continuous; yet the general homogeneity of the infill suggests that we are not dealing with much time, perhaps weeks to a few years.

Four species of carnivores, represented by about 11 individuals, have been found in the three dens: the bear dog *Daphoenodon superbus*, the unnamed temnocyonine bear dog, a small canid *Phlaocyon annectens*, and the mustelid *Paroligobunis simplicidens*. Two species of bear dogs were found by us in place in the burrows: the aged *Daphoenodon* in den 3 and the adult temnocyonine in den 2. Because of the proximity of these dens to an early Miocene braided stream (*11*), flooding of the burrows is the most likely cause of death of younger animals.

In summary, this unusual site appears to represent a den complex of early Miocene amphicyonid, canid, and mustelid carnivores that was developed during an unknown time interval in the channel floodplain of a wide, shallow, ephemeral stream which was subject to intense periodic floods, probably seasonal in nature. *Daphoenodon* was the principal occupant of the dens, but other bear dogs, canids, and mustelids used them for shelter, and *Daphoenodon* females probably used the dens to care for their young. Before the den complex was buried by stream sediments, floodwaters may have drowned juvenile and young adult bear dogs (including a mother-cub pair) in their dens. The carcasses decomposed in the burrows and were disturbed by scavengers and additional floods. As shifting episodic streamflow resulted in channel migration over the dens, ash-rich sediment gradually filled the burrows, thereby preserving a remarkable association of young, mature, and aged extinct Carnivora.

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References and Notes

1. The age of the den complex is determined from two volcanic tuffs, Sioux County, Nebraska: (i) Agate Ash, about 10 m stratigraphically below the dens and main Agate bone bed, 21.3 million years, KA sample 481 [J. Evernden *et al.*, *Am. J. Sci.* **262**, 145 (1964)]; (ii) Eagle Crag Ash [location: W½, NW¼, SW¼, SE¼, section 27, T.32N, R. 56W], either stratigraphically above or about the same level as the dens, dated at 19.2 ± 0.5 million years (1 standard deviation), fission track (zircon) by R.M.H.
2. E. Riggs [*Fieldiana Geol.* **8**, 59 (1942); *ibid.* **9**, 69 (1945)] reported finding partial skeletons of two small carnivores, each found separately in the burrows of fossorial rodents, early Miocene, Harrison Formation, Sioux County, Nebraska, in 1906. They were not in burrows of their own making. R. J. Emry (personal communication) has discovered bones of two individuals of the small early Oligocene canid *Hesperocyon*, as well as bones of many smaller mammals, probably prey of the canid, in an early Oligocene burrow in Wyoming. He believes that the burrow was occupied by, but probably not excavated by, *Hesperocyon*.
3. See M. Voorhies, in *The Study of Trace Fossils*, R. Frey, Ed., (Springer-Verlag, New York, 1975), p. 336.
4. Older vertebrate burrows that contained resident fossil mammals are those of extinct fossorial rodents [O. Peterson, *Mem. Carnegie Mus.* **2**, 139 (1905)], early Miocene, Harrison Formation, Sioux County, Nebraska. These burrows preserved not only rodents but also two carnivores (*2*). Somewhat older Oligocene burrows (*3*) from the Brule Formation, Nebraska, did not contain mammal bones.
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9. On basis of development of living wolves (*8*), the juvenile den 1 *Daphoenodon* is 6 months to 1 year old, since its canines are in final eruption, and long bone epiphyses are still unfused. In wolves, permanent canines are about half erupted at 6 months; epiphyses fuse about 1 year from birth.
10. The adult and juvenile *Daphoenodon* were found in 1905 by O. Peterson [(*7*); *Ann. Carnegie Mus.* **4**, 51 (1907)], who discovered and named quarry 3 and reported from it three or four other individuals of *Daphoenodon*, a small canid, and mustelid. Despite the number of carnivores and the absence of herbivores, Peterson did not identify the site as a den complex. To determine why only carnivores were found, we relocated Peterson's original site. By screening topsoil at the most probable locality at Beardog Hill in September 1981, we found a proximal *Daphoenodon* tibia that fit a bone fragment collected by Peterson in 1905. In Fig. 1, quarry 3 could have been limited to meter squares B–D 5–6, an area of 6 m^2, where the bone fragment was found.
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Intracellular *p*H Regulates Transitions Between Dormancy and Development of Brine Shrimp (*Artemia salina*) Embryos

