# Kinetics of Formation and Dissociation of Manganese-Bovine Carbonic Anhydrase B

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**Abstract:** The second-order rate constant ($k_f$) for formation of the manganese(II) complex of bovine carbonic anhydrase B from Mn(II) ion and apoenzyme has been measured at 25° by a spectral-pH method. The rate constant increases from pH 6.1 to 7.7. The first-order rate constant ($k_d$) for dissociation of the manganese-carbonic anhydrase into apoenzyme and Mn(II) ion has been determined through the use of either (a) $Zn^{2+}$ ion or (b) phenanthroline as scavenging agents for apoenzyme or $Mn^{2+}$, respectively. The reactions were monitored by a catalytic assay method involving the hydrolysis of *p*-nitrophenyl acetate. The value of $k_d$ (at low concentrations of scavenging agent) decreases from pH 6.1 to 7.7. The values of $k_f$ and $k_d$ at pH 6.25 are 9.0 $M^{-1}$ sec$^{-1}$ and $1.1 \times 10^{-3}$ sec$^{-1}$ and at pH 7.75 are 56 $M^{-1}$ sec$^{-1}$ and $3.5 \times 10^{-4}$ sec$^{-1}$, respectively. Combination of $k_f$ with $k_d$ gives an apparent formation constant ($K$) for the formation of manganese-carbonic anhydrase, $\log K = -2.2 + 1.0$ pH. $Zn^{2+}$ ion accelerates the removal of Mn from the holoenzyme most effectively at higher pH, while the accelerating effect of phenanthroline is more pronounced at lower pH. The implications of the results are discussed.

Carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) contains one zinc atom per molecule of the metalloenzyme.[1] The zinc can be removed from carbonic anhydrase by dialyzing the native enzyme against 1,10-phenanthroline (phen) in acetate buffer at pH 5.[2,3] The metal-free carbonic anhydrase is stable in the pH range 5–9 for long periods.[2,3] Addition of zinc ion to the apoenzyme restores completely the original activity. A number of bivalent metal ions added to apocarbonic anhydrase form metallo derivatives which, apart from the cobalt(II) form, have zero or near zero activity. There seems little doubt, however, that the metal ion is located near or at the active site of the enzyme.[2–5]

The formation constants of the metallocarbonic anhydrases have been determined at pH 5.5 by an equilibrium dialysis technique.[6,7] A full study of the variation of the formation constant of native carbonic anhydrase with pH has been made. Over the pH range 5–10, the formation constant $K$ varies according to the relationship

$$\log K = 5.0 + 1.0 \text{ pH} \qquad (1)$$

and at neutral pH the reaction approximates to

$$Zn^{2+} + \text{apo}^{n+} \rightleftharpoons (\text{Zn-apo})^{(n+1)+} + H^+ \qquad (2)$$

The bovine B and human B and C isoenzyme forms[1] give a similar p$K$ *vs.* pH profile.[7]

The kinetics of binding of zinc ion by bovine apocarbonic anhydrase B have been studied in detail by Henkens and Sturtevant.[8] The reaction has been monitored by a variety of methods which lead to similar results. The degree of variation of the second-order formation rate constant with pH depends on the ionic strength of the medium, but in all cases is a relatively small one. It follows then that the pH dependence of the apparent formation constant shown in (1) must reside mainly in a strong variation of the dissociation rate constant with pH.[1] Rough calculations indicate that the times for removal of zinc from bovine carbonic anhydrase by the phenanthroline treatment[2,3] are somewhat shorter than those from estimated dissociation rate constants.[1] It appears then that the phenanthroline *possibly* provides an additional path for the removal of the metal, in addition to disturbing the equilibrium (2) in continually removing zinc(II) as the phen complex from the dialysis bag. It would be worthwhile to confirm these interesting points by direct measurement of the dissociation rate constant in the presence and absence of phenanthroline at a variety of pH values. Unfortunately, dissociation half-lives are inordinately long (~18 months at pH 7), a situation which is true of several of the other holoenzymes also.[1]

The least stable metalloform is the manganese–enzyme,[7] and associated formation and dissociation rate constants are undoubtedly more ameniable to measurement. Furthermore, scavenging agents such as phenanthroline are likely to remove the metal without recourse to dialysis procedures since indications are that the manganese–phenanthroline complex is more stable than manganese–carbonic anhydrase. We therefore have measured dissociation and formation rate constants for manganese–bovine carbonic anhydrase B, assessed the effect of pH 6.0–7.8 on both, and studied the role of zinc ion and phenanthroline on the rate of demetalation of the manganese–protein.

