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Hydroxo-Complexes of Hg^{2+} Chelates as Models of Hydrolytic Metalloenzymes

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Hydroxo-complexes of chelates of Hg^{2+} were found to catalyze hydrolysis of active esters and amides. Thus, 10^{-2} *M* of the hydroxo-complex of Hg^{2+} pentamethyl diethylenetriamine (Hg–PDETA) at pH 7 enhanced the rate of hydrolysis of *p*-nitrophenyl esters and of cinnamoyl imidazole by factors of 400 and 1900, respectively. In the latter case the rates of reaction were linear with catalyst concentration. The hydroxo-complexes of Hg^{2+} phenanthroline (Hg–Phen) exhibited kinetic specificity toward *p*-nitrophenyl carbalkoxyglycinates. With these specific substrates acceleration factors of 1000 and more were obtained at 5×10^{-3} *M* Hg–Phen, pH 8. The dependence of rates upon catalyst concentration was found to be curvilinear. This latter behavior was attributed to attack of one molecule of Hg–Phen, in the form of a hydroxo-complex, on a ternary complex Hg–Phen-substrate. The general features of metal-ion-catalyzed hydrolytic reactions are discussed and compared with the mode of action of hydrolytic metalloenzymes such as carboxypeptidase A and carbonic anhydrase.

INTRODUCTION

Metal ions play an important role in the mechanism of action of hydrolytic metalloenzymes, such as leucine aminopeptidase (*1*), carboxypeptidase A (*2*) and carbonic anhydrase (*3*), in which the metal ion is embedded in the protein whose functional groups serve as ligands.

Rate enhancement caused by metal ions in enzymes is attributable to one or more of the following factors: 1) Introduction of a positive charge in a favorable position serving as a "sink" (*4*) for the electron density developing on the substrate carbonyl during hydroxide ion attack, an effect achieved either through the acyl residue of the substrate (as in leucine aminopeptidase (*1*)), or through polarization of its carbonyl group (as suggested in the case of carboxypeptidase A (*5*)); 2) the metal ion serving as carrier of hydroxide ion to the substrate (*6*); 3) the metal ion serving as a template for the reaction and orienting properly the catalytic group of the protein relative to the labile bond of the substrate (*4*, *7*); 4) the metal ion exerting a stabilizing effect on a reactive intermediate in the reaction pathway (*4*); 5) binding of the metal ion to one of the reaction products but not to the substrate (*4*).

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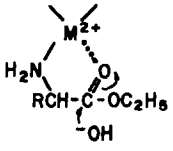
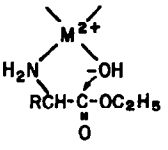
Models of hydrolytic metalloenzymes studied to date fall into two categories: 1) Models in which the substrate is bound to the metal ion. In metal-ion-catalyzed hydrolysis of amino acid esters (*8–11*), two kinetically equivalent pathways are available: Attack by external hydroxide on metal-chelated substrate (**I**) or attack by metal-bound hydroxide on metal-attached substrate (**II**).



I



II

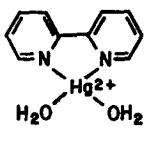
2) Models in which hydroxo-complexes of metal-ion chelates catalyze the hydrolysis of unbound active esters by direct transfer of a hydroxide ion from the metal ion to the substrate.

The Cu^{2+}-glycylglycine hydroxo-complex was studied as a catalyst for the hydrolysis of pNPA (*12*); in this system, however, the kinetic scheme is complicated by formation of an inactive dimer of the catalyst, the monoolate:

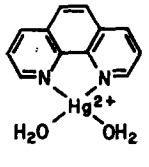
$$2Cu(GlyGly)OH^- \rightleftarrows (CuGlyGly)_2OH^- + OH^-. \qquad [1]$$

Accordingly further models of this category were studied by us (*13*) in an attempt to obtain both high catalytic factors and a kinetic scheme uncomplicated by a dimerization process.

Hydroxo-complexes of chelates of Hg^{2+} with triamines meet either one or both of these requirements. Very high catalytic factors, combined with specificity toward a certain class of substrates, carbalkoxyglycine esters, are obtained with hydroxo-complexes of the aromatic diamines, dipyridyl (**III**) and 1,10-phenanthroline (**IV**) (*14, 15*). These systems are shown to involve attachment of the substrate to the chelated metal ion, thus behaving ultimately like models of the first category with respect to specific substrates.



III



IV

Abbreviations used are: pNPA, *p*-nitrophenyl acetate; NPAG, *p*-nitrophenyl acetyl glycinate; NPCMG, *p*-nitrophenyl carbomethoxyglycinate; NPZG, *p*-nitrophenyl carbobenzoxyglycinate; NPCMGO, *p*-nitrophenyl carbomethoxyglycolate; CI, cinnamoyl imidazole; DETA, diethylenetriamine; PDETA, pentamethyl diethylenetriamine; MES, 2-(*N*-morphilino) ethane sulfonate; HEPES, 2-hydroxyethyl-piperazino ethane sulfonate; ML, metal–ligand complex.

EXPERIMENTAL

Materials. Reagent-grade inorganic materials and buffer compounds were used with deionized fresh glass-distilled water for the solutions. Nitrate salts were used instead of halide ions in order to avoid complexing of Hg^{2+}. $Hg(NO_3)_2$ was purchased from Baker (Analyzed Reagent), phenanthroline, dipyridyl and collidine from Fluka, purest grade. DETA from Aldrich (highest purity), PDETA from Eastman (white label), MES and HEPES from Sigma. The substrates used were from previous studies (*16*, *17*).

Methods. Kinetic runs were carried out at 30°C, with ionic strength kept at 0.055 with $NaNO_3$. Stock solutions of $Hg(NO_3)_2$ (0.1 *N*) contained 0.025 *N* HNO_3 to prevent hydrolysis of Hg^{2+} (*17*). The buffers (at 5×10^{-3} *M* or 1×10^{-2} *M*) were MES (pH 6–6.5), HEPES (pH 7–7.5), collidine (pH 8–8.5), borate (pH 8.5–9) and carbonate (pH 9), none of which affected the rates of reaction of the metal chelates with the substrates.

