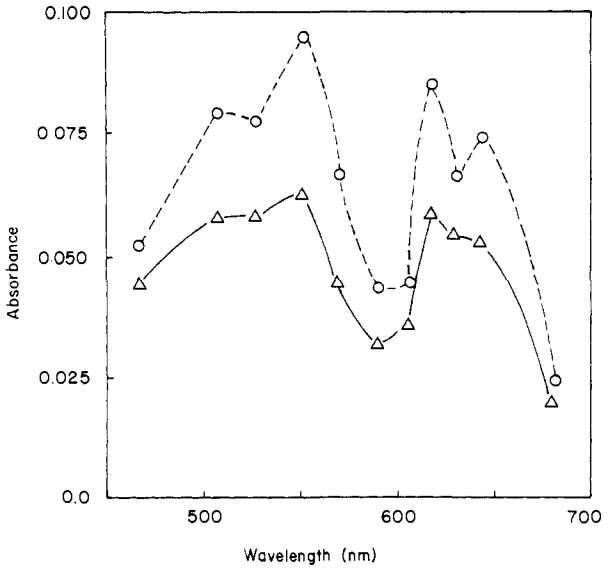

Figure 2. Absorbances at (△) 8 ms and (○) greater than 200 ms after the mixing of Co(II)-substituted bovine carbonic anhydrase III and CO_2. Conditions were the same as described in Figure 1 (Top). Lines are estimates of the spectra based on the 11 wavelengths measured; lines were not determined experimentally.

computer. One drive syringe contained CO_2, and the second contained enzyme and buffer and in some cases indicator. The status of the cobalt at the active center was determined from the absorbances at 551 and 642 nm in the absence of indicator; the progress curve and initial velocity for CO_2 hydration were determined by the rate of change of absorbance of phenol red at 559 nm by methods described earlier.[10,11]

Figure 1 (top) shows the absorbances at 551 and 642 nm as a function of time after mixing of Co(II)-isozyme III wtih CO_2. After the dead-time of our instrument, ~5 ms, we were able to observe a decreased absorbance resulting from the status of cobalt at steady state. As the hydration reaction proceeded to equilibrium, the absorbances at 642 and 551 nm approached their values at chemical equilibrium. To correlate this with the catalysis, we present in Figure 1 (bottom) the absorbance of the pH indicator phenol red and the calculated CO_2 concentration during the progress curve in a solution identical with that of Figure 1 (top) except for the addition of phenol red at 7.5×10^{-6} M. By initial velocity measurements at pH 7.5 using the method of ref 10, 11, we determined for the hydration of CO_2 catalyzed by bovine Co(II)-carbonic anhydrase III (data not shown) $k_{cat}^{CO_2} = (1.3 \pm 0.1) \times 10^3\ s^{-1}$ and $k_{cat}^{CO_2}/K_m^{CO_2} = (1.4 \pm 0.1) \times 10^5\ M^{-1}\ s^{-1}$. The analogous values for catalysis by the native zinc-containing bovine isozyme III are $3 \times 10^3\ s^{-1}$ and $3 \times 10^5\ M^{-1}\ s^{-1}$.[2,8]

The experiments of Figure 1 were repeated at 10 other wavelengths with results shown in Figure 2. The points at times greater than 200 ms represent the spectrum at equilibrium which is similar to the spectrum of Engberg and Lindskog.[4] Although we have not observed the spectrum of Co(II)-isozyme III fully in its low pH form, we anticipate that it will be similar to the corresponding spectrum for isozyme II. Oxygen-18 exchange between CO_2 and water catalyzed by Co(II)-carbonic anhydrase III (by the method of ref 12) showed no substrate or product inhibition at pH 7.5 up to a concentration of 80 mM for the sum of all species of CO_2. This suggests equilibrium dissociation constants too large to have significant enzyme-substrate or enzyme-product complexes at equilibrium under the conditions of this study. Our sample of Co(II)-carbonic anhydrase III had as large as 10% contamination by the native zinc-containing carbonic anhydrase III, the latter of which has steady-state rate constants larger than those for Co(II)-substituted isozyme III. This means our experimental values of $k_{cat}^{CO_2}$ and $k_{cat}^{CO_2}/K_m^{CO_2}$ can be considerably greater than the true values. The usual chelating agents have not proved useful in preparing the apoenzyme of isozyme III,[4] indicating that isozyme III has a greater affinity for zinc than does isozymes I and II.