## Experimental Section

**Materials.** Bovine carbonic anhydrase (Sigma Chemical Co.) was chromatographed on a Whatman DE 52 DEAE-cellulose column according to the method of Lindskog.[9] The purity of the leading fraction (termed the B isoenzyme) was checked by gel electrophoresis. The apoenzyme was prepared from this fraction

(1) For a recent review, see S. Lindskog, L. E. Henderson, K. K. Kannan, A. Liljas, P. O. Nyman, and B. Strandberg, *Enzymes, 3rd Ed.*, **5**, 587 (1971).
(2) S. Lindskog and B. G. Malmstrom, *J. Biol. Chem.*, **237**, 1129 (1962).
(3) S. Lindskog, *J. Biol. Chem.*, **238**, 945 (1963).
(4) J. E. Coleman, *Biochemistry*, **4**, 2644 (1965).
(5) J. E. Coleman, *J. Biol. Chem.*, **242**, 5212 (1967).
(6) B. G. Malmstrom, *Arch. Biochem. Biophys.*, **46**, 345 (1953).
(7) S. Lindskog and P. O. Nyman, *Biochim. Biophys. Acta*, **85**, 462 (1964).
(8) R. W. Henkens and J. M. Sturtevant, *J. Amer. Chem. Soc.*, **90**, 2669 (1968).
(9) S. Lindskog, *Biochim. Biophys. Acta*, **39**, 218 (1960).

by dialysis against phen in 0.1 $M$ acetate buffer at pH 5.0–5.2 for 7 days.[2,3] Reagent grade chemicals were used without further purification. Solutions containing buffers or ionic strength electrolyte were extracted with dithizone in carbon tetrachloride to remove traces of transition metal ions.

**Kinetic Runs.** Stock solutions containing $Mn^{2+}$ or $Zn^{2+}$ ions were standardized with EDTA using Eriochrome Black T as indicator. Protein concentrations were determined from absorbances at 280 nm ($\epsilon$ 5.7 $\times$ $10^4$ $M^{-1}$ $cm^{-1}$).[7] All runs were carried out at 25.0° using an ionic strength of 0.1 $M$, made up of reactants, buffer, and sodium chloride. For the *dissociation runs* the reaction solution was prepared from apocarbonic anhydrase B, buffer (either $10^{-2}$ $M$ Hepes or Pipes), NaCl, and $MnCl_2$. The Mn(II) concentration was sufficient ($10^{-2}$ $M$) to complex at least 90% of the apoenzyme (4 $\times$ $10^{-5}$ $M$). The reaction solution was then forced through a column of Baker CGC-241 cation exchange resin 200–400 R, in the $Na^+$ form, which had been preequilibrated with a buffer solution A of the same concentration, pH, and ionic strength as that of the reaction mixture. The column was rinsed with A (apoenzyme concentration at end = 2 $\times$ $10^{-5}$ $M$). This treatment removes free $Mn^{2+}$ ion ($\leq 10^{-6}$ $M$ from esr measurement) from the reaction mixture, which is then quickly treated with the scavenger species (either $Zn^{2+}$ or phen). At specified times after addition of the scavenger, aliquots were removed and assayed for esterase activity with *p*-nitrophenyl acetate.[10] For the runs using $Zn^{2+}$ this activity is a direct measure of the carbonic anhydrase produced during the dissociation. In phenanthroline-containing runs, it was necessary to add $Zn^{2+}$ ($10^{-4}$ $M$) to the assay mixture. Zinc ion combined rapidly with the apoenzyme which had formed during the dissociation reaction, but was insufficient in concentration to cause noticeable direct replacement of manganese from the metalloprotein by zinc during the assay. After the reaction was complete, the pH was determined using a Corning Digital 112 Research pH meter. Good first-order plots were obtained and the associated rate constants are shown in Table I. For the study of the *formation* of manganese-carbonic anhydrase, a solution containing $Mn^{2+}$, buffer (2 or 4 $\times$ $10^{-4}$ $M$), indicator (2 $\times$ $10^{-5}$ $M$ Phenol Red or Chlorophenol Red), and NaCl was treated with apocarbonic anhydrase ($10^{-5}$ $M$) in a Cary cell using a mixing wand[11] or in the Gibson–Durrum stopped-flow spectrometer. Reaction was $\geq$95% complete in all conditions. The buffer concentration limited the pH change to one of 0.05–0.10 pH unit during the reaction, and this was monitored by an appropriate indicator. The pH of the reaction was measured on its completion. The absorbance of the indicator was almost linear with pH over this small range of pH and pseudo-first-order rate plots were linear over at least 3 reaction half-lives. Similar rate plots were obtained if the metal ion was added instead to the apoenzyme–buffer–indicator solution. The pseudo-first-order rate constants, which are the mean of several determinations, are shown in Table II. Their values were independent of protein concentrations, and were directly proportional at pH 7.0 to $[Mn^{2+}]$, thus substantiating the second-order rate law. Rate constants are accurate to ±4%. In some runs, not reported in Tables I and II, the mixture of A and B isozymes was used. Similar results were obtained as with the pure B form except that the first-order plots tended to curve slightly toward the end of the reaction, which could reflect a slower reacting A form.