Kinetics was followed spectrophotometrically as described previously (*16*). The nature of the reaction products was determined by the hydroxamic acid method for esters and with the aid of the spectral properties of cinnamoyl derivatives in the case of CI.

In order to avoid the slow drift of chloride anions from the calomel electrode during pH measurements and potentiometric titrations, agar–KNO_3 bridges were used with the electrode, and one end of the bridge was immersed in a saturated solution of KNO_3 and the other end in the sample solution.

Calculations. The first-order constant k_{obs} was calculated from the absorbance changes as described previously (*16*).

$$d[S]/dt = -k_{obs}[S] = -(k_0 + k_{ML}[ML])[S]. \quad [2]$$

At each pH, k_{obs} values were measured at several metal–ligand concentrations [ML]. In those cases where k_{obs} was linear with [ML], the slope was taken as the apparent second-order constant, k_{ML}, at the specific pH, and the intercept yielded k_0 ($= k_{OH}[OH^-]$). Where this behavior was not observed, a special kinetic analysis was resorted to (see below). In cases where k_{ML} was found to follow a titration curve, pH-independent constants k_{ML-OH} were obtained by dividing k_{ML} values by the fraction of dissociated ML–OH (Eq. [3]) present in solution at the pH in question. If the undissociated form ML–H_2O was active, k_{ML-H_2O} constants could be obtained by dividing k_{ML} values by the fraction of undissociated ML–H_2O.

$$ML\text{–}H_2O \rightleftarrows H^+ + ML\text{–}OH. \quad [3]$$

p*K* values were determined by plotting the values of $\Delta T_{OH}/\Delta$ pH vs pH, where T_{OH} is the amount of hydroxide added to the solution, after correction for titration of a blank solution. The maximum of such a plot is the p*K* of the titrated compound (*18*).

RESULTS

Hydroxo-Complexes of Mercury (II)-Chelates with Triamines

pH-rate profile of reactions of Hg–DETA (v). Hydroxo-complexes of chelates of Hg^{2+} with various triamines (such as the DETA chelate **V**) catalyze hydrolysis of

p-nitrophenyl esters and other active substrates (*13*). Catalysis by (V) is effective in the 5–9 pH range and the reactions can be followed spectrophotometrically. With CI as substrate plotting of k_{ML} against pH yields a titration curve with pK_a 7.15 (Fig. 1).

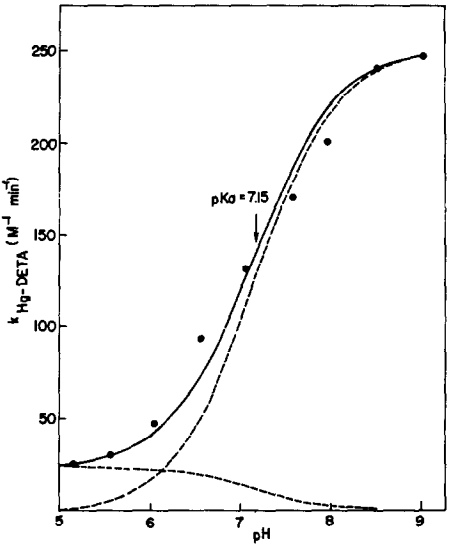
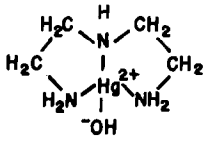
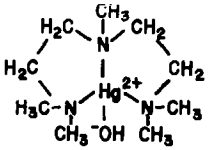


FIG. 1. pH-rate profile for Hg–DETA-catalyzed hydrolysis of CI at 30°C, $I = 0.055$. Full curve is sum of the two dotted curves, and is calculated according to $k_{ML} = k_{ML-H_2O}[a_H/(a_H + K_a)] + k_{ML-OH}[K_a/(a_H + K_a)]$; p$K_a = 7.15$, $k_{ML-H_2O} = 27$ min^{-1} M^{-1}, $k_{ML-OH} = 250$ min^{-1} M^{-1}.



V



VI

In the case of the *p*-nitrophenyl esters NPAG and NPCMGO plots of k_{obs} vs [ML] were linear only in the range 1×10^{-4}–1×10^{-3} M and became concave at higher concentrations.[3] The pH-rate profile obtained in the lower concentration range of NPCMGO is shown in Fig. 2. The pK_a values and rate constants of the active species

[3] The deviations from linearity observed at [ML] $> 1 \times 10^{-3}$ were analyzed on the assumption of a dimerization process yielding an inactive dimer:

$$2ML(H_2O)^{2+} \overset{K_D}{\rightleftharpoons} [(ML)_2OH]^{3+} + H^+.$$

K_D is obtainable from the concave dependence of k_{obs} on [ML] (Appendix I). For the two *p*-nitrophenyl esters studied $K_D = (3.43 \pm 0.23) \times 10^{-5}$. The fact that the same value of K_D is obtained for two substrates is consistent with the assumption that the dimer is inactive. Further evidence of a dimerization process was obtained by potentiometric titration of $[Hg(DETA)H_2O]^{2+}$ at concentrations between 5×10^{-3} and 1.5×10^{-2} M. The pH dependence failed to follow a simple titration curve due to superimposition of the dimerization and ionization processes. Analysis of the titration data yields values of pK_a and K_D comparable to those derived from the kinetic data.

