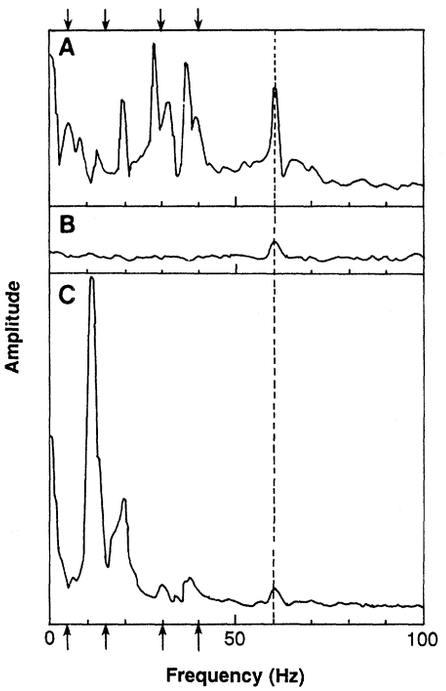

Fig. 5. Tracings of power spectra of swimming prey. Power spectra (plots of the distribution of the intensity of acoustic signals as a function of frequency) of digitized recordings (1.25-s interval) of (**A**) a brine shrimp nauplius in 25 μl of seawater, (**B**) 25 μl of seawater alone, and (**C**) an adult brine shrimp in 0.2 ml of seawater. Arrows mark the optimal frequencies for stimulating the discharge of nematocysts in mucin-treated cnidocytes (Fig. 3). Electrical noise was noted at 60 Hz (dashed line).

in certain free-standing hair cells is correlated with the length of the stereocilia. Hair cells having longer stereocilia respond to lower frequencies (*19*).

In sea anemones, the cilium of each cnidocyte mechanoreceptor originates from the cnidocyte, whereas the stereocilia and the receptors for N-acetylated sugars are located on supporting cells (*9*). Supporting cell chemoreceptors for N-acetylated sugars tune mechanoreceptors involved in discharging nematocysts, possibly by inducing a change in the length of the stereocilia (Fig. 1). Thus, cnidocyte–supporting cell complexes select prey by discriminating among potential prey according to a combination of the chemical and physical cues they present. Our findings raise the possibility that, in other cnidarians as well, preferred prey are selected on the basis of an interplay between chemical and mechanical sensing systems such that chemical substances derived from the prey tune cnidocyte mechanoreceptors to frequencies that match those produced by the moving prey.

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Major Enhancement of the Affinity of an Enzyme for a Transition-State Analog by a Single Hydroxyl Group

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The compound 1,6-dihydropurine ribonucleoside, prepared by reduction of nebularine in the presence of ultraviolet light, is bound by adenosine deaminase approximately 10^8-fold less tightly than 6-hydroxy-1,6-dihydropurine ribonucleoside, a nearly ideal transition-state analog. This difference in affinities, which is associated with the presence of a single hydroxyl group in the second compound, suggests the degree to which one or a few hydrogen bonds may stabilize the transition state in an enzyme reaction of this type.

THE CATALYTIC FUNCTION OF AN enzyme depends on its ability to discriminate between the substrate in the ground state and the altered substrate in the transition state, binding the latter species more tightly and diminishing the difference in free energy that limits the rate of reaction. These two forms of the substrate tend to be broadly similar in structure, so that discrimination is probably based on a few differences in the immediate neighborhood of the bonds that are formed and broken during the reaction. It is shown that the presence of a single hydroxyl group appears to contribute −9.8 kcal/mol to the free energy of binding of a transition-state analog by adenosine deaminase.