**Table I.** Rate Constants for Dissociation of Manganese–Bovine Carbonic Anhydrase B at 25°

| (a) Using $Zn^{2+}$ to remove metal | | | (b) Using phen to remove metal | | |
|---|---|---|---|---|---|
| $[Zn^{2+}]$, m$M$ | pH | $10^3k$, $sec^{-1}$ | [phen], m$M$ | pH | $10^3k$, $sec^{-1}$ |
| 0.82 | 6.26 | 1.3 | 0.27 | 6.26 | 1.65 |
| 1.42 | 6.27 | 1.5 | 0.71 | 6.23 | 2.5 |
| 0.82 | 6.53 | 0.95 | 0.35 | 6.78 | 1.2 |
| 2.4 | 6.51 | 0.95 | 0.71 | 6.75 | 1.6 |
| 3.2 | 6.55 | 0.96 | 1.05 | 6.72 | 2.0 |
| 1.0 | 6.79 | 0.62 | 0.65 | 7.37 | 1.0 |
| 1.7 | 6.77 | 0.69 | 0.96 | 7.32 | 1.03 |
| 0.24[a] | 7.33 | 0.33 | 1.29 | 7.32 | 1.6 |
| 0.61[a] | 7.30 | 0.57 | 1.59 | 7.30 | 1.65 |
| 1.27[a] | 7.26 | 0.96 | 0.85 | 7.62 | 0.67 |
| 2.6[a] | 7.30 | 1.5 | 1.27 | 7.58 | 1.1 |
| 0.24 | 7.33 | 0.34 | 1.29 | 7.63 | 0.94 |
| 0.61 | 7.33 | 0.54 | 1.58 | 7.57 | 1.3 |
| 1.23 | 7.34 | 1.1 | 1.20 | 7.78 | 0.95 |
| 2.4 | 7.29 | 1.65 | 1.44 | 7.75 | 1.12 |
| 0.26 | 7.67 | 0.62 | 1.70 | 7.71 | 1.22 |
| 0.52 | 7.62 | 0.96 | | | |
| 0.66 | 7.66 | 1.14 | | | |
| 0.78 | 7.64 | 1.31 | | | |

[a] Solution contains 1.1 $\times$ $10^{-2}$ $M$ $Mn^{2+}$

**Table II.** Rate Constants for Formation of Manganese–Bovine Carbonic Anhydrase B at 25°

| $[Mn^{2+}]$, m$M$ | pH | $k$, $sec^{-1}$ | $k_f$, $M^{-1}$ $sec^{-1}$ |
|---|---|---|---|
| 28.4 | 6.16 ± 0.03[a] | 0.24 | 8.5 |
| 28.4 | 6.34 ± 0.02[a] | 0.27 | 9.4 |
| 28.4 | 6.55 ± 0.04[a] | 0.38 | 13.4 |
| 28.4 | 6.71 ± 0.06[a] | 0.46 | 16 |
| 0.95 | 6.95 ± 0.05[b] | 0.021 | 22.5 |
| 0.95 | 7.00 ± 0.05[b] | 0.023 | 24 |
| 0.95 | 7.02 ± 0.05[b] | 0.024 | 25 |
| 1.4 | 7.02 ± 0.04[b] | 0.036 | 25 |
| 1.9 | 7.00 ± 0.02[b] | 0.045 | 24 |
| 1.4 | 7.31 ± 0.03[b] | 0.047 | 33 |
| 1.9 | 7.44 ± 0.02[b] | 0.092 | 49 |
| 1.9 | 7.51 ± 0.02[b] | 0.11 | 57 |
| 0.95 | 7.56 ± 0.03[b] | 0.052 | 55 |
| 0.95 | 7.74 ± 0.01[b] | 0.053 | 56 |

[a] Using 4 $\times$ $10^{-4}$ $M$ Pipes buffer and 2 $\times$ $10^{-5}$ $M$ Chlorophenol Red indicator, and stopped-flow technique. [b] Using 2 $\times$ $10^{-4}$ $M$ Hepes buffer and 2 $\times$ $10^{-5}$ $M$ Phenol Red indicator and Cary 14.