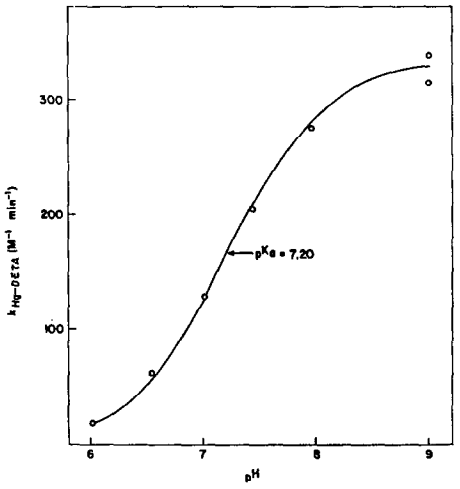


FIG. 2. pH-rate profile for Hg–DETA-catalyzed hydrolysis of CMGOP at 30°C, $I = 0.055$ (calculated curve for $pK_a = 7.20$).

are summarized in Table 1. The term k_{ML-H_2O} occurs only in the case of CI and may be due to equilibrium between CI and its protonated species ClH^+ (cf. (*19*)):

$$v = k_{ML-H_2O} \times [ML\text{–}H_2O][CI] = k_{ML-OH} \times [ML\text{–}OH][ClH^+]. \quad [4]$$

The active species in the above system is the hydroxo-complex (V) and the pK of its conjugate acid is 7.15.

TABLE 1

VALUES OF pK_a AND OF RATE CONSTANTS FOR HYDROLYSIS REACTIONS CATALYZED BY ACTIVE SPECIES OF Hg–DETA AT 30°C, $I = 0.055$

Substrate	CI	NPAG	NPCMGO
pK_a of pH-rate profile[a]	7.15	7.10	7.20
k_{MLOH} ($min^{-1}\ M^{-1}$)	250 ± 20	86 ± 9	333 ± 25
k_{ML}–H_2O ($min^{-1}\ M^{-1}$)	27 ± 3	0[b]	0[b]

[a] Potentiometrically determined $pK = 7.19 \pm 0.03$.
[b] Not measurable.

Reactions of Hg-DETA. With CI as substrate, the reaction catalyzed by Hg–DETA yielded hydrolytic products in all cases. This was determined, under a variety of conditions, both by the hydroxamic acid method for esters (*20*, *16*) and from the spectral properties of the products (*19*, *16*). The rate constant for the reaction was almost independent (<20% deviation) of the substrate concentration over a 20-fold range (including substrate concentrations higher than those of the catalyst).

The temperature dependence of the reaction of Hg–DETA with CI was determined at pH 7.0, between 20 and 40°C, and yielded (using a standard state of 1 M) $\Delta H^+ = 6.8$ kcal/mole and $T\Delta S^+ = -10.4$ kcal/mole, as expected for a bimolecular reaction (*21*).

Reactions of Hg–PDETA (VI). An attempt to suppress the dimerization process was made by using a bulkier ligand than DETA. The Hg^{2+} complex of the pentamethyl derivative of DETA, $[Hg–(PDETA)H_2O]^{2+}$ (**VI**) exhibits a normal ionization behavior with $pK_a = 6.80 \pm 0.03$. Kinetic experiments were carried out with NPAG, NPCMG and NPCMGO in the 6–9 pH range. For the three *p*-nitrophenyl esters k_{obs} was linear with [ML] at least up to 7×10^{-3} *M*. The data obtained for $pK_a = 6.80$ measured potentiometrically were consistent with a two-term expression:

$$k_{ML} = k_{ML-OH} \times [K_a/(a_H + K_a)] + k_{ML-H_2O} \times [a_H/(a_H + K_a)]; \quad [5]$$

i.e., both the hydroxo-complex and its conjugate acid are active with respect to the substrates. The constants derived from this analysis are listed in Table 2.

TABLE 2

RATE CONSTANTS FOR HYDROLYSIS REACTIONS CATALYZED BY ACTIVE SPECIES OF Hg–PDETA AT 30°C, $I = 0.055$

Substrate	NPAG	NPCMG	NPCMGO
k_{MLOH} (min^{-1} M^{-1})	81.5	70.5	422
k_{ML-H_2O} (min^{-1} M^{-1})	7.2	6.11	22.3

Hydroxo-Complexes of Mercury (II) Chelate with Dipyridyl and Phenanthroline

Reactions of Hg–DP (III). The rates of reaction of Hg–DP with NPAG and NPZG were measured in the 7–9 pH range. The rate constant vs catalyst concentration plot was linear in the case of NPAG and upward curved in that of NPZG (Fig. 3). Kinetic analysis of the data for NPZG revealed substrate binding to the catalyst with the resulting complex subsequently hydrolyzed by a second molecule of the latter, the most effective nucleophile in solution [Eq. [6]) (*15, 17*). The kinetic expression is given in Appendix I.

$$ML + S \overset{K_{MLS}}{\rightleftharpoons} MLS, \quad [6a]$$

$$MLS + ML \xrightarrow{k_{MLS}} 2ML + P. \quad [6b]$$

The results for NPZG are summarized in Table 3. For NPAG at pH 7.95, $k_{ML} = 22.5$ min^{-1} M^{-1} which is lower by a factor of 20 than the value $k_{MLS} = 465$ min^{-1} M^{-1}

TABLE 3

KINETIC PARAMETERS FOR REACTION OF Hg–DP WITH NPZG AT 30°C, $I = 0.055$

pH	K_{MLS} (M^{-1})	k_{MLS} (min^{-1} M^{-1})
6.97	137	84.8
7.95	61.7	465
8.90	61.7	1175

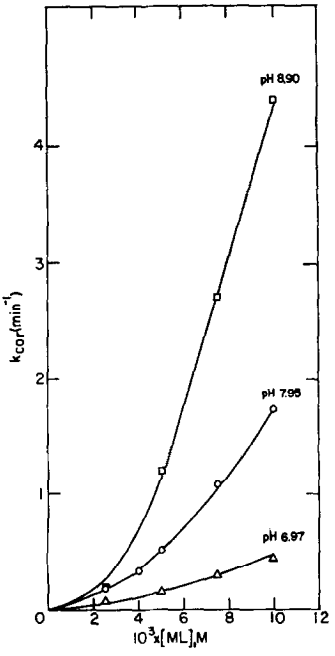


FIG. 3. Dependence of hydrolysis rate of NPZG on Hg–DP concentration at different pH values, 30°C, $I = 0.055$.

obtained for NPZG, a result of the binding effect in the latter case. The dependence of the k_{MLS} values upon pH is compatible with dissociation of a single group with $pK = 8.20$, which agrees with the potentiometrically determined $pK = 8.25$. Thus, the whole catalytic activity of Hg–DP may be assigned to the monodissociated form of (**III**), i.e., to attack of the monohydroxo-complex of Hg–DP on catalyst–substrate complex.

