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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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

inner cylinder rotates in the opposite direction before coming to rest. The exponential decay of the angular position of the inner cylinder as a function of time is monitored during the recoil period. The decay time, or retardation time τ, is directly proportional to the product of the intrinsic viscosity and the molecular weight of the DNA (*13–15*). Shear-induced degradation is avoided by not isolating the DNA and by making measurements on cell lysates at very low shear rates. The viscoelastic response is determined by the extremely large DNA in the lysates (*13*). Effects of various agents that damage mammalian cell DNA have been examined with viscoelastometry, under both alkaline (*15*) and neutral (*16*) lysing conditions.

9L rat brain tumor cells were treated with DFMO for 48 hours and then exposed to various doses of x-rays, after which the viscoelastic recoil of DNA in lysates of irradiated control and treated cells was determined. Figure 1 shows plots of τ as a function of x-ray dose. The values of τ for untreated control cells increased at low x-ray doses, reached a maximum value at 1000 rads, and then dropped sharply at higher doses, a profile very similar to that observed for untreated 24-hour-old 9L cells (*16*). The increase in the value of τ at low x-ray doses has been interpreted as the effect of radiation-induced single- and double-strand breaks on the relaxation of supercoiled or otherwise conformationally constrained regions of DNA (*15, 16*). Relaxation of supercoiled DNA domains increases the hydrodynamic volume of DNA, which results in an increase in the intrinsic viscosity of the lysate and hence an increase in the value of τ. The lower values for τ caused by x-ray doses higher than 1000 rads probably reflect the accumulation of enough strand breaks to cause DNA degradation, which would reduce both the molecular weight and the intrinsic viscosity.

Curves obtained for DFMO-treated cells were similar in shape to the curves for untreated controls, but the position of the curve was shifted to higher x-ray doses. In comparison with control cells, the x-ray dose had to be doubled to produce a maximum value for τ, which proved to be higher than the value for control cells. As found for control cells, DFMO-treated cells to which exogenous putrescine had been added had maximum τ values at 1000 rads; however, the maximum value of τ for putrescine-treated cells was significantly smaller than the value for polyamine-depleted cells.

The observation that polyamine depletion shifts the x-ray response curve to higher doses suggests that DNA from these cells may be organized into smaller supercoiled domains than the DNA from control cells. This conclusion is based on simple target theory (*17*) and the fact that a single nick suffices to relax a supercoiled region. Consequently, if the DNA is arranged into smaller units, more strand breaks are required to attain the maximum value for τ, corresponding to the loss of all supercoiling.