Abstract. *The intracellular* pH (pH_i) *of encysted gastrula-stage embryos of the brine shrip,* Artemia, *as previously shown by in vivo phosphorus-31 nuclear magnetic resonance spectroscopy, increases by more than 1 unit during arousal from cryptobiotic dormancy and decreases by the same amount during reinduction of dormancy. These changes in* pH_i *are now shown to be a fundamental regulator of the transitions between dormancy and metabolism: acidification of activated embryo* pH_i *by more than 1 unit with carbon dioxide induced a state comparable to natural dormancy, while alkalinization of dormant embryo* pH_i *with the weak base ammonia terminated natural dormancy. This demonstration of* pH_i*-mediated regulation of cryptobiotic dormancy extends the known scope for* pH_i *as a regulator of development to include multicellular stages of the metazoan life cycle.*

Cellular dormancy, a spectrum of hypometabolic states involving inhibition of both catabolic and anabolic processes, is common to some stage of the life cycles of most organisms. Arousal from the comparatively modest dormancy of the sea urchin egg is regulated in part by a moderate (~ 0.4 unit) alkalinization of intracellular *p*H (*p*H_i) (*1, 2*). Even larger increases in *p*H_i (~ 1 unit) accompany the transitions between dormancy and metabolism of bacterial (*3*) and perhaps also yeast spore (*4, 5*) germination, but the physiological significance of these larger *p*H_i changes is not yet clear (*6*). The largest *p*H_i changes observed under biologically meaningful conditions (> 1 unit) accompany transitions between profound dormancy (cryptobiosis) and development in encysted gastrula-stage embryos (cysts) of the brine shrimp, *Artemia salina* (*7*). While the extreme degree of dormancy of *Artemia* cysts is unusual among metazoans, its true reversibility, enabling study of both initiation and termination of dormancy, offers opportunities to further test the role of *p*H_i in metabolic regulation.

The *Artemia* cyst displays two distinguishable but related forms of dormancy (*8*). Newly released from the ovisac, the cyst remains developmentally arrested for extended periods. Under natural conditions, such aerobic dormant cysts, which are so called because the embryos remain dormant even in the presence of oxygen, are activated to resume metabolism and development by desiccation and subsequent aerobic rehydration. When rehydrated in the absence of oxygen, however, they remain dormant, or reenter dormancy if oxygen is withdrawn after aerobic rehydration; we term these "anaerobic dormant" cysts because they normally remain dormant only under anoxic conditions. Aerobic dormancy, anaerobic dormancy, and aerobic development thus represent three alter-

native physiological states of a single developmental stage, the late gastrula, modified in some fashion by the process of desiccation and rehydration.

As observed by in vivo ^{31}P nuclear magnetic resonance spectroscopy (^{31}P-NMR), changes in pH_i accompany the reversible transitions between aerobic development ($pH_i \geq 7.9$) and anaerobic dormancy ($pH_i = 6.3$) (*7*). In order to assess the physiological significance of the pH_i decrease accompanying induction of dormancy, we first tested the effect of intracellular acidification on cyst aerobic metabolism. We used simultaneous ^{31}P-NMR and O_2 polarography to determine both pH_i and respiration rate in activated (that is, desiccated and rehydrated) cysts incubated under constant oxygen partial pressure and increasing partial pressures of the membrane-permeant weak acid, CO_2 (Fig. 1). The CO_2 depresses pH_i from its normal aerobic value of ≥ 7.9 to 6.8 (60 percent CO_2) and inhibits respiration in a pH_i-dependent manner by up to 70 percent over this pH_i range. Respiratory inhibition is reversible upon removal of CO_2.