Reactions of Hg–Phen (IV) with glycine derivatives. High catalytic efficiency, directed specifically toward *p*-nitrophenylcarbalkoxyglycinates $ROCONHCH_2COOPhNO_2$ was shown to be an outstanding feature of the reaction of catalyst (**IV**) with active substrates (*15*). The dependence of the rates of reaction on catalyst concentration showed a curved pattern with "specific" substrates (*15*) and linearity with "nonspecific" ones as in the case of **III**.

The rates of reaction of Hg–Phen with the specific substrates NPCMG and NPZG and with the nonspecific substrates NPAG and NPCMGO were measured in the 6.5–9 pH range. The data for the specific substrates were analyzed according to the same model as for **III** and the results for all four are summarized in Table 4.

Analysis of the pH dependence of the kinetic parameters showed that in this case the catalytic activity cannot be assigned to a single species of hydroxo-complex, and a modified expression is called for:

$$k_{ML} \text{ or } k_{MLS} = k_{MLOH}[MLOH] + k_{ML(OH)_2}[ML(OH)_2]. \quad [7]$$

The kinetic data for NPCMGO yielded the pK values of 6.95 for $ML(H_2O)_2$ and approximately 9.0 for $ML(H_2O)OH$. The data for NPCMG and NPZG were consistent

TABLE 4

KINETIC PARAMETERS FOR REACTIONS OF Hg–PHEN WITH SPECIFIC AND NONSPECIFIC SUBSTRATES AT 30°C, $I = 0.055$

	k_{ML} (min^{-1} M^{-1})				K_{MLS} (M^{-1})	
pH	NPAG	NPCMGO	NPCMG	NPZG	NPCMG	NPZG
6.45	13.4	27.5	526	1,300	50.5	38.5
6.95	30.4	49.5	1,000	2,320	85.5	66
7.45	58.5	88.5	2,740	3,610	120.5	142
7.95	87.5	186	5,000	6,140	175.5	323
8.45	194	418	16,670	15,900	118.5	204
8.90	—	665	—	—	—	—

with these pK values, but because of the extremely rapid reaction at pH levels above 8 no reliable constants could be obtained.

Reactions of Hg-Phen with other specific substrates. In addition to the *p*-nitrophenyl esters of carbalkoxyglycine (*15*), it was found that those of carbalkoxy derivatives of other amino acids are also specific substrates. In fact, the rates of reaction of alanine and phenylalanine derivatives, as well as that of NPZG, also showed an upward-curved pattern, amenable to analysis according to the model of Eq. [6]. The results of such an analysis for a series of substrates are listed in Table 5. The very high susceptibility of

TABLE 5

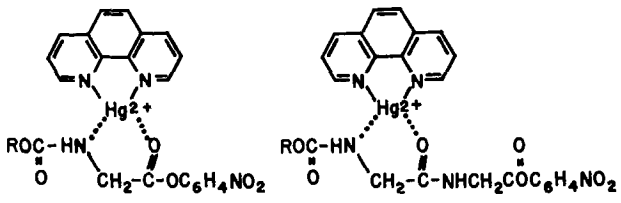
KINETIC PARAMETERS FOR REACTIONS OF Hg–PHEN WITH SPECIFIC SUBSTRATES AT pH 7.95, 30°C, $I = 0.055$

Substrate	$10^{-2} \times k_{MLS}$ (min^{-1} M^{-1})	K_{MLS} (M^{-1})
$CH_3OCOGlyOC_6H_4NO_2$	50	175
$C_6H_5CH_2OCOGlyOC_6H_4NO_2$	61	323
$C_4H_9COOGlyOC_6H_4NO_2$	40	120
$C_6H_5CH_2OCOAlaOC_6H_4NO_2$	71.5[a]	250
$CH_3OCOPheOC_6H_4NO_2$	62.5[b]	98
$C_6H_5CH_2OCOGlyGlyOC_6H_4NO_2$	5.1	935

[a] For this substrate, $k_0 = 7 \times 10^{-3}$ min^{-1}.
[b] For this substrate, $k_0 = 9 \times 10^{-3}$ min^{-1}.

these substrates is readily explained by the previously proposed mechanism (*15*) for this specific catalysis involving binding of specific substrates to the mercury of the chelate (**VII**). It is apparent that the side chain of the amino acid in the substrate affects neither the catalytic constant nor the binding constant, whereas the latter is doubled upon introduction of a phenyl group in the carbalkoxy moiety. The glycylglycine derivative has the highest binding constant and the lowest catalytic constant of all.

In this case the carbonyl group of the susceptible bond is further removed from the mercury ion, making for less favorable interaction. On the other hand, chelation of the substrate is probably enhanced in the presence of an additional amide group (**VIII**).