## Results and Discussion

**Dissociation of the Mn–Carbonic Anhydrase.** The results shown in Table I indicate that both phenanthroline and Zn ion can promote dissociation of manganese from the manganese–protein, although the effects of pH differ in the two cases. Extrapolation of the dissociation rate constants to zero concentration of scavenger gives an intercept which is considered to be the spontaneous dissociation rate constant of the manganese–enzyme ($k_0$). The zinc-promoted dissociation gave in general better linear plots than those for phenanthroline (see, *e.g.*, Figure 1). More reliance was therefore placed on the values for the dissociation rate constants $k_0$ obtained from the Zn(II)-scavenging results (see Table III). The slowness inherent in the "batch" method of monitoring limited the range of pH which could be conveniently studied to about 6–7.8.

**Phenanthroline-Promoted Dissociation.** Phenanthroline enhances the dissociation of manganese–carbonic anhydrase and, over a limited range of phen concentrations, the observed first-order dissociation rate constant $k_{obsd}$ is given by

$$k_{obsd} = k_0 + k_1[\text{phen}] \quad (3)$$

The values for $k_0$ and $k_1$ at various pH's are listed in Table III. The phenanthroline presumably acts by forming a transient ternary complex involving manganese, protein ligands (see below), and phenanthroline. The manganese–protein bonds will dissociate more rapidly in such an arrangement, just as higher metal complexes release ligands more rapidly, generally, than lower complexes.[12] The manganese–phenanthroline bond is also

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(11) J. H. Swinehart, *J. Chem. Educ.*, **44**, 524 (1967).

(12) R. G. Wilkins, *Accounts Chem. Res.*, **3**, 408 (1970).

Table III. Values of Rate Constants for Unassisted, $k_0$(Zn) or $k_0$(phen), and Assisted, $k_1$(phen) or $k_2(Zn^{2+})$, Dissociation of Manganese–Carbonic Anhydrase into Apoenzyme at 25°

| pH | $10^3k_0$(Zn), $sec^{-1}$ | $k_2$(Zn), $M^{-1}$ $sec^{-1}$ | $10^3k_0$(phen), $sec^{-1}$ | $k_1$(phen), $M^{-1}$ $sec^{-1}$ |
|---|---|---|---|---|
| 6.25 | 1.1 ± 0.05 | 0.27 ± 0.05 | 1.15 ± 0.05 | 1.9 ± 0.2 |
| 6.53 | 0.94 ± 0.01 | 0.01 ± 0.005 | | |
| 6.76 | 0.51 ± 0.03 | 0.11 ± 0.02 | 0.8 ± 0.3 | 1.1 ± 0.1 |
| 7.32 | 0.21 ± 0.02 | 0.62 ± 0.06 | 0.4 ± 0.2 | 0.8 ± 0.2 |
| 7.58 | | | 0.2 ± 0.1 | 0.6 ± 0.1 |
| 7.65 | 0.27 ± 0.01 | 1.3 ± 0.01 | | |
| 7.75 | | | 0.35 ± 0.03 | 0.5 ± 0.1 |

weakened, since no substantial amounts of the ternary complex are formed. This is also observed for the native carbonic anhydrase; the determination of the formation constant[7] and the ability of the enzyme to catalyze[1] are not modified by the presence of phenanthroline, as they certainly would be if a ternary complex had any appreciable stability. However, experiments on the interaction of apocarbonic anhydrase with cobalt(II)– and nickel(II)–phenanthroline complexes show that the metal complex reaches the active site pocket intact, and only then releases the ligand.[13,14] The ternary complex is thus a real, if transient, species and its presence a reasonable explanation for the effect of phen on the removal of metal from the metal–enzyme.

