et al.[36] to give TNP-BSA. The product (100 μg) was dissolved in 0.1 M sodium phosphate (pH 7.4) (1 mL) and carrier-free $Na^{125}I$ (1 mCi) was added followed by chloramine T (250 μg) as described by Greenwood et al.[37] The solution was thoroughly mixed and after 3 min sodium metabisulfite (625 μg) was added to terminate the reaction and the solution was dialyzed against saline.

(iii) **Measurement of Antibody Levels by Radioimmunoassay.** ^{125}I-Labeled TNP-BSA (20 μL in saline; 30 000 cpm) was added to the mouse serum (20 μL) and the mixture was diluted with normal rabbit serum (0.5 mL) and 0.2 M Tris buffer (pH 7.4) (0.5 mL). The solution was stirred and was left at 4 °C overnight and then 7.5% poly(ethylene glycol)-6000 (1 mL) in 0.2 M Tris buffer (pH 7.4) was added, as described by Desbuquois and Aurbach.[38] The mixture was centrifuged at 2000 rpm for 45 min and the supernatant and precipitate were separated. The precipitate was washed once with 0.2 M Tris buffer (pH 7.4) (1 mL) and the washings were added to the supernatant. The activity (cpm) in the precipitate and supernatant was determined using a Packard γ-counter (Model 3002). The percentage of activity precipitated gave a measure of the anti-TNP antibody level in the serum. The results for the various mouse strains are given in Table I.

Acknowledgment. The authors thank Dr. C. M. Kay (Department of Biochemistry, University of Alberta) for providing the CD spectra.

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Communications to the Editor

Theoretical Study of Binding and Proton-Labilizing Properties of Zn^{2+}

Sir:

In the active site of carbonic anhydrase,[1] which catalyzes the hydration of CO_2, x-ray crystallography shows[2] that a Zn^{2+} ion is coordinated to three imidazoles from histidine residues and to a water molecule in a distorted tetrahedral geometry. To account for the fact that the enzyme activity is controlled by ionization of a group having a $pK_a = 7$, close to the Zn^{2+} ion,[1] various mechanisms have been proposed, assuming either ionization of an imidazole (bound[3] or not[4] to Zn^{2+}) and nucleophilic attack on CO_2 by the coordinated imidazolate anion (directly[5] or through water[6]), or ionization of the Zn^{2+}-coordinated water and nucleophilic attack of CO_2 by OH^-.[7-9] Furthermore, it was proposed by analogy to the imidazole inhibitor that "CO_2 binds weakly to the fifth coordination site of the metal ion in the hydrophobic region of the active site".[8] Others have proposed that CO_2 binds to Zn^{2+} in a transient five-coordinate intermediate.[10]

As a first step in a theoretical study of the problems raised by these proposals, we have performed ab initio molecular orbital calculations, with high quality basis sets, on the binding of Zn^{2+} with the molecules imidazole (ImH), water (OH_2), and carbon dioxide (CO_2), as well as with the anions imidazolate (Im^-) and hydroxyl (OH^-). Leaving aside other factors present in the enzyme, this allows us to ascertain (i) the nature and *intrinsic* characteristics of Zn^{2+} binding with each ligand, (ii) the aptitude of Zn^{2+} to bind CO_2, (iii) the effect of Zn^{2+} binding on the ease of deprotonation of ImH and OH_2. Gaussian basis sets taken from the literature[11] were contracted single ζ for the core and double ζ for the valence shell.[12] We added a diffuse p and a diffuse d set to the zinc basis ($\zeta = 0.2539$ and 0.2313), and a diffuse p set on oxygen ($\zeta = 0.08$, optimized for OH^-) and nitrogen ($\zeta = 0.067$).[13] Diffuse p functions on carbon ($\zeta = 0.047$) were found to have little in-