Rate of reaction versus Hg:Phen ratio. The rates of reaction of Hg–Phen with the nonspecific substrates NPAG and NPCMGO and the specific substrates NPZG and NPCMG were measured over a range of Hg:Phen ratios, 1:1–1:3 (Fig. 4). The initial increase of the rate constant, with a peak at M:L = 1:2, is attributable to the active

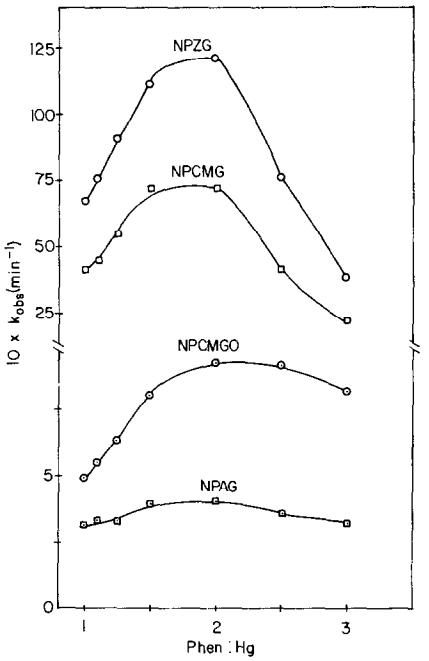


FIG. 4. Dependence of reaction rate of Hg–Phen with different substrates on L:M ratio, pH 7.95, 30°C, $I = 0.055$.

species ML_2OH derived from the ML_2 complex, while at higher M:L ratios increasing amounts of the inactive species ML_3 are presumably obtained (*22*) ($\log K_3$-formation constant of ML_3 is 3.7). The plot of the rate of reaction of Hg–$(Phen)_2$ with NPZG vs ML_2 concentration again showed an upward curvature as in the case of the ML

complex. The data were analyzed according to Eq. [6], and, whereas the rate constant for NPAG was $k_{ML_2} = 99\ min^{-1}\ M^{-1}$, that for NPZG (bound to ML_2) was $k_{ML_2S} = 8330\ min^{-1}\ M^{-1}$, with binding constant $K_{ML_2S} = 247\ M^{-1}$. Thus, in this case binding causes rate enhancement by a factor of 80.

Inhibition of reactions of Hg–Phen with substrates. It was found that benzyl carbobenzoxyglycinate is not hydrolyzed by Hg–Phen at pH 8 and could act as a competitive inhibitor for the reaction with NPZG. Analysis of the relevant rate constants at different inhibitor concentrations [I] (from 0.8 to $2.6 \times 10^{-4}\ M$), under the assumption that MLI is an inactive species and the rate constants are proportional to the "free" (noninhibited) complex ML (see Appendix II), yields the inhibition (association) constant $K_{MLI} = 2280\ M^{-1}$.

Carboxylate anions constitute another class of inhibitors for the reactions of Hg–Phen with all substrates. K_{MLI} values, calculated from data for NPZG and NPAG at pH 8 and $[ML] = 10^{-3}\ M$, were as follows: acetate $35 \pm 10\ M^{-1}$, phenylpropionate $70 \pm 20\ M^{-1}$, acetylglycinate $30 \pm 10\ M^{-1}$, carbobenzoxyglycinate $370 \pm 60\ M^{-1}$. In contrast to the close values of the inhibition constants of acetate and acetylglycinate (derived from a nonspecific substrate), that of carbobenzoxyglycinate (derived from a specific substrate) is more than tenfold higher. Moreover, the inhibition constants of this anion at pH 7.95 ($K_{MLI} = 370 \pm 60\ M^{-1}$) and at pH 7.45 ($K_{MLI} = 100 \pm 20\ M^{-1}$) compare well with the binding constants of the parent substrate (Table 4), indicating similarity of binding of the substrate and inhibitor.

TABLE 6

RATES OF REACTION OF Hg–PHEN WITH NPZG AND NPCMGO IN THE PRESENCE AND ABSENCE OF H_2O_2 AT PH 6.95, 30°C, $I = 0.055$

Substrate	k_{ML} (min^{-1})[a]	$k_{H_2O_2}$ (min^{-1})[b]	$k_{(ML+H_2O_2)}$ (min^{-1})[c]
NPZG	2.45	0.58	5.87
NPCMGO	0.18	2.54	2.24

[a] At $[Hg–Phen] = 4.9 \times 10^{-3}\ M$.
[b] At $[H_2O_2] = 9.8 \times 10^{-3}\ M$.
[c] Hg–Phen and H_2O_2 concentrations as above.

Participation of additional nucleophile in reactions of Hg–Phen. According to the model proposed for the reactions of Hg–Phen, the MLS complex is attacked by a second molecule of ML (Eq. [6b]). This reaction prevails, since under the conditions in question MLOH is the best nucleophile in solution. However, by using a good nucleophile which does not compete too strongly for free sites on the metal ion, it was shown that the MLS complex is susceptible to attack by other nucleophiles as well. The anion of H_2O_2 was found to meet these requirements. As can be seen from Table 6, the nucleophile does not enhance the rate of reaction of Hg–Phen with the nonspecific substrate NPCMGO (actually a decrease is observed, probably due to some complexing of HOO^- by Hg–Phen, as was also observed with Cu–DP (*23*)), while an increase is found in the case of the specific substrate NPZG. Moreover, the specific contribution

to the rate constant, obtained by subtracting the values of k_{ML} and $k_{H_2O_2}$ from that of $k_{(ML+H_2O_2)}$, was linear with the concentration of H_2O_2 in the range 2×10^{-3}–1×10^{-2} M. The second-order constant for attack of H_2O_2 on MLS (obtained after dividing these values by the concentration of bound substrate, using K_{MLS} at pH 7), was found to be 1800 min^{-1} M^{-1}, which is comparable with the value $k_{MLS} = 2300$ min^{-1} M^{-1} at pH 7 (Table 4).