The effectiveness of phen shown by $k_1$ values (Table III) increases, although not markedly, as the pH is lowered. This enhancement cannot reside in the nature of the ligand species, which remains unprotonated over the pH 6–7.5 range. The structure of carbonic anhydrase has been determined. Three imidazoles from histidyl residues in the protein coordinate to the zinc and (probably) a coordinated water molecule makes up a distorted tetrahedron around the metal.[15] We have no knowledge of the coordination environment around the metal in the manganese form of the protein. The small formation constant[7] does suggest that the metal is not filling the coordination pocket very well, and indeed even anions which bind to the manganese may replace one of the (imidazole) ligands binding to the metal.[16] There appears to be one water molecule directly linked to the metal at higher pH, but none at lower pH.[16] The easier removal of metal in acid pH, both in the presence and absence (Table III) of phen, may therefore originate in a reduction of the coordination around the manganese by detachment of one or more imidazoles through protonation.

If the behavior of the manganese form can be extrapolated to the zinc–enzyme, it is clear why the most effective condition for preparing the apobovine and human form is the highest feasible acidity.[17,18] The dissociation rate constant and the dissociative enhancement by phen are largest here.

**Zinc Ion Promoted Dissociation.** Early results appeared to indicate that the dissociation rate constant was dependent on the concentration of free $Mn^{2+}$ ion, used to effect formation of the manganese–enzyme. Free manganese ion was therefore removed by ion exchanger prior to the reaction runs. Later work (Table I) showed that $10^{-2}$ $M$ $Mn^{2+}$ had no effect on the rate constant. Zinc ion accelerates the dissociation of manganese from manganese–carbonic anhydrase. The effect is barely noticeable at pH's around 6, but is important at pH 7.3–7.7 (Table III). The observed dissociation rate constant $k_{obsd}$ is given by

$$k_{obsd} = k_0 + k_2[Zn^{2+}] \quad (4)$$

The second-order removal of metal ions from simpler complexes by aquated metal ions is well documented.[19] The replacement of nickel by copper from the nitrilotriacetate complex ($NiNTA^-$) from pH 2.6 to 4.9 obeys the rate law[20]

$$V = k[NiNTA^-] + k'[NiNTA^-][Cu^{2+}] + k''[NiNTA^-][H^+] \quad (5)$$

Equation 5 contains terms all of which are duplicated in the present metal interchange study. The second term is associated mechanistically with a rate-determining reaction of a copper–nickel binuclear complex containing NTA bridging elements, formed reversibly from reactants. A similar mechanism for the observed rate law (eq 4) is difficult to envisage for the manganese–carbonic anhydrase. The attacking zinc ion may coordinate to the imidazole(s) which are weakly attached or not attached to the manganese,[16] particularly at higher pH. Alternatively, zinc ion may coordinate to one of the histidines in the active site region.[15] In either case, although the former may be

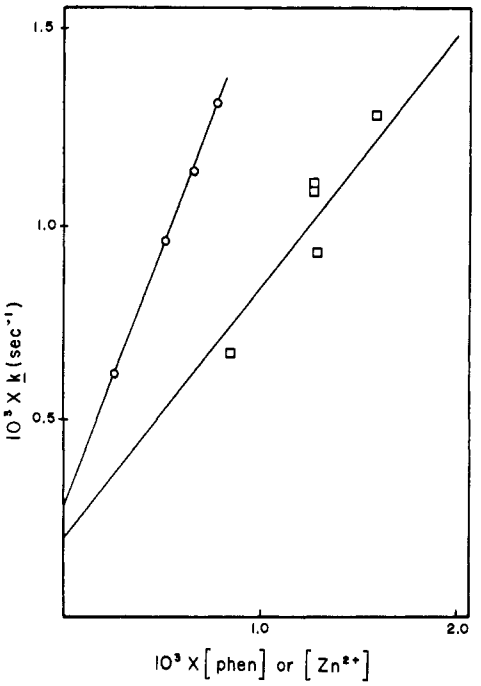


Figure 1. Plot of dissociation rate constant ($k$, $sec^{-1}$) *vs.* concentration of scavenger at 25° [(○) Zn ion; (□) phen] for Mn–bovine carbonic anhydrase B; pH 7.6.