We conclude that the hypothesis of a rate-contributing proton transfer in the maximum velocity of catalysis of CO_2 hydration involving the aqueous ligand of the metal in carbonic anhydrase III remains viable, for both the native and cobalt-substituted forms. The cobalt-bound water and cobalt-bound HCO_3^- forms of isozyme III, in analogy with isozyme II, are anticipated to have similar absorption spectra with weak absorbance at 640 nm. Therefore, these results cannot differentiate between a rate limitation by step 3 of eq 1 and step 4 of eq 2. It is known, however, that there is a significant H/D solvent isotope effect of 2.5 on the turnover number for CO_2 hydration catalyzed by isozyme III.[5,6] This suggests a change in bonding to hydrogen and favors step 4 of eq 2 as a rate-contributing event. Although the proton donor in this transfer is zinc- or cobalt-bound water, the proton acceptor is not known. Unlike carbonic anhydrase II, isozyme III is not enhanced in CO_2 hydration activity by buffers in solution,[5,6] indicating that proton transfer to buffer is not a rate-contributing event. Moreover, unlike carbonic anhydrase II, isozyme III does not have a nonliganded histidine at postion 64 to act as a proton shuttle residue. Residue 64 in bovine isozyme III is lysine.[13] The catalysis by carbonic anhydrase III is slow enough that water itself could serve as proton acceptor.

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3-(2-Deoxy-β-D-erythropentofuranosyl)-6,7-dihydro-6,7-dihydroxyimidazo[1,2-*a*]purin-9(3*H*)-one, a Major Deoxyguanosine Adduct Formed from a Novel Diazo Hydroxide Product of α-Hydroxylation of the Carcinogen *N*-Nitrosomorpholine

George F. Palladino,*[1a,b] Fung-Lung Chung,[1c] and Stephen S. Hecht[1c]

Division of Chemical Carcinogenesis
Naylor Dana Institute for Disease Prevention
Valhalla, New York 10595

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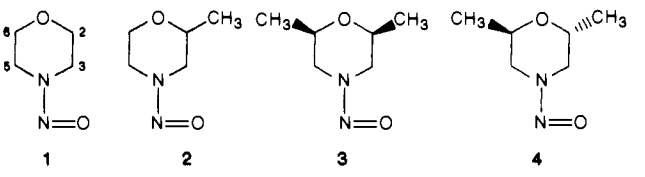
N-Nitrosomorpholine (**1**) and its methylated analogues **2**-**4** are an important group of remarkably potent and versatile carcinogens.[2] For example, in Syrian golden hamsters, **1** causes mainly



1 **2** **3** **4**

nasal cavity and tracheal tumors, **2** induces tumors of the nasal

(1) (a) Department of Chemistry, United States Military Academy, West Point, NY. (b) Visiting Scientist, Naylor Dana Institute for Disease Prevention, 1983-1984. (c) Division of Chemical Carcinogenesis, Naylor Dana Institute for Disease Prevention, Valhalla, NY. This is Paper No. 97 In *A Study of Chemical Carcinogenesis*.
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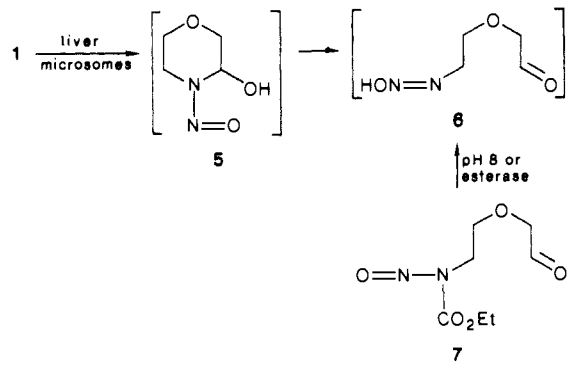
cavity, trachea, liver, and lung, while **3** produces tumors of the pancreas and liver and is more potent than its trans isomer **4**.[3,4] In contrast, **1** is a potent liver carcinogen in rats, and **4** is a stronger carcinogen than **3** in rats, giving tumors of the esophagus and nasal cavity.[2,5] The reasons for these differing activities are not well understood. DNA modification is believed to be the key step in the initiation of the carcinogenic process. Structure–activity and metabolic studies suggest that 3- or 5-hydroxylation of **1** would be a likely first metabolic step leading to DNA modification via the intermediates **5** and **6**.[6-9] It is well established that carci-