Development, too, is reversibly arrested by intracellular acidification with CO_2 (Fig. 2). In contrast with aerobically rehydrated controls incubated without CO_2, in which hatching commences by 11 hours and is completed by about 72 hours (dashed line in Fig. 2), cysts incubated under either 11 or 60 percent CO_2 (pH_i about 7.4 or 6.8) do not hatch for at least 110 hours in the presence of O_2; acidification of pH_i thus mimics aerobic dormancy. When CO_2 is removed (time zero in Fig. 2) hatching proceeds to normal levels and with normal kinetics in cysts previously treated with 60 percent CO_2 but proceeds substantially more synchronously in cysts previously incubated under 11 percent CO_2. This latter observation suggests that some intermediate step of preemergence development is unusually sensitive either to depression of pH_i or to its metabolic consequences and offers the possibility that CO_2-induced synchronization of development may prove useful in biochemical studies, which have been complicated by the developmental asynchrony apparent in Fig. 2. It is important to note that synchronization appears not to involve cell cycle regulation, since preemergence development in *Artemia* occurs without cell division (*9*).

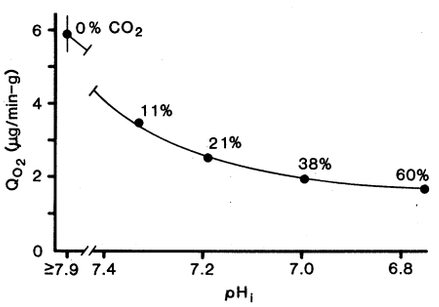

Fig. 1. Dependence of respiratory rate (Q_{O_2}, in micrograms of oxygen consumed per minute per gram dry weight) on intracellular pH. The pH_i was manipulated by increasing superfusion CO_2 partial pressure as indicated (at constant oxygen partial pressure). Points represent the mean from two experiments. Bar represents two standard errors of the mean, where this is larger than the graphical representation of the mean (*16*).

Table 1. Termination of aerobic dormancy with NH_3. Aerobic dormant cysts were collected in the laboratory soon after release from females collected near San Francisco. Precautions were taken to avoid desiccation. Cysts were incubated at low density on filter paper disks supported on the meniscus of the indicated buffer in petri dishes held over reservoirs of the same buffer in sealed hygrostats at ~ 23°C. Control (NH_3-free) buffer was 200 m*M* NaCl and 50 m*M* Tricine (*N*-[tris(hydroxymethyl)methyl]glycine), pH 8.5 at 23°C. The NH_3 buffer was 40 m*M* NH_4Cl, 160 m*M* NaCl, and 50 m*M* Tricine, pH 8.5 at 23°C. After 20 hours, the cysts incubated in NH_3 and their filter paper disk were rinsed and transferred to a petri dish and hygrostat containing control buffer. Desiccated controls were dried for 48 hours over anhydrous $CaSO_4$, rehydrated 2 hours in ice-cold control buffer, and incubated as controls. Hatched embryos developed to the free-swimming nauplius stage. Each result is the mean (and standard deviation) of three counts of 100 to 200 cysts each.

Incubation (hours)	Mean hatch (%)		
	Control	Incubated in NH_3	Desiccated and rehydrated
30	3.4 (1.1)	5.5 (0.5)	
44	5.6 (2.3)	10.5 (1.3)	
68	4.9 (0.8)	33.4 (2.1)	26.1 (4.4)