(13) R. G. Wilkins, *Pure Appl. Chem.*, 33, 583 (1973).
(14) R. J. McQuate and R. G. Wilkins, unpublished experiments.
(15) A. Liljas, K. K. Kannan, P. C. Bergsten, I. Waara, K. Fridborg, B. Strandberg, U. Carlbom, L. Jarup, S. Lovgren, and M. Petef, *Nature (London), New Biol.*, 235, 131 (1972).
(16) A. Lanir, S. Gradsztajn, and G. Navon, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, 30, 351 (1973).
(17) S. Lindskog and B. Malmstrom, *Biochem. Biophys. Res. Commun.*, 2, 213 (1960).
(18) E. E. Rickli and J. T. Edsall, *J. Biol. Chem.*, 237, PC 258 (1962).
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(20) T. J. Bydalek and M. L. Blomster, *Inorg. Chem.*, 3, 667 (1964).

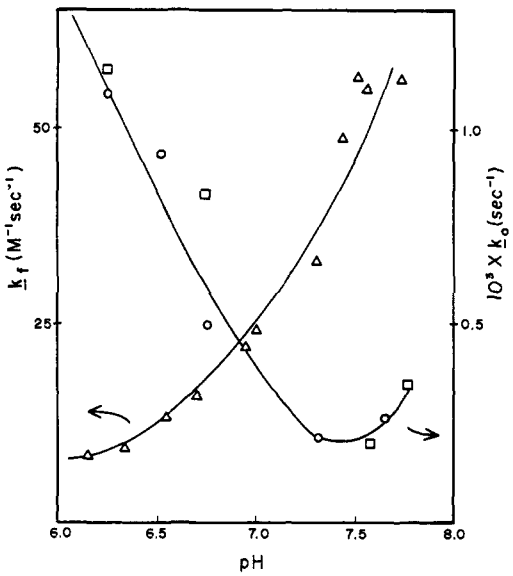


**Figure 2.** Plots of dissociation rate constant ($k_0$, $sec^{-1}$) and formation rate constant ($k_f$, $M^{-1}$ $sec^{-1}$) *vs.* pH at 25° for Mn-bovine carbonic anhydrase B: (○) Zn ion; (□) phen.

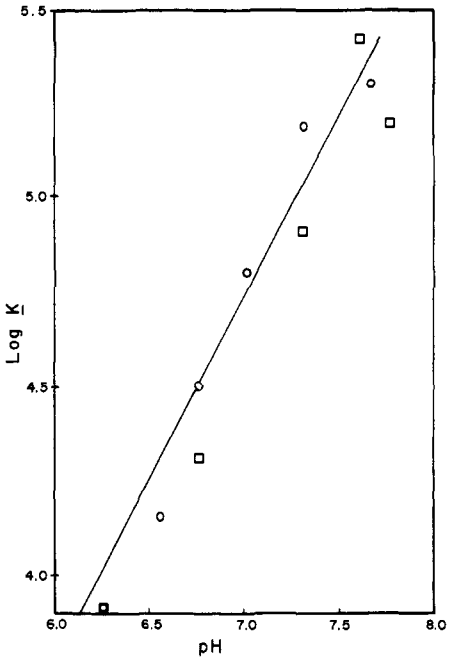


**Figure 3.** Plot of log (formation constant) of Mn-bovine carbonic anhydrase B *vs.* pH at 25°: (○) data from Zn scavenging; (□) data from phen scavenging.

more likely, the proximity of the zinc to the manganese coordination sphere may underly its action.

An even closer resemblance to the enzyme system is that of metal ion interaction with the metalloporphyrins where the metal is firmly and rigidly held in the porphyrin ring. The replacement of Zn by $Cu^{2+}$ in the zinc complex of tetrakis(*N*-methyltetrapyridyl)porphine at pH 3.0–4.4 is a second-order reaction. Here the role of the entering metal ion is paramount, "spontaneous" dissociation being negligible.[21]

The dissociation rate constant $k_0$ appears to reach a limiting (low) value at pH ~7.5 (Figure 2). This value may be associated with a basic form of the manganese–enzyme.[16] The increase of $k_0$ with decreasing pH is observed also with simpler systems, eq 5, and may likewise arise from protonation of detached ligands and thus prevention of reattachment to the manganese.