Table I

Species	R, Å	θ, deg	Δ(BE)[a]	BE[b]
$Zn^{2+}\cdots OH_2$	1.8	0.0	1.37	
	1.89		0.0	−104.1
	2.0		2.34	
	1.89	15	1.22	
	1.89	30	4.95	
	1.89	60	20.77	
	1.89	90	50.43	
$Zn^{2+}\cdots OH^-$	1.55		12.06	
	1.65		0.96	
	1.70		0.0	−410.7
	1.75		1.23	
	1.70	15	−.04	
	1.70	30	+.06	
	1.70	60	4.03	
	1.70	90	21.34	
$Zn^{2+}\cdots ImH$	1.8		1.10	
	1.87		0.0	−169.4
	1.9		0.26	
	2.0		3.46	
	2.1		9.13	
	1.87	15	1.75	
	1.87	30	6.72	
	1.87	60	24.6	
$Zn^{2+}\cdots Im^-$	1.65		9.49	
	1.75		1.10	
	1.82		0.0	−366.4
	1.85		0.42	
	1.95		4.63	
	1.82	15	0.91	
	1.82	30	3.40	
$Zn^{2+}\cdots OCO$[c]	1.8		0.5	
	1.85		0.0	−79.4
	1.9		0.5	
	2.0		3.5	
	2.1		8.2	
	2.3		19.3	
	2.5		30.3	−48.9
	1.85	15	1.1	
	1.85	30	4.7	

[a] Δ(BE), energy with respect to optimum geometry (kilocalories/mole). [b] BE, binding energy of $Zn^{2+}\cdots L$; BE = $E(Zn^{2+}\cdots L) - E(Zn^{2+}) - E(L)$ (kcal/mol). [c] No diffuse p functions were used on the neutral CO_2 ligand. Calculations on $Zn^{2+}\cdots OH_2$ without diffuse p functions on OH_2 give a BE of −112.8 kcal/mol, and the same equilibrium geometry.

fluence on the results. With this basis, the occupied molecular orbital energies for the anions are all below −0.10 au and the proton affinity calculated for OH^-, 392.1 kcal/mol, is satisfactory (experiment: 390 kcal/mol[14]). Without putting too much emphasis on this agreement we take it as indicating that the basis set is qualitatively correct.

Experimental geometries were used for the ligands;[15] OH^- was optimized (R(O-H) = 0.98 Å). These geometries were kept frozen. For each complex the metal–ligand distance was first optimized. Then angular distortion was allowed (Figure 1).

It is seen (Table I) that the bond strength increases in the order $CO_2 < OH_2 < ImH \ll Im^- < OH^-$, following a leading electrostatic term in the binding energy. Our results indicate also a strong polarization of the ligands by Zn^{2+} and some charge transfer to empty Zn^{2+} orbitals: 4s, 4pσ, and a little 4pπ. There is no metal-to-ligand π-electron back-donation in these d^{10} systems, but some mixing occurs between metal 3dσ and high-lying ligand σ orbitals.

The computed bond length fall near 1.85–1.90 Å, a satisfactory value for a single ligand compared with the 2.0 Å distance usually found in crystals,[16-18] where Zn^{2+} has several ligands. The shorter length in $Zn^{2+}\cdots OH^-$ seems due to the small size of the ion, which allows stronger coulomb attraction by closer approach. Further discussion of bonding in these complexes will be presented elsewhere.

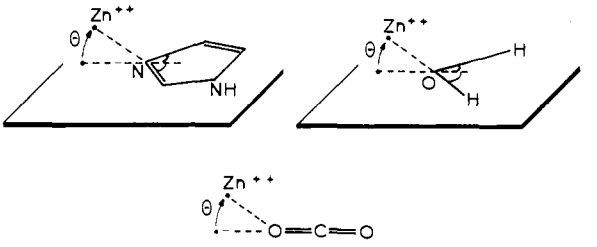

Figure 1.