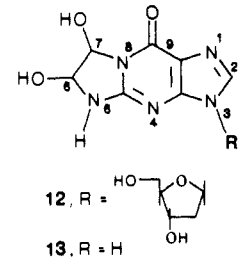
nogenic dialkylnitrosamines such as **8** undergo an initial metabolic α-hydroxylation to **9** followed by spontaneous formation of an aldehyde **10** and an alkyldiazo hydroxide **11**.[2] The DNA al-

$$\underset{\mathbf{8}}{RCH_2N(N{=}O)R'} \rightarrow \underset{\mathbf{9}}{RCHOHN(N{=}O)R'} \rightarrow \underset{\mathbf{10}}{RCHO} + \underset{\mathbf{11}}{[R'N{=}NOH]}$$

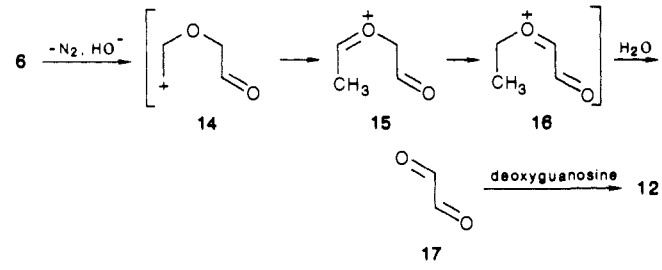
kylating properties of **11** have been thoroughly studied in cases where R is a simple alkyl group.[10] However, there have been no previous reports of the reactions with deoxyribonucleosides of heteroatom-substituted diazohydroxide aldehydes like **6**, which are the apparent key intermediates leading to the carcinogenic effects described above. In this paper, we describe the formation of the tricyclic deoxyguanosine adduct **12** by reaction with deoxyguanosine of **6**, generated in situ from **7**. This is the first example of deoxyribonucleoside modification by a heteroatom-substituted diazohydroxide aldehyde.

[2-(Carbethoxynitrosoamino)ethoxy]ethanal (**7**) was synthesized as a stable precursor to **6**. Bromoacetaldehyde diethyl acetal was added with cooling to a mixture of ethanolamine and NaH in THF. After it was refluxed for 3–6 h, the organic extract was distilled to give (2-aminoethoxy)ethanal diethyl acetal: bp 78–85 °C (0.5 torr); 27%; NMR δ 4.61 (1, t, C*H*CH_2), 3.6 (6, m, CH_3CH_2O + $CHCH_2O$), 2.84 (2, t, CH_2NH_2), 1.79 (2, br s, NH_2), 1.20 (6 t, CH_3CH_2).[11] This was allowed to react with $ClCO_2Et$ and triethylamine in CH_2Cl_2.[12,13] The product, [2-(carbethoxyamino)ethoxy]ethanal diethyl acetal, bp 117–125 °C (0.5 torr), was obtained in 40–60% yield. It was nitrosated by treatment with N_2O_4 and $NaHCO_3$ in Et_2O at −76 °C.[13] The reaction mixture was washed with H_2O and purified by preparative TLC (silica, 4/1 Et_2O/hexane), yielding pure **7** (28%, R_f = 0.17) and the corresponding diethyl acetal (49%, R_f = 0.52). NMR of **7**: δ 9.60 (1, s, CHO), 4.52 (2, q, $CO_2CH_2CH_3$), 4.0 (4, s + t, $OHCCH_2O$ + $CH_2N(NO)$), 3.51 (2, t, OCH_2CH_2), 1.44 (3, t, $CO_2CH_2CH_3$).

