHe is maintained at 0.3–0.4 units. At the end of 66 h of perfusion with dry air, two of the three snails were exposed to elevated ambient humidity for 1 h (triangle, inset to Fig. 2). Of these, one showed no change in pHi (remained at 7.23), whereas the other showed an increase in pHi from 7.16 to 7.61. These observations demonstrate the variability in the timing of arousal noted in studies of respiration (Herreid, 1977), but suggest that, in those animals that do arouse, the intracellular acidification is quickly reversed.

In the only other study of intracellular pH of land snails, Barnhart and McMahon (1988) used the weak acid 5,5-dimethyl-oxazolidine-2,4-dione (DMO) to measure pHi in non-estivating *Otala lactea*. This snail has a pHi of about 7.69 [calculated from equation 3 of Barnhart and McMahon (1988) for a hemolymph P_{CO_2} of 1.79 kPa (Barnhart, 1986)], which agrees well with the values reported here for non-estivating *Oreohelix strigosa*. The difference between pHi and pHe (7.86; Barnhart, 1986), however, is only 0.17 units in *O. lactea*, or about half of the value noted for *O. strigosa*. This discrepancy could be a species difference in the magnitude of the transmembrane pH gradient or a result of differing techniques for the measurement of pHi. When non-estivating *O. lactea* were exposed to elevated levels of ambient CO_2, pHi declined in parallel with pHe (Barnhart and McMahon, 1988). We have now demonstrated that in *O. strigosa* pHi and pHe decline in parallel during entry into estivation. Furthermore, during this transition the temporal nature of the change in pHi is very similar to alterations of metabolism in this species (Rees and Hand, 1990), supporting the hypothesis that intracellular acidosis may be one factor involved in metabolic suppression during estivation (for evidence for the involvement of other factors, see Rees and Hand, 1991).

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