The $Zn^{2+}\cdots$ligand bond, while strong, is easy to distort. Stretching by 0.10 Å or bending up to 30° is achieved at the cost of only a few kilocalories/mole, a small percentage of the total bond energy. Thus, even if an imidazole ligand was constrained to occupy a position far from equilibrium, its binding energy to Zn^{2+} might still be sizable. Bending is easiest with the anionic ligands, and the optimal angle in $Zn^{2+}\cdots OH^-$ may be slightly different from zero. These angular results appear supported by structural data indicating angles θ up to 30° for imidazole complexes,[16] and by the failure of attempts to synthetize sandwich imidazolate complexes.[19]

We find that Zn^{2+} can bind CO_2, although less strongly than the other ligands studied. The calculated binding energy of 79.4 kcal/mol at equilibrium is appreciable, and reasonable,[20] and the values computed at large distances (e.g., −48.9 kcal/mol at 2.5 Å) seems to indicate that, in the active site of carbonic anhydrase, where a close approach of CO_2 to Zn^{2+} might be prevented, its interaction with the metal as a fifth-distant[8] ligands is still favorable. The energy required to bend CO_2 by 15° (3.2 kcal/mol) is not affected by its binding to Zn^{2+}.

As to the effect of Zn^{2+} on the ease of deprotonation of water and imidazole, the results are the following (in kilocalories/mole):

$$OH_2 \rightarrow OH^- + H^+ \qquad 392.1$$

$$ImH \rightarrow Im^- + H^+ \qquad 369.5$$

$$Zn^{2+}\cdots OH_2 \rightarrow Zn^{2+}\cdots OH^- + H^+ \qquad 85.5$$

$$Zn^{2+}\cdots ImH \rightarrow Zn^{2+}\cdots Im^- + H^+ \qquad 163.4$$

Thus free imidazole is more easily ionized than free water. Binding to Zn^{2+} greatly facilitates the process for both species, *but in such a way as to reverse the ease of ionization.*[21] We thus show explicitely (1) that the Zn^{2+} ion does facilitate to a considerable extent the ionization of a water or imidazole bound to it, and (2) that ionization of water is *intrinsically* much more favored than ionization of imidazole.

The bearing of these elementary results on the mechanism of action of carbonic anhydrase will be tested by computations on more elaborate models of the Zn^{2+} binding segment of the active site in the light of recent experimental data.[22,23]

Acknowledgment. This research was supported by the National Foundation for Cancer Research (U.S.A.) and by ATP 2646 of the CNRS.

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(13) The final basis sets follow: for Zn, 12s, 6p, 4d → 6s, 4p, 3d; for the first row atoms, 7s, 4p → 3s, 3p; and for hydrogen, 4s → 2s. With the program IBMOL H a single computation on the complex ImH-Zn^{2+} takes 34 min of CPU time on an IBM 370/168.
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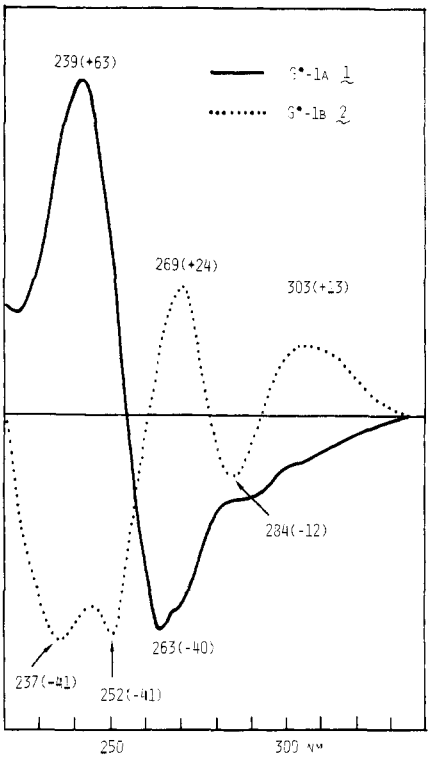

Figure 1. CD in 5% aqueous MeOH, extrema in nanometers ($\Delta\epsilon$).