Adenosine deaminase catalyzes the hydrolytic removal of ammonia and other leaving groups from 6-substituted purine ribonucleosides. Hydrolysis cannot occur when hydrogen is present at the 6-position, so that nebularine [purine ribonucleoside (**I** in Scheme 1, where R is ribose] is bound by adenosine deaminase as a competitive inhibitor, exhibiting an enzyme affinity comparable with that of the substrate adenosine (*1*). Until recently, nebularine had appeared to be a typical substrate analog, but ^{13}C nuclear magnetic resonance (NMR) and ultraviolet spectra strongly suggest that nebularine is actually bound in a form that is sp^3-hybridized at the 6-position (*2*). The bound form is probably the rare species [1 part in 10^7 in dilute solution (*3*)] that is covalently hydrated at the 1,6-positions (**II**) (*4*). The apparent affinity of this species for the enzyme (apparent inhibition constant $K_i = 3.0 \times 10^{-13} M$) exceeds the affinities of substrates,

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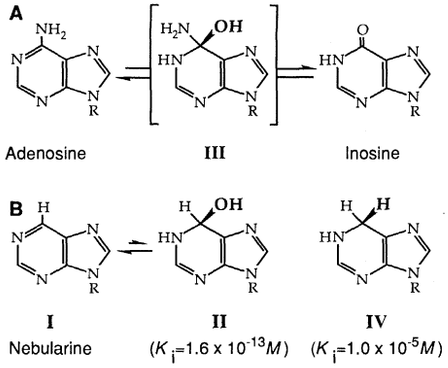
products, and unmodified nebularine by factors greater than 10^8. Other evidence (*5, 6*) suggests that water attacks adenosine directly in the reaction catalyzed by adenosine deaminase, proceeding through the tetrahedral intermediate **III**. Compound **II** differs from **III** only in the replacement of the variable leaving group by hydrogen, and this substitution is expected to be well tolerated



Scheme 1. (**A**) Reaction catalyzed by adenosine deaminase, proceeding through hydrated intermediate **III**. (**B**) Hydration of nebularine to produce inhibitor **II**, which is compared with 1,6-dihydronebularine (**IV**). R = D-ribofuranose.

because the enzyme exhibits little specificity for the variable leaving group.

With an enzyme affinity exceeding that of the substrate adenosine by many orders of magnitude, compound **II** captures most of the added binding affinity expected (*7*) of an ideal transition-state analog for this reaction. The forces of attraction that stabilize its complex with adenosine deaminase are likely to be analogous to forces that are present during the catalytic process, so that it would be desirable to know which of the distinctive structural features of **II** are responsible for its remarkable binding affinity. These factors include the presence of a proton at N-1, the presence of a hydroxyl group at C-6, and the distortion of the purine ring (*8*) away from its normal planar structure. To assess the relative importance of these contributions, it seemed desirable to determine the affinity of the enzyme for 1,6-dihydropurine ribonucleoside (**IV**), which resembles **II** in being protonated at N-1 and distorted away from the normal planar structure of the purine ring. Differences in observed binding affinity between **II** and **IV** should thus be largely attributable to the presence of the 6-hydroxyl group in **II**.

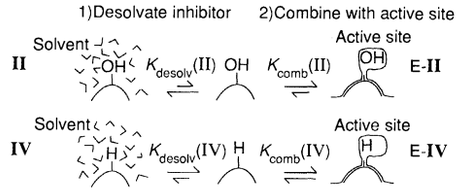
Compound **IV** does not appear to have been described before. We were not able to prepare **IV** by conventional methods of reduction, but we were able to obtain **IV** by irradiating nebularine (0.8 mmol) in the presence of $NaBH_4$ (2.4 mmol) in aqueous ethanolamine (0.05*M*; 50 ml) in a rotating quartz reaction vessel for 2 hours with four 15-W germicidal lamps. The major product, isolated in 25% yield by preparative reversed-phase high-performance liquid chromatography, eluting with 12% methanol in water, gave the expected mass and ^1H NMR spectra. Photoreduction with $NaBD_4$, followed by oxidation with phenyltrimethylammonium tribromide (*8*), yielded **I** containing 50% deuterium at C-6, confirming that reduction had occurred at C-6. The cation of **IV** exhibited a pK_a value of 6.5, and the ultraviolet absorption spectrum of the neutral species (wavelength of maximum absorbance λ_{max} = 299 nm, logarithm of the extinction coefficient ϵ_{max} = 3.55) was similar to the spectra of 6-alkylated 1,6-dihydropurine ribonucleosides (*5*). Compound **IV** proved to be a linear competitive inhibitor of adenosine deaminase, with a K_i value of 5.4 ($\pm$0.5) $\times$ $10^{-6}M$. Inhibition was instantaneous and did not change when **IV** was preincubated with enzyme over extended periods of time. Accordingly, compounds **II** and **IV** differ in binding affinity by a factor of 1.8×10^7. This factor, equivalent to the equilibrium constant associated with exchanging **II** for **IV** at the active site, indicates that the 6-hydroxyl group of **II** contributes −9.8 kcal/mol to the free energy of binding of **II**.