DISCUSSION

Catalytic Efficiency of Hydroxo-Complexes of Mercury (II)-Chelates

In Table 7 the catalytic efficiency of hydroxo-complexes of Hg–DETA and Hg–PDETA is evaluated by comparing their performances to spontaneous hydrolysis (k_0) at pH 7. The catalytic enhancement factor $F = k_{ML}/k_0$ at 10^{-2} M catalyst is 70–90

TABLE 7

COMPARISON OF RATES OF HYDROLYSIS OF DIFFERENT SUBSTANCES BY HYDROXO-COMPLEXES OF CHELATES OF Hg^{2+} AT PH 7, 30°C, $I = 0.055$

Substrate	pNPA	NPAG	NPCMG	NPCMGO	CI
$10^2 \times k_{OH}$ (min^{-1})	0.016	1.18	0.14	0.64	0.3
$10^2 \times k_{Hg-DETA}$[a] (min^{-1})	1.29	16.0	9.9	56.9	118
$10^2 \times k_{Hg-PDETA}$[a] (min^{-1})	—	44.9	35.6	256.4	576[b]

[a] $[ML] = 9.5 \times 10^{-3}$ M.

[b] In this system the dependence on complex concentration was not linear.

for Hg–DETA and 300–400 for Hg–PDETA with *p*-nitrophenyl ester substrates,[4] whereas with CI they are 400 and 1900, respectively. Comparison of the F values with the Cu–glycylglycine system (*12*) shows that Hg–DETA is at least 20-fold and Hg–PDETA 100-fold more efficient with respect to pNPA.

However, DETA chelates with Cu^{2+} and Ni^{2+} are much poorer catalysts than Hg–DETA. Thus, at pH 8 with $[ML] = 1 \times 10^{-2}$ M, the following rate constants were obtained with CI as substrate: Hg–DETA = 2.04, Cu–DETA = 0.218, and Ni–EDTA = 0.048 min^{-1}.

Nature of Chelate Systems Exhibiting Specificity Toward a Certain Class of Substrates

No other metal ion is comparable to Hg^{2+} with respect to rate enhancement obtained with specific substrates. The rate constants for the reactions of metal-Phen with NPZG, at a ratio M:L = 1:2 ($[M] = 3 \times 10^{-3}$ M), pH 8, are as follows: Cu–$(Phen)_2$ = 0.098, Zn–$(Phen)_2$ = 0.031, Cd$(Phen)_2$ = 0.15, Hg$(Phen)_2$ = 8.59 min^{-1}. With Mn^{2+}, Co^{2+} and Ni^{2+} the values were even lower than for Zn^{2+}. Moreover, in the case of Cd$(Phen)_2$ the rate constants were linear with concentration for all substrates, and in

[4] The relatively high value of k_{OH} for NPAG is due to oxazolinone formation rather than to hydrolysis (*24*).

the case of $Cu(Phen)_2$ there was even a downward curvature because of formation of diolates (*25*).

The unique catalytic specificity of Hg–DP and, even more, of Hg–Phen towards substrates such as NPZG is also apparent when comparing F values obtained (at pH 8) with various ligands (Table 8). Thus, two methyl groups in the vicinity of the metal-coordinated nitrogens, or a tridentate ligand such as terpyridine, are "harmful" modifications of the catalytic complex, in particular with specific substrates. This comparison also shows that Hg–DP and Hg–Phen form hydroxo-complexes whose reactivity is comparable to that of Hg–DETA and Hg–PDETA toward nonspecific substrates.

TABLE 8

COMPARISON OF VALUES OF F FOR REACTION OF SPECIFIC AND NONSPECIFIC HYDROLYTIC CATALYSTS WITH DIFFERENT SUBSTRATES AT PH 7.95, 30°C, $I = 0.055$

	F[a]				
Catalyst	NPAG[b]	NPCMGO	NPCMG	NPZG	CI
Hg–DP	7.4	—	—	43	22
Hg–Phen	28	15.0	1000	1500	42
Cu–Phen	1.9	1.9	—	4	—
Hg–dimethyl Phen	<2.1	2.3	—	10	4
Hg–terpyridine	<2.0	2.0	—	4	9.5
Hg–DETA	20.0	18.5	14.7	14.8	76
Hg–PDETA	34.5	32.3	25.1	20	116

[a] At $[ML] = 5 \times 10^{-3}$ M.

[b] In the case of NPAG the k_{OH} value used is assumed to be equal to that of NPCMG; because of oxazolinone formation in the case of NPAG, the apparent k_{OH} is too high (*24*).

Kinetic Specificity Factor

The specificity of the reactions of Hg–DP and Hg–Phen with the carbalkoxy derivatives of amino acid esters is reflected in high catalytic factors, $F > 1000$ (Table 8). Thus, kinetic specificity is exhibited, in analogy with enzymatic reactions (*26*), and accordingly a specificity factor f_s can be defined, $f_s = F_s/F_{ns}$ where F_s and F_{ns} are the catalytic factors for specific and nonspecific substrates, respectively.

This factor represents the additional rate enhancement obtained by binding of specific substrates to a specific model and corrects for the "conventional" rate enhancement caused by any hydroxo-complex, which ranges from 10–35 at 5×10^{-3} M chelate (see below). Thus, for Hg–Phen $f_s = 100$ is obtained when calculated for the specific substrate NPZG and the nonspecific compound NPCMGO, whereas dimethylphenanthroline has $f_s = 4$ and terpyridine, $f_s = 2$. With a different metal ion, namely Cu–Phen instead of Hg–Phen, f_s is reduced from 100 to 2, reflecting the inability of the Cu^{2+} chelate to bind the specific substrates, partially because of formation of diolates (*25*).

General Features of Metal-Ion-Catalyzed Hydrolytic Reactions

The present study provides an insight into some of the factors governing metal-ion-catalyzed hydrolytic reactions. Hydroxo-complexes of metal chelates are known to be

nucleophilic reagents with respect to *p*-nitrophenyl esters (*12*), metal-bound esters (*27*), and amides (*28*) of amino acids. Their tendency to form inactive dimers, diolates (*25*), was shown in this study to depend both on the metal ion and on the ligand. Thus, Hg^{2+}, presumably due to its larger ionic radius (*29*), shows less tendency than Cu^{2+} to form diolates (for example, with Phen as ligand). The importance of the structure of the ligand can be evaluated by comparing Hg–PDETA and Hg–DETA. Thus, substitution of the cordinating nitrogen atoms of the ligand by methyl groups precluded diolation, presumably through steric interference.