Because CO_2 is an important metabolic intermediate, its inhibitory effect on respiration and development cannot be unambiguously attributed to its effect on pH_i. The shell enclosing the *Artemia* embryo is impermeable to agents other than low molecular weight gases and water (*8*), thus preventing the use of other weak acids or ionophores to acidify pH_i, and pH_i is effectively independent of buffer pH for the same reason (*7*). However, if aerobic dormancy is due to a depression of pH_i, then alkalinization with a volatile weak base such as NH_3 should terminate aerobic dormancy. Using previously described techniques (*7*), we observed that NH_3 penetrates the cyst shell and alkalinizes anaerobic dormant cysts: incubation in buffer containing NH_4Cl (40 m*M*, pH 8.5) increases pH_i by about 0.9 unit. When aerobic dormant embryos are first incubated for 20 hours in this same buffer and then transferred to a medium without NH_3 (Table 1), 33 percent hatch normally to yield free-swimming nauplius larvae, while < 5 percent of control embryos (without NH_3) hatch during the same interval [$P < 0.005$, $t(4) = 22.2$]. Aerobic dormant cysts activated by the conventional method (desiccation and rehydration) display significantly lower hatchability than NH_3-activated cysts [$P < 0.05$), $t(4) = 2.6$], but the desiccation technique used probably was not optimal (*10*). In otherwise similar experiments in which cysts are continuously incubated in NH_4Cl, preemergence development is similarly activated, but all embryos died before release from the hatching membrane [E_2 stage (*8*)]. Why NH_3 or NH_4^+ are toxic during hatching (but not before) is unclear, but we note that many metabolic characteristics of the embryo change at this point in development—for example, cell division recommences at this time (*9*) and diguanosine tetraphosphate utilization, which has an acidic pH optimum (*11*), increases (*12*).

We conclude that large pH_i changes similar to those accompanying reversible anaerobic dormancy–aerobic development transitions in *Artemia* also play a fundamental role in the regulation of aerobic dormancy. We cannot yet directly determine the pH_i of aerobic dormant embryos, which are not readily available

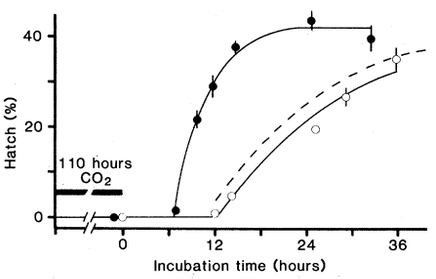

Fig. 2. Inhibition and synchronization of development by acidification of pH_i with CO_2. Desiccated cysts were hydrated on ice and incubated at room temperature for 110 hours under gas mixtures composed of 40 percent O_2 and either 11 percent (closed circles) or 60 percent (open circles) CO_2, the balance being N_2; note that no hatching occurred during CO_2 treatment. The gas phase was then changed to 40 percent O_2 and 60 percent N_2 at time zero. Controls were incubated only under this second gas mixture (dashed line, data points omitted). Each point is the mean of four counts of 100 to 200 cysts each and bars represent two standard errors of the mean (*17*).

in the large quantities required by the NMR technique. Nevertheless, our observation that manipulation of pH_i with a weak acid or base respectively imposes or terminates aerobic dormancy indicates that pH_i is a primary effector of metabolism (and, thus, development) in the *Artemia* gastrula as it is in the fertilized sea urchin egg. This may have practical consequences for investigators who use the isolated transcriptional or translational machinery of *Artemia* cysts at arbitrary *p*H values, as well as for aquaculturalists, for whom the hatchability of *Artemia* cysts is of economic concern. Our observations also expand on the role for pH_i as a regulator of development as understood from studies of sea urchin fertilization and bacterial spore germination. In the *Artemia* embryo, but not in the urchin egg (*1, 2*) or bacterial spore (*6*), alkalinization alone is sufficient to evoke normal metabolism and completion of the suspended developmental program, indicating that pH_i is a primary effector of dormancy in this multicellular system.

The regulation of two rather different forms of dormancy by pH_i in organisms as distantly related as echinoderms and crustaceans raises the possibility that pH_i may play a role in the regulation of other hypometabolic states (for example, the diapause of many other arthropods, the dormancy of plant seeds, or the hibernation of mammals) (*13*). All known instances of pH_i changes accompanying metabolic activation involve alkalinizations (*14*), the relative magnitudes of which appear to be directly related to the relative increases in the metabolic rate achieved (*15*). Our observations on *Artemia* embryos, which combine a large physiologically significant increase in pH_i with a pronounced metabolic activation, extend these correlations. Indeed, the intimate participation of protons in numerous aspects of energy metabolism suggests that pH_i may be a primitive and fundamental indicator of cellular energy balance, accounting for its increasingly apparent utility as a pleiotropic regulator of metabolism and development (*1*).