It is reasonable to inquire whether this

Table 1. Apparent contributions of hydroxyl substituents to the free energies of binding, as determined by comparing the affinities of hydroxylic ligands and their deoxy analogs.

OH ligand	H analog	ΔΔG* (kcal/mol)	Reference
	Glycogen phosphorylase		
d-Glucose	1-Deoxy	−1.0	(*16*)
	2-Deoxy	−1.6	(*16*)
	3-, 4-, or 6-Deoxy	−3.0	(*16*)
	β-*Glucosidase*		
d-Glucose	1-Deoxy	+0.6	(*17*)
	2-Deoxy	−0.1	(*17*)
	6-Deoxy	−0.4	(*17*)
d-Galactose	6-Deoxy	+0.8	(*17*)
	2-Deoxy	−0.6	(*17*)
	Acetylcholinesterase		
Phenol	Benzene	+0.4	(*18*)
	Leucine amino-peptidase		
Amastatin	Deoxyamastatin	−4.8	(*19*)
	Pepsin		
Pepstatin	Dideoxypepstatin	−5.1	(*20*)†
	Adenosine deaminase		
II	**IV**	−9.8	

*$\Delta\Delta G = RT \ln[K_i(OH)/K_i(H)]$, where $\Delta\Delta G$ is the change in the free energy, R is the gas constant, T is the absolute temperature, and $K_i(OH)$ and $K_i(H)$ are, respectively, the inhibition constants for hydroxyl and hydrogen. †Two hydroxyl groups are responsible for this difference.



Scheme 2. Compound **IV** is removed from water approximately 5 orders of magnitude more readily than **II**; K_{desolv} and K_{comb} are, respectively, the equilibrium constants for desolvation and combination with the active site. In watery surroundings, **II** is found to be more tightly bound than **IV** by roughly 8 orders of magnitude. Accordingly, the affinity of the enzyme for desolvated **II** presumably exceeds its affinity for desolvated **IV** by roughly 13 orders of magnitude [see text and (*13*)].

level of discrimination, which is large in comparison with other examples of oxy-deoxy discrimination in the literature (Table 1), can be explained without postulating hydrogen bonds of unusual strength (*9*). That question can be addressed by assuming that water is absent from regions of close contact between the enzyme (E) and an inhibitor (I) in their E-I complexes, and that the free energy of desolvating the active site itself is roughly the same for either inhibitor. The relative affinities of the desolvated ligands for the active site can then be estimated as shown in Scheme 2. Comparison of the vapor pressures of secondary alcohols over their aqueous solutions with those of the corresponding alkanes suggests that desolvation of **IV** is expected to occur with an equilibrium constant approximately 10^5-fold more favorable than that of **II** (*10*). In an aqueous environment, **II** is more tightly bound than **IV** by a factor of 6.3×10^7 in water. Accordingly, the affinities of the desolvated ligands would differ by a large factor, amounting to $(10^5)(6.3 \times 10^7) = 6.3 \times 10^{12}$, or −17.5 kcal/mol favoring the binding of **II**. Mass spectrometric experiments (*11*) and force-field calculations (*12*) indicate that the association of a single water or alcohol molecule with a formate or acetate anion in the vapor phase is accompanied by a decrease in enthalpy of −17 to −20 kcal/mol. Accordingly, it seems possible to explain the present results without postulating the presence of hydrogen bonds with unusual properties. A single hydrogen bond between the 6-hydroxyl group of **II** and a charged Asp or Glu residue at the active site, or several weaker hydrogen bonds, would suffice (*13*). The crystal structure of adenosine deaminase, which is currently under investigation (*14*), should constrain these alternatives.