The order of catalytic efficiency of the hydroxo-complexes of the metal ions, $Hg^{2+} > Cu^{2+}$, $Cd^{2+} > Zn^{2+} > Co^{2+} > Mn^{2+} > Ni^{2+}$, does not parallel that of stability of their chelates which for Phen is: $Hg^{2+} > Ni^{2+} > Cu^{2+} > Co^{2+} > Zn^{2+} > Cd^{2+} > Mn^{2+}$ (*30*). On the other hand, it agrees well with the strength of OH^- binding (*31*) by these metal ions, thereby accounting for the low efficiency of Ni^{2+} ions (which have very little affinity for hydroxide ions) in contrast with the high efficiency of Cd and Hg ions, which bind OH^- strongly.

It is apparent that the metal ion is the main factor in determining the pK of the hydroxo-complex, since replacement of the ligand caused less change in the pK than replacement of the central cation. One deduced effect of the nature of the ligand is that the strength of binding of hydroxide ions is increased, i.e., the pK drops, on provision of a hydrophobic environment (see also below) for the metal ion; for example, replacement of DETA with PDETA as ligand.

Another striking feature of these complexes is that, although the pK_a of their bound water is eight units lower than that of free water, their nucleophilic effect is not proportionately lower. Thus, the hydroxo-complexes of Hg–DETA and Hg–PDETA are at least 10^4–10^5-fold better nucleophiles than would be expected from their reduced basicity. Hg–Phen, which has a specificity factor of ~100 toward specific substrates, is $\sim 10^7$-fold better nucleophile than expected from basicity alone. The same phenomenon was observed in the case of the inert complexes of Co^{3+} (*28*) and is attributable to a "charge" effect in nucleophilic reactions (*32*).

A very interesting finding of this study is the effect of the environment of the metal ion on the nature, efficiency, and specificity of reactions catalyzed by hydroxo-complexes. Thus, the presence of ligand methyl groups in the vicinity of the metal cation in Hg–PDETA was shown to affect the properties of the nucleophile attached to it. In addition to preclusion of diolation and lowering of the pK_a of the water molecule bound to Hg, this chelate exhibits a higher catalytic efficiency than the parent Hg–DETA (Table 7). Moreover, in this case both the hydroxo-complex and its conjugate acid, $Hg(PDETA)H_2O$, were found to be reactive toward *p*-nitrophenyl esters, apparently because of the "environmental" effect provided by the methyl groups on the ligand. However, the best example of the environmental effect is the kinetic specificity ($f_s \sim 100$) exhibited by Hg–Phen toward carbalkoxy derivatives of *p*-nitrophenyl esters of amino acids, which is most probably related to the extent of electron delocalization operative in such systems (*33*). The same argument could explain why 1:2 complexes of Hg–Phen with the coordination number of Hg^{2+} probably increased to 6, are better catalysts than their 1:1 counterparts (Fig. 4). Phenanthroline–metal ion complexes were found to exhibit special reactivity in other types of reactions (*34–36*). The detailed mechanism of this specificity is not fully understood. On the basis of a

series of analogous substrates (*15*), we previously suggested that the urethane nitrogen of the substrate provides a binding site for Hg^{2+}. In other chelates the mercury cation did not exhibit this affinity, which must therefore be considered as a consequence of the environment of Hg^{2+} in the chelate. As for the second binding site of these substrates, it is apparently provided by the carbonyl of the scissile bond. This assumption is supported by two observations: 1) The inhibition constants for the anions of the parent carboxylic acids were equal to the binding constants of the substrates; 2) the *p*-nitrophenyl ester of ZGlyGly, the hydrolysis rate of which was lower than that of NPZG by a factor of 12 (Table 5), was bound better than NPZG, probably because the second binding site in this case is an amide instead of an ester group (**VIII**).

The equality of the binding constants of the substrates and the inhibition constants of the parent carboxylate anions suggests extensive bond-breaking in the leaving group in the transition stage, as observed in nucleophilic reactions of oxyanions with activated esters (*37*). Since only nucleophilic attack is involved, it is also clear that hydroxo-complexes would not be efficient catalysts toward substrates whose leaving groups have a pK much higher than that of formation of the catalytically active species. This explains why the benzyl ester (pK of benzyl alcohol = 15.1 (*38*) of carbobenzoxyglycine is an inhibitor and not a substrate for the hydroxo-complexes of Hg–Phen.[5] On the other hand, the reactivity of CI (pK of imidazole = 14) toward hydroxo-complexes can be rationalized in terms of its known susceptibility to oxyanions reactions (*16, 17*).

That nucleophilic attack on the ternary complex Hg–Phen–substrate is involved can also be deduced from the fact that a powerful nucleophile, such as the hydroperoxide anion, was capable of competing with the hydroxo-complex of Hg–Phen for the substrate (Table 6). In general, the hydroxo-complexes of Hg^{2+} chelates act as a source of hydroxide anions at pH values around neutrality. These hydroxide ions can act as nucleophiles with respect to substrates with good leaving groups, whereby the hydroxide ion is transferred from the metal ion to the substrate.