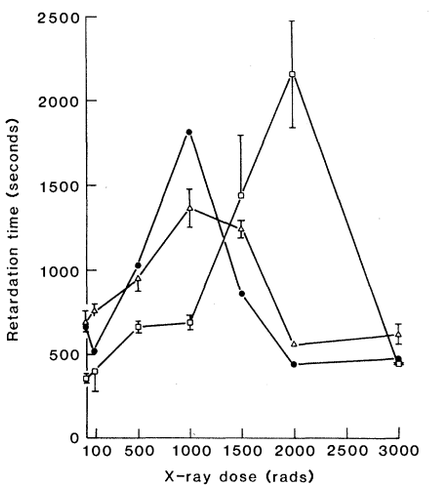

Fig. 1. Plot of τ versus x-ray dose for polyamine-depleted (□) and control (●) 9L cells. In these experiments, 5×10^5 9L cells were seeded into flasks containing 14.5 ml of minimal essential medium (MEM) with 10 percent fetal calf serum and incubated for 24 hours at 37°C in a humidified atmosphere containing 5 percent CO_2 and 95 percent air. Cells were then treated with either 0.5 ml of 30 m*M* DFMO in Hanks balanced salt solution (HBSS) (final DFMO concentration of 1 m*M*) or with 0.5 ml of HBSS alone (controls). Both DFMO-treated and control cells were incubated for 48 hours, during which time putrescine and spermidine levels in the DFMO-treated cells declined to less than 5 percent of control levels while spermine levels remained essentially unchanged. After 48 hours of incubation, DFMO-treated and control cells were x-irradiated at doses of 0, 100, 500, 1000, 1500, 2000, 2500, or 3000 rads. Cells were then rinsed to remove DFMO, trypsinized, suspended in a volume of MEM that was sufficiently large to stop trypsin digestion, centrifuged at 1000 rev/min for 10 minutes at 4°C, resuspended in a phosphate-buffered saline solution (0.15*M* NaCl, 0.007*M* Na_2HPO_4, and 0.003*M* NaH_2PO_4, *p*H 7.1), filtered through a 20-μm-pore Nitex filter, and diluted in phosphate-buffered saline to a concentration of 2×10^4 cells per milliliter. A portion (1.5 ml) of the suspension of treated cells was placed in the cassette of a Couette-type viscoelastometer and treated with 1.5 ml of buffer (0.25*M* Na_2HPO_4, 0.032*M* Na_4EDTA, and 0.85*M* NaCl, adjusted to *p*H 7 with HCl) and 0.3 ml sodium dodecyl sulfate in distilled water (2 percent, weight to volume); the cassette was rapidly inverted twice, and cells were lysed at 26°C for 2 hours. The same conditions were used in putrescine reversal experiments, except that after the 48-hour DFMO treatment, cells were treated for 24 hours with 0.5 ml of 31 m*M* putrescine in HBSS (final putrescine concentration 1 m*M*, a physiologic concentration for 9L cells) before being irradiated (△). Error bars represent standard error of the mean for duplicate or triplicate experiments.

This structural change appears to be separate from purely electrostatic effects on DNA conformation arising from polyamine binding. The latter manifests itself in the increase in the maximum τ value for polyamine-depleted cells in comparison with cells in which polyamines were restored. With decreased polyamine levels, neutralization of the negative charges on the DNA backbone is decreased, which leads to a stiffening of the DNA helix and a consequent increase in hydrodynamic volume and intrinsic viscosity (*18*). Addition of exogenous putrescine to polyamine-depleted cells increases intracellular putrescine, spermidine, and spermine to ≧ 200, 80, and 125 percent of control levels, respectively. In this case, an increase in charge neutralization results in a more compact conformation, which decreases the maximum value of τ.

Our studies show that depletion of intracellular polyamines dramatically alters the conformation of DNA in lysates of 9L cells. We have presented one possible explanation for this effect; other mechanisms may be involved. Although these findings support our hypothesis that increased cell death caused by chloroethylnitrosoureas and decreased cell death caused by *cis*-platinum are the result of polyamine-induced changes in DNA structure, additional work is needed to further define this phenomenon.

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An Altered aroA Gene Product Confers Resistance to the Herbicide Glyphosate

Abstract. *The hypothesis that the herbicide glyphosate (*N*-phosphonomethylglycine) acts on plants and microorganisms by inhibiting synthesis of 5-enolpyruvyl-3-phosphoshikimate, a precursor to aromatic amino acids, was tested.* Salmonella typhimurium *was treated with ethyl methanesulfonate, and mutants mapping at the aroA locus, which encodes 5-enolpyruvyl-3-phosphoshikimate synthetase, were isolated by selection for glyphosate resistance. One of the mutants results in the synthesis of a 5-enolpyruvyl-3-phosphoshikimate synthetase that is resistant to inhibition by glyphosate. The mutant aroA gene and the corresponding wild-type allele were cloned. The mutation confers high resistance to glyphosate when introduced in* Escherichia coli *in the presence or absence of the wild-type aroA allele.*

The herbicide phosphonomethylglycine (common trade name: glyphosate) is used as a broad spectrum weed killer. It appears to inhibit the shikimic acid pathway (*1–4*), which in plants and bacteria provides a precursor for the synthesis of aromatic amino acids. The glyphosate-sensitive step is the conversion of phosphoenolpyruvate and 3-phosphoshikimic acid to 5-enolpyruvyl-3-phosphoshikimic acid catalyzed by the enzyme 5-enolpyruvyl-3-phosphoshikimate synthetase (E.C. 2.5.1.19).