The high level of expression of the intrinsic (*15*) hydrogen-bonding potential of the 6-hydroxyl group of **II**, as measured by the

difference in binding affinity between **II** and **IV**, probably hinges in part on the fact that this hydroxyl group is fixed in position, so that it can be bound without substantial loss in freedom of movement with respect to the rest of the nucleoside. Other entries in Table 1, in which the effect of hydroxyl substitution is less, have considerably greater freedom of rotation. Experiments are currently under way to characterize entropic aspects of the hydration of nebularine and of its binding by adenosine deaminase. The present results imply that under favorable circumstances a single hydroxyl substituent can enhance the binding affinity of an enzyme inhibitor by a very large factor. Analogously, formation of one or two hydrogen bonds in the transition state might suffice to account for the catalytic efficiency of an enzyme of this type.

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Direct Measurements of Sliding Between Outer Doublet Microtubules in Swimming Sperm Flagella

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The relative motion of 40-nanometer gold beads bound to the exposed outer doublet microtubules of demembranated sea urchin sperm flagella has been observed and photographed during adenosine triphosphate (ATP)–reactivated swimming. This direct demonstration and measure of sliding displacements between outer doublet microtubules in actively bending flagella verifies the original sliding microtubule model for ciliary bending that was established by electron microscopy of fixed cilia and provides a new, functional measure for the diameter of the flagellar axoneme of 132 ± 8 nanometers.

SLIDING BETWEEN OUTER DOUBLET microtubules has been assumed to occur during, and to be responsible for, the bending of cilia and flagella. The occurrence of sliding during bending was inferred from electron micrographs of fixed cilia (*1*), from observations of sliding during flagellar disintegration (*2*), and from the characteristics of certain unusual bending patterns (*3*). Direct visualization and measurement of sliding in actively bending flagella of ATP-reactivated, demembranated, sea urchin spermatozoa has now been achieved by using gold beads as markers of positions on the outer doublet microtubules.

Preparations of demembranated spermatozoa were incubated in suspensions containing gold beads and then diluted into solutions containing MgATP for observation of motility (*4–8*). Beads [or bead aggregates, which cannot be easily distinguished from individual beads by light microscopy (*9, 10*)] attached to the flagellar axoneme and remained in position for hundreds of beat cycles; they were not dragged or propelled to either end of the flagellum. Demembranated sperm flagella decorated with beads swam with nearly normal bending patterns, similar to those of undecorated flagella in the same preparation (Fig. 1A).

Under ideal circumstances, where a pair of beads is separated by a distance of 1 μm or less along the axoneme, direct inspection of a sequence of photographs (for example, Fig. 1B) revealed that the two beads moved closer together and then farther apart as the flagellum bent. When low MgATP concentrations were used to obtain flagellar beat frequencies of 1 Hz or less, these movements were also seen by direct visual observation, with the use of dark-field light microscopy. The relative movements of three beads indicating three independently sliding doublet microtubules can be seen in Fig. 1D, in which portions of a photograph were cut and realigned for direct comparison of the positions of bead images at the extremes of the beat cycle.

Computer-assisted measurements of the distances between beads were carried out on digitized portions of the photographic negatives (*11*). Usually, measurements were made only when a pair of beads, separated by 1 to 4 μm, was located in a straight region between bends on the flagellum (Fig. 2A). The angle between these straight regions and the axis of the sperm head was the measure of shear angle. Amplitudes of oscillation of bead separation ranging from 0 to 0.4 μm were measured. Occasionally, measurements on closely spaced beads were made throughout the bend cycle (Fig. 2C); in these cases the small error caused by measuring straight-line distance between the beads rather than distance along the curve of the flagellum was ignored.

Relative movement of beads attached to outer doublet microtubules could be caused either by interdoublet sliding or by contraction and elongation of outer doublets (Fig. 3). Two properties of the movements clearly discriminate between these two possibilities. First, bead movements resulting from interdoublet sliding have peak amplitudes in phase with either positive or negative shear angle, as shown by the sliding model in Fig. 3 and the data in Fig. 2. Bead movements resulting from length changes have peak

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