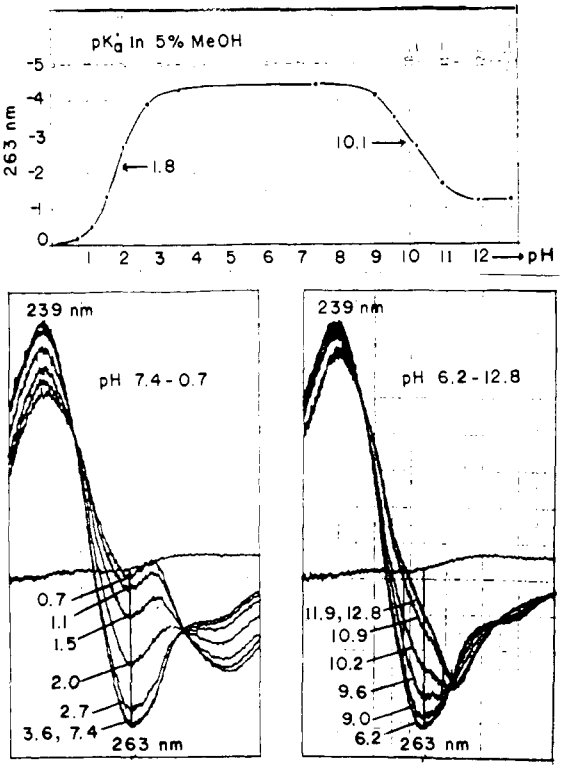

Figure 2. Change in CD of adduct **1** and plot of CD $\Delta\epsilon_{239}$ with pH, in 5% aqueous methanol. Owing to the instability of **1** to acidic conditions, the initial solution was neutralized to pH 7.4 and acidified to pH 0.7. A JASCO J-40 instrument was used.

Structures of 7,12-Dimethylbenz[*a*]anthracene 5,6-Oxide Derivatives Linked to the Ribose Moiety of Guanosine

Sir:

7,12-Dimethylbenz[*a*]anthracene (DMBA) is one of the most potent carcinogens which requires cell mediated activation before it can react with cellular macromolecules.[1] The K-region oxide (DMBA 5,6-oxide) has been implicated as one of the possible intermediates in the carcinogenic and/or mutagenic process.[2,3] Since chromatographic mobilities of the four known guanosine-DMBA 5,6-oxide adducts[4] formed under pH 5–6 did not coincide with the rat liver tissue culture products, we have prepared six other adducts G*-**1a**, -**1b**, and II–V by reacting the oxide with guanosine in acetone–water (2:1) at pH 9.5. About 15% of the guanosine reacted to yield in decreasing amounts G*-**1a**, III, II, G*-**1b**, IV, and V, which were isolated and purified on a Sephadex LH-20 column followed by HPLC.[4,5] Comparisons of the chromatographic behavior of the products with those isolated from the RNA of rat liver cells treated with [^{3}H]-DMBA showed that three of the products, G*-**1a**, -**1b** and II, which constituted less than ~10% of the total nucleoside-[^{3}H]-DMBA adduct, were detected in the cell culture.[5]

Structural studies of G*-**1a** and -**1b** (carried out on ~1 mg of each) showed that they can be expressed by **1** (or **3**) and **2** (or **4**), which, unlike other arene oxide and diol epoxide–nucleic acid base adducts identified so far,[4,6-8] are characterized by a unique ribose–DMBA link. Moschel et al.[9] have recently shown by fluorescence methods that the binding of DMBA to DNA mostly occurs after metabolic activation in the angular ring rather than the K region (C-5, C-6). The differences could possibly be attributed to different experimental procedures (e.g., RNA vs. DNA) and also to the fact that the present