A mixture of **7** (98 mg, 0.48 mmol) and deoxyguanosine (125 mg, 0.46 mmol) in 25 mL of pH 8 phosphate buffer was heated for 24 h at 37 °C in a metabolic shaking incubator. After neutralization and extraction with Et_2O, the aqueous portion was concentrated to dryness in vacuo. The residue was extracted with 1/1 MeOH/EtOH, and the extracts were concentrated, redissolved in H_2O, and analyzed by HPLC by using a μBondapak C_{18} column with a 45 min gradient from 0 to 30% CH_3OH in H_2O at a flow rate of 1 mL/min and UV detection (254 nm). A single major adduct peak (25–30% yield based on deoxyguanosine) was observed, retention time 16.5 min. Deoxyguanosine eluted in 27.5 min. No major peaks eluted later than deoxyguanosine. A similar result was obtained upon incubation of **7** and deoxyguanosine at 37 °C, pH 7, in the presence of porcine liver esterase. The adduct was identified as 3-(2-deoxy-β-D-erythropentofuranosyl)-6,7-dihydro-6,7-dihydroxyimidazo[1,2-*a*]purin-9(3*H*)-one (**12**) by comparison of its HPLC retention time, UV, MS, and NMR, as well as the UV and NMR of its acid-hydrolysis product with those of references **12** and **13**. References **12** and **13** were unambiguously prepared by reaction of deoxyguanosine with glyoxal (**17**).[14,15]



The formation of **12** from **7** can be rationalized by initial hydrolysis of **7** to diazohydroxide **6**. Loss of N_2 and hydroxide ion from **6** would give the carbonium ion **14**, which could be expected to rearrange to O-ethylglyoxal (**16**). The latter hydrolyzes to glyoxal (**17**) which reacts with deoxyguanosine to give adduct **12**. The formation of **12** as the single major deoxy



guanosine adduct produced upon decomposition of the diazo hydroxide **6** is striking. This result clearly demonstrates the major effect of the ether oxygen atom and aldehyde group of **6** in controlling its reactivity and suggests that the mechanisms of DNA modification by nitrosamines such as **1**–**4** may be markedly different from those of simple dialkyl or alicyclic nitrosamines. Simple alkyldiazo hydroxides react primarily at the 7-position of deoxyguanosine.[10] (4-Oxobutyl)diazo hydroxide, which has the aldehyde group but lacks the ether oxygen of **6**, reacts with deoxyguanosine to give mainly cyclic 1,N^2-propanodeoxyguanosine adducts identical with those formed by reaction of crotonaldehyde with deoxyguanosine.[16] The present results suggest that adducts

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such as **12** should be formed in vivo in DNA upon metabolism of **1**.[17] Experiments designed to detect **12** or related adducts in vivo are currently in progress.

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(17) We have recently determined that glyoxal is a metabolite of **1**, which is in agreement with the present results.

Convergent Functional Groups Provide a Measure of Stereoelectronic Effects at Carboxyl Oxygen

J. Rebek, Jr.,* R. J. Duff, W. E. Gordon, and K. Parris

Department of Chemistry, University of Pittsburgh Pittsburgh, Pennsylvania 15260

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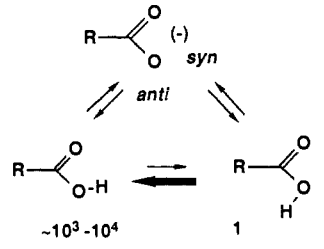
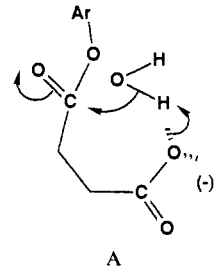
Acyl transfer reactions and aldol chemistry have provided a number of experimental methods by which stereoelectronic effects at carboxyl carbon may be assessed. By contrast, much less is known of these effects as they pertain to reactions at carboxyl *oxygen*. For carboxylic acids (eq 1) the *Z* form is regarded as



(1)

some 5 kcal more stable than the *E* form.[1a-c] This difference has biochemical implications; Gandour[1d] has observed that carboxylates found at the active sites of enzymes generally employ the more basic syn lone pairs rather than the less basic anti lone pairs. Evaluating the importance of these effects through equilibrium measurements of lone pair basicity has not been possible because of the lack of suitable model systems, and even kinetic analyses[2] of intramolecular general base catalysis, A, involve only the less basic anti lone pairs.