We now describe the isolation of glyphosate-resistant mutants in *Salmonella typhimurium* that map in the locus, aroA, coding for 5-enolpyruvyl-3-phosphoshikimate synthetase. The aroA gene from one of the mutants was cloned in *Escherichia coli* and the mutation or mutations characterized as a structural alteration of the enzyme encoded by the aroA locus. The properties of this mutant gene make it potentially useful for the introduction and expression of herbicide resistance in plant cells, which is our long-range goal.

If the aroA gene product is the primary target of glyphosate inhibition, glyphosate-resistant mutants which map in the aroA locus may be isolated by selection on glyphosate. Such mutations were identified by tight cotransduction of glyphosate resistance and aroA into the aroA-deficient *S. typhimurium* strain A1 (*5*). Ethyl methanesulfonate mutagenesis (*6*) was used to increase the frequency of glyphosate-resistant mutations, large numbers of which were screened by preparing mixed P22 lysates from thousands of pooled mutants (Table 1).

Strain CTF3 is a representative mutant obtained by mutagenesis and selection at a glyphosate concentration of 350 μg/ml. Although resistance of this mutant to glyphosate was not very high, it cotransduced 97 percent with the aroA mutation of *Salmonella* strain A1. A second cycle of mutagenesis on CTF3 and selection at 1000 μg/ml yielded a series of aroA related mutants very resistant to glyphosate. They grew well on glyphosate at 2000 μg/ml. All cotransduced 95 to 99 percent with the independent *S. typhimurium* aroA-deficient mutations aroA1, aroA124 (*7*), and aroA148 (*5*). One of these mutants, designated CT7, was chosen for further characterization.

Resistance to glyphosate mediated by a mutation at the aroA locus could result from altered regulation leading to overproduction of 5-enolpyruvyl-3-phosphoshikimate synthetase or to structural changes in the enzyme. To distinguish between these possibilities we analyzed enzyme preparations from the mutant strain CT7 and an isogenic wild-type strain, STK1, prepared by transducing the wild-type aroA allele from *S. typhimurium* strain TA831 into strain A1.

At saturating substrate concentrations, CT7 enzyme preparations had twice the specific activity of those from STK1 (*8*). Also, they were more resistant to glyphosate than those from STK1 over a range of different substrate and inhibitor concentrations. Neither the mutant nor the wild-type enzyme was overproduced in response to glyphosate-induced stress.

To study the mutation or mutations in the aroA locus of mutant strain CT7, we cloned in *E. coli* both mutant and wild-type alleles. A cosmid bank was constructed from each strain in the low-copy cosmid vector pVK100 (*9*). The chromosomal DNA was partially digested with restriction endonuclease Sau 3A, ligated

Table 1. Frequency of glyphosate-resistant mutants. Strain TA831 is a *his*$^-$ derivative of *S. typhimurium* LT2; strain CTF3 is a mutant strain originated from experiment 2, resistant to glyphosate at 350 μg/ml and mapping in the aroA locus. It was constructed by transducing an aroA-glyphosate–resistant allele from experiment 2 in strain Al (*5*). EMS, ethyl methanesulfonate.

Experiment	Strain	EMS	Glyphosate* (μg/ml)	Frequency	
				Glyphosate	aroA-Glyphosate resistance†
1	TA831	−	350	10^{-8}	$< 10^{-9}$
2	TA831	+	350	10^{-4}	10^{-6}
3	CTF3	+	1000	10^{-6}	10^{-9}

*A commercial preparation of glyphosate was used. †Glyphosate-resistant mutations mapping at the aroA locus were identified by cotransduction. Since lysates were prepared from pooled groups of mutants the ratio of glyphosate-resistant aroA cotransductants to the total number of aroA transductants was taken as a close estimate of the ratio of aroA-encoded to aroA-unrelated mutations in the original mutagenized culture. To assure that the estimates were meaningful, random cotransductants were further tested for tight cotransduction.