Comparison with Hydrolytic Metalloenzyme Mechanisms of Action

One of the proposed mechanisms for the action of carbonic anhydrase involves attack by the hydroxo-complex of Zn^{2+} of the enzyme on the substrate (CO_2 or active esters, such as pNPA) (*40*). The hydroxo-complexes of Hg^{2+} chelates in this study appear to be appropriate models of this type of reaction. The common features of the enzyme and model reactions described here are: 1) Catalysis of CO_2 hydration[6] and hydrolysis of "active" esters only (*41*); 2) the pK controlling the reaction is close to neutrality (*42*); 3) similar enhancement factors: for the enzyme, $F = 600$ with *p*-nitrophenyl propionate (*41*); for Hg–PDETA, $F = 400$ with NPCMGO (Table 7). Although the cation in the models is Hg^{2+} whereas Zn^{2+} is a very poor substitute, it should be borne in mind that the environment in the enzyme might decrease the pK of the Zn^{2+}–H_2O to lower values than in models. Reduction of this pK to 7 would make the hydroxo-complex in the enzyme a powerful catalyst toward active esters. However, from nmr studies, it was recently suggested that the pK observed at pH 7 is not that of a

[5] Esters with poor leaving groups were not substrates for any hydroxo-complex, such as that of Hg–DETA (*39*).

[6] In preliminary experiments (*39*) it was shown that the hydroxo complex of Hg–DETA catalyzes the hydration rate or CO_2 at 6°C.

water molecule bound to the Zn^{2+} ion of the enzyme, and that the active species contains an undissociated molecule (Zn^{2+}–H_2O) (*43*, *44*). Pocker and Storm (*41*) had previously suggested that the latter comprises the active group of the enzyme and that an imidazole group (pK of conjugate acid = 7) causes general base catalysis during attack of the bound H_2O on the substrate. They also observed higher catalytic activity at higher pH, which they ascribed to formation of the hydroxo-complex. This behavior would be similar to that observed in the case of Hg–PDETA, where the hydroxo-complex and its conjugate acid (namely, Hg(PDETA)–H_2O) were active species (Table 2), as a consequence of the hydrophobic environment provided by the methyl group of the ligand. (In this context, it is pertinent to mention that the CO_2-binding site in carbonic anhydrase is thought to be hydrophobic (*45*)).

As for the specific models, Hg–DP and Hg–Phen, the role of their metal ions is in part similar to that ascribed to the Zn ion of carboxypeptidase A (*2*), i.e., polarization of the substrate carbonyl. Although Hg^{2+}–carboxypeptidase A is inactive towards peptides, it is known to be more active than the Zn^{2+}–enzyme toward ester substrates (*46*). The complex substrate–metal chelate (or enzyme) is attacked both in the model and in the enzyme by additional nucleophiles (*2*, *3*). In the model, the nucleophile is usually a second molecule of metal chelate in hydroxo-complex form, whereas in the enzyme all additional nucleophilic groups are provided by itself. It was argued recently (*47*, *48*) that a water molecule bound to the Zn ion of carboxypeptidase A might play an important role in the mechanism of action of this enzyme. If both polarization of the substrate carbonyl and nucleophilic attack on it by Zn^{2+}-bound hydroxide ions are operative, then the Hg–DP and Hg–Phen chelates are appropriate models. It might also be noted that two of the Zn^{2+} ligands in carboxypeptidase A (*2*, *3*) and three Zn^{2+} ligands in carbonic anhydrase (*3*, *40*) are imidazole side chains of histidine residues. This type of environment, found both in models and in metalloenzymes, might endow the chelated cation with the special properties for its catalytic reaction.

APPENDIX I

The kinetic expression for the reaction of the chelate ML with the complex MLS (Eq. [6]) is:

$$v = k_{\mathrm{MLS}} \times [\mathrm{MLS}] \times [\mathrm{ML}]. \qquad [1A]$$

At steady-state:

$$[\mathrm{MLS}] = K_{\mathrm{MLS}} \times [\mathrm{ML}]_f \times [S]_f. \qquad [2A]$$

Since $[\mathrm{ML}]_T \gg [S]_T$ it turns that $[\mathrm{ML}]_f = [\mathrm{ML}]_T = [\mathrm{ML}]$ and $[S]_f = [S]_T - [\mathrm{MLS}]$, where subscript f denotes free species and T the total amount, and in analogy to an enzymic reaction

$$[\mathrm{MLS}] = ([\mathrm{ML}] \times [S]_T)/(K_m + [\mathrm{ML}]), \qquad [3A]$$

where K_m denotes $1/K_{\mathrm{MLS}}$. Thus

$$v = \frac{k_{\mathrm{MLS}} \times [\mathrm{ML}]^2 \times [S]_T}{K_m + [\mathrm{ML}]}, \qquad [4A]$$

and, since $k_{\mathrm{obs}} = v/[S]_T$,

$$k_{\mathrm{obs}} = k_{\mathrm{MLS}} \times [\mathrm{ML}]^2/(K_m + [\mathrm{ML}]). \qquad [5A]$$

A double reciprocal plot of Eq. [5A]:

$$([ML]/k_{obs}) = (1/k_{MLS}) + (K_m/k_{MLS}) \times (1/[ML]) \qquad [6A]$$

yields then $1/k_{MLS}$ as the intercept and K_m/k_{MLS} as the slope.

APPENDIX II

For the reaction of an inhibitor with the chelate ML,

$$K_{MLI} = [MLI]/[ML]_f[I]_f, \qquad [7A]$$

where

$$[ML]_f = [ML]_T - [MLI] \quad \text{and} \quad [I]_f = [I]_T - [MLI].$$

For a nonspecific substrate $k_{cor} = k_{obs} - k_0$ is proportional to $[ML]_f$, and therefore

$$[ML]_f = k_{cor}/k_{ML}. \qquad [8A]$$

$[ML]_f$ yields the values of $[MLI]$ and $[I]_f$, which in turn yield K_{MLI} according to Eq. [7A]. In the case of a specific substrate, where the inhibitor-free concentration of ML is no longer proportional to k_{cor}, $[ML]_f$ is obtained from:

$$[ML]_f = \frac{1}{2}\left[\frac{k_{cor}}{k_{MLS}} + \left(\left(\frac{k_{cor}}{k_{MLS}}\right)^2 + 4 \times \frac{k_{cor}}{k_{MLS}} \times K_{MLS}\right)^{1/2}\right]. \qquad [9A]$$

This expression is derived from Eq. [5A] in Appendix I.

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