A

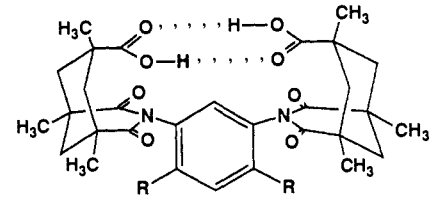
We recently introduced[3] the first molecular system having the appropriate structure to approach these issues. In these molecules, **1a**, **2**, and **3**, remote steric barriers *enforce the convergence of two carboxylic acids*, a feature which permits some control of the microenvironment of the syn lone pairs of a carboxylate. Here we report how these structural limitations affect ionization behavior.

Table I. Equilibrium Acidities of Carboxylic Acids (25 °C)

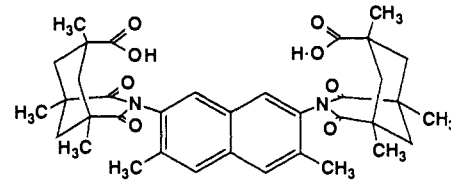
struct	pK_1	pK_{a2}	ΔpK_a	solvent	ref	dist,[a] Å
1a	4.8	11.1	6.3	$EtOH/H_2O$	this work[b]	3
1b	5.1	7.7	2.6			>8
2	5.5	7.5	2.0			5.8
3	6.5	7.8	1.2			8
4	4.7	7.6	2.9	$MeOH/H_2O$	4	3
5	6.0	6.9	0.9	$EtOH/H_2O$	5	4.5
6 (oxalic)	2.0	5.5	3.5	$EtOH/H_2O$[c]	6	3
7 (malonic)	3.4	7.1	3.7		6	3
8 (succinic)	5.0	6.8	1.8		6	6
9	6.3	8.0	1.7	Me_2SO/H_2O[d]	7	>8

[a] Approximate distance between oxygen atoms of the dicarboxylate as measured by CPK or Dreiding models. [b] Titration curves (30-50 points) were obtained in $EtOH/H_2O$ (1:1, w/w) at 0.01 M KCl, and regression analysis[8] was used to calculate pK_a's. Chemical shifts of the NMR of **3** in hydroxylic media indicate the dominance of the zwitterionic form shown.[9] [c] 40% EtOH by weight. [d] 65% Me_2SO by volume.

Dissociation constants of representative dicarboxylic acids in H_2O/alcohol mixtures are reported in Table I; all values have been corrected for statistical effects. While other diacids are available for evaluation, the selections below were intended to include structures in which the two $C_\alpha \rightarrow CO_2^-$ bonds are parallel (**4**, **5**), divergent (**1b**, **6**, **7**), and convergent (**1a**, **2**, **3**).



1a R = CH_3
1b R = H



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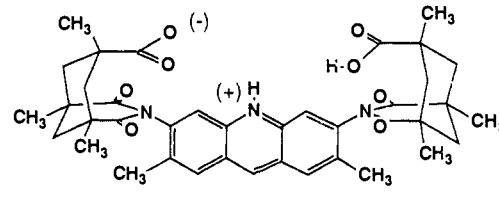

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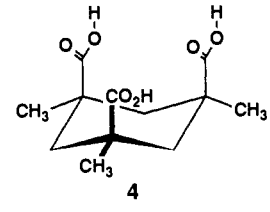

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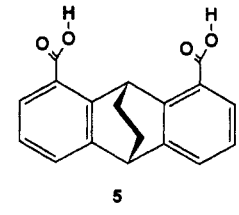

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The specific issue concerns the effect of orientation (rather than distance) on ΔpK_a. When about 3 Å separates the carboxylate oxygens (**4**, **6**, **7**), a ΔpK_a of 3 units is generally observed. In **6** and **7** the carboxyl groups diverge and, at worst, only the anti lone pairs can be directed toward each other in the dianion. In **4** the planes of the carboxyl groups are roughly parallel and only the π electrons of each may come into contact; intramolecular hydrogen bonds are not likely.[3] With **1a** the corresponding distance

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