**Formation of the Mn–Carbonic Anhydrase.** The second-order rate constant for the formation of manganese–carbonic anhydrase increases with increasing pH (Figure 2). This is a characteristic shared with the $Zn^{2+}$– and $Co^{2+}$–apoenzyme systems[8,22] and with the $Ni^{2+}$–apoenzyme interaction (from pH 5.0 to 9.0).[23] The small increase in rate constant with increasing pH for reaction of $Zn^{2+}$ with apobovine carbonic anhydrase B has been interpreted in terms of $Zn^{2+}$ reacting with diprotonated (p$K$ = 5.4), monoprotonated (p$K$ = 7.2), and unprotonated forms of apocarbonic anhydrase as well as $ZnOH^+$ reacting with the unprotonated form.[8] The associated rate constants differ only moderately, namely $10^3$, $8 \times 10^3$, $3 \times 10^4$, and $4 \times 10^4$ $M^{-1}$ $sec^{-1}$ at 25°, respectively. It would be possible to treat our data similarly in terms of reaction of a mono- and unprotonated apoenzyme, but until the reasons for the different rate–pH profiles are known, it seems premature to analyze the system in detail.

The apparent formation constant $K$ of the manganese–enzyme defined as in (6) can be estimated at

$$K = [\text{Mn–enzyme}]/[Mn^{2+}][\textstyle\sum\text{enzyme forms}] = k_f/k_0 \quad (6)$$

each pH from the ratio of formation $k_f$ and dissociation $k_0$ rate constants. The result, plotted as a variation of log $K$ with pH, is shown in Figure 3. The line drawn with unity slope accounts quite well for the experimental points. For the manganese complex

$$\log K = -2.2 + 1.0\ \text{pH} \quad (7)$$

from pH 6–7.5, illustrating the similarity to the behavior of the Zn–enzyme, eq 1. Our value for log $K$ at pH 5.5 is 3.3 and is in reasonable agreement with that determined for manganese–human carbonic anhydrase (3.8 at pH 5.5).[7,24] The value for log $K$ at pH 7.0 is 4.8 and much higher than that obtained for the bovine B form (3.4) by Lanir and Navon,[25] whose conditions differed from ours only in that they used 0.1 $M$ Tris sulfate buffer. In our formation runs at pH 7.0, $10^{-3}$ $M$ $Mn^{2+}$ effected ≥95% consumption of apoenzyme ($10^{-5}$ $M$ starting concentrations) from assay of the equilibrium mixture. The value of $K$ must be therefore $\geq 10^{4.3}$, consistent with our $k_f/k_d$ value.

Finally, it is worth commenting that the higher thermodynamic stability of the zinc over the manganese–holoenzyme by more than seven orders of magnitude resides (say at pH 7 and 25°) in a (a) somewhat larger Zn formation rate constant, $10^4$ $M^{-1}$ $sec^{-1}$ compared with $2.5 \times 10$ $M^{-1}$ $sec^{-1}$ and (b) much smaller Zn dissociation rate constant $10^{-8}$ $sec^{-1}$ compared with $10^{-3}$ $sec^{-1}$. Kinetically the effect of dissociation is obviously more important than formation in dictating thermodynamic stability. The relative spread of for-

(21) H. Baker, P. Hambright, L. Wagner, and L. Ross, *Inorg. Chem.*, 12, 2200 (1973).
(22) S. A. Rudolph and J. M. Sturtevant, *Biophys. Soc. Abstr.*, 189a (Feb 1970); S. A. Rudolph, Ph.D. Thesis, Yale University, 1971.
(23) F. Ng and R. G. Wilkins, unpublished results.
(24) The values of $K$ for the bovine form of carbonic anhydrase are similar to those of the human forms, although perhaps 0.2–0.4 log unit lower when a direct comparison is made.
(25) A. Lanir and G. Navon, *Biochemistry*, 13, 3536 (1972).

mation constant for other metal ion–carbonic anhydrase complexes,[7] combined with a narrower range of formation rate constants,[8,13,22,23] indicates that this may be a general rule for metallo derivatives of this particular enzyme.

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# Communications to the Editor

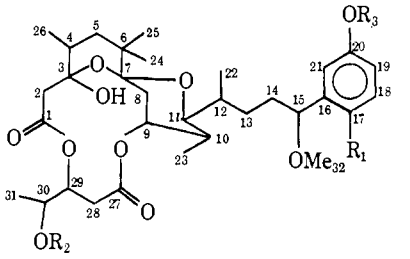
## Aplysiatoxin and Debromoaplysiatoxin, Constituents of the Marine Mollusk *Stylocheilus longicauda* (Quoy and Gaimard, 1824)

*Sir:*

Accounts of toxicity of sea hares, which are gastropod mollusks without shells, date to Roman times,[1] but attempts to characterize the toxin, initiated by Flury in 1915,[2] have been few[3–6] and have shed no light on its chemical nature. In contrast, recent investigations into secondary metabolites of sea hares, irrespective of physiological activity, have yielded a rich harvest of mono-, sesqui-, and diterpenoids, most of them halogenated.[7–10] The early literature[1,2] mentions several toxic sea hare secretions, but Winkler, *et al.*,[4] and Watson[5] linked the animal's toxicity to its digestive or midgut gland. Watson[5] distinguished two toxic entities by solubility and bioactivity, one soluble in water and one in ether.