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16. Great Salt Lake cysts were used for ^{31}P-NMR as previously described (*7*), except that superfusion buffer was 0.25*M* NaCl and the superfusion chamber was modified to permit sampling of effluent buffer uncontaminated by contact with air. The buffer reservoirs were preequilibrated and bubbled with gas mixtures composed of 40 percent O_2, the percentage of CO_2 indicated in Fig. 1, and the balance N_2. Simultaneous with spectroscopic determination of pH_i, the buffer O_2 tension of the inlet and outlet streams was determined with a flow-through Clark-type electrode. Aerobic pH_i in the nominal absence of CO_2 slightly exceeds the upper limit of detection by ^{31}P-NMR (*7*) and is arbitrarily reported as ≥ 7.9. Except for the nominally CO_2-free data point, where Q_{O_2} increased slowly with time, each data point represents the steady-state values of Q_{O_2} and pH_i achieved within ~ 0.5 hour after the introduction of CO_2.
17. Hatching was assayed as previously described (*7*) except that the hygrostat was sealed with a glass plate to permit observation, and humidified gas was introduced through a side arm at 200 ml/min. Hatched embryos developed to the free-swimming nauplius stage.
18. Supported by National Sea Grant R/A-47 and NSF grant PCM 80-04720.

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Depletion of Intracellular Polyamines May Alter DNA Conformation in 9L Rat Brain Tumor Cells

Abstract. *Depletion of polyamines in 9L rat brain tumor cells by treatment with α-difluoromethylornithine dramatically altered DNA conformation as measured by viscoelastometry. The reduction of intracellular putrescine and spermidine concentrations to less than 5 percent of their concentrations in control cells decreased the sensitivity of 9L cell DNA to x-irradiation and increased the maximum viscoelastic retardation time of the DNA. Both of these phenomena were reversed by addition of exogenous putrescine.*

The polycationic aliphatic amines putrescine, spermidine, and spermine are present in all eukaryotes and are essential for the growth of both normal and neoplastic tissue (*1*). Polyamines can cause cell-free DNA to condense into compact structures (*2*) and are involved in the packing of DNA in T7 and λ phage heads (*3*). Polyamines protect DNA from methylation (*4*), enzymatic degradation (*5*), and thermal- or x-ray-induced denaturation (*6*). Little is known, however, about the effects of polyamines on the conformation and structure of mammalian cell DNA.

We have reported that depletion of intracellular polyamines in 9L rat brain tumor cells by treatment with α-difluoromethylornithine (DFMO), an enzyme-activated, irreversible inhibitor of the polyamine biosynthetic enzyme ornithine decarboxylase (*7*), increases the cytotoxicity of chloroethylnitrosoureas (CENU's) (*8*), antitumor agents that alkylate and cross-link DNA (*9*), and decreases the cytotoxicity of the cross-linking agent *cis*-diaminodichloroplatinum II (*cis*-platinum) (*10*). We have speculated that polyamine-related destabilization of the helical structure of DNA changes the spatial orientation of DNA nucleophilic sites in such a way that reactions with CENU-reactive moieties are increased—probably by increasing the number of interstrand cross-links—and that displacement reactions on platinum, which form both inter- and intrastrand cross-links, are made less favorable (*10*). Using the sister chromatid exchange assay, we have shown that DFMO-induced polyamine depletion alters the induction of damage to chromosomes caused by these cytotoxic agents (*11*). Aberrations in a polyamine-depleted Chinese hamster ovary cell line grown in the absence of polyamines have been described (*12*). Our experiments on the effects of DFMO-induced polyamine depletion on the viscoelasticity of DNA from 9L rat brain tumor cells were conducted to define more fully the effects that polyamine depletion may have on DNA structure.

Viscoelastometry, developed by Zimm and his co-workers (*13*), is a hydrodynamic technique that measures the recovery, or recoil, from a shear-induced strain in solutions of high molecular weight polymers such as DNA. In practice, a solution of DNA to be analyzed is placed in the region between the surfaces of two concentrically placed cylinders. The inner cylinder is rotated through a specific angular displacement by an externally applied electromagnetic torque. When the applied torque is removed, the