We now wish to report that we have isolated from *Stylocheilus longicauda* Watson's ether-soluble toxin ($LD_{100}$ 0.3 mg/kg, ip mouse; 0.025% of wet whole animal) as a difficultly separable oily mixture of aplysiatoxin (**1**) and debromoaplysiatoxin (**2**) and that we have determined their structures. The homogeneity of all compounds was verified by tlc, mass, and nmr spectrometry. Two parallel noncrystalline nontoxic acetates, **3** and **4**, accompany the toxins. The phenolic hydroxyl and the tertiary hydroxyl at C-3 render the toxins labile above pH 7 and below pH 4. Diazomethane treatment furnished stable anisoles **5–8**, but the C-3 hydroxyl was readily eliminated under many experimental conditions and by active adsorbents. The ensuing artifacts, four C-3,4 olefins corresponding to **1–4**, doubled the components of the natural mixture.

Chemical ionization mass spectrometry on **8** with ammonia as the carrier gas[11] led to an unambiguous molecular weight of 648, while mass spectrometry under all other conditions had furnished only M − 18. Composition was determined by high resolution mass spectroscopy[11] on the anhydrophenyl acetate of **4**, 658.3377 (calcd for $C_{36}H_{50}O_{11}$ 658.3353).



**1**, $R_1$ = Br; $R_2$ = $R_3$ = H
**2**, $R_1$ = $R_2$ = $R_3$ = H
**3**, $R_1$ = Br; $R_2$ = Ac; $R_3$ = H
**4**, $R_1$ = H; $R_2$ = Ac; $R_3$ = H
**5**, $R_1$ = Br; $R_2$ = H; $R_3$ = Me
**6**, $R_1$ = $R_2$ = H; $R_3$ = Me
**7**, $R_1$ = Br; $R_2$ = Ac; $R_3$ = Me
**8**, $R_1$ = H; $R_2$ = Ac; $R_3$ = Me

The molecular architecture of the aplysiatoxins (**1**, **2**), which are bislactones of 3,4-dihydroxyvaleric acid and of 4,6,6,10,12-pentamethyl-3,7,9,11,15-tetraoxy-15-phenylpentadecanoic acid, encompassing a symmetrical trioxacyclododecane was revealed by the following major degradations and spectral data.

Principal evidence for the aromatic portion of the toxins includes phenolic uv in MeOH at 283 nm (ε 1950), shifted to 290 nm (ε 3000) in 0.1 *N* NaOH–MeOH, and typical mass spectral fragments resulting from benzylic cleavage. Nmr chemical shifts (δ 6.28, 6.97, 7.40) and coupling constants (<1.0, 3.1, 8.0 Hz) of **1** are those of a 1,2,4-trisubstituted benzene, mass spectral data indicate that the three substituents are Br, OH, and the aliphatic moiety, and the chemical shifts of **5** calculated from aromatic substituent effects[12] matched those of a 1-Br-2-R-4-$OMeC_6H_3$. The more complex four-spin system of **2** was confirmed by matching nmr spectra in $CDCl_3$ and in benzene-$d_6$ with computer generated spectra.

From 1 *N* KOH (EtOH) or $NaBH_4$ (EtOH) treatment of 3,4-anhydro-**8** we isolated after acetylation 4-acetoxy-*trans*-2-pentenoic acid (ir 3400–2500, 1720–1700 $cm^{-1}$; nmr ($CDCl_3$, δ) H-28 5.96 (1 H, dd, 16.0, $J$ = 1.5 Hz), H-29 6.95 (1 H, dd, 16.0, $J$ = 5.0 Hz), H-30 5.45 (1 H, m), H-31 (3 H, d, $J$ = 6.5 Hz), $MeCO_2$– 2.10 (3 H, s)), thus describing the five carbons of the valeric acid moiety.

The principal carbon chain of the aplysiatoxins, comprising C-1 to C-26 and C-32, was revealed after pro-

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