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SOME ASPECTS OF THE BIOINORGANIC CHEMISTRY OF ZINC

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I. Introduction

A. Importance of Zinc in Biological Systems

Zinc is present at a relatively high concentration in the cells of most organisms, indeed among the $3d^n$ elements its concentration is second

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only to that of iron. As study of the various biological functions of zinc proceeds, its extreme importance in life processes is constantly being emphasized.

It is an essential element for the normal functioning of most organisms, and its deficiency can lead to reduction of normal growth, impaired bone development, hindered maturation and function of reproductive organs and processes, impaired protein and carbohydrate metabolism, and retardation of learning capacity. The metal is thought to be effective in promoting wound healing and to be of therapeutic value in the treatment of atherosclerosis. It is probably involved in the photochemistry of vision (*1*).

Although nutritional studies of zinc were made as early as the nineteenth century (*2*) it was not until 1940 that carbonic anhydrase was isolated from mammalian red blood cells (erythrocytes) by Keilin and Mann (*3*) and the protein was shown to contain 0.33% of zinc, which was essential for activity.

As the number of zinc metalloenzymes that have been studied in considerable detail approaches 20, it is becoming clear that probably at least a further 60 or 70 await detailed characterization.

Recently it has been found that zinc may also have a role in genetics: studies of the zinc content of nucleic acid preparations and chromatin by sensitive analytical methods (*4*) suggest that zinc may have a function in DNA replication and transcription as well as in protein synthesis. A striking demonstration of a role for zinc in this area is exemplified by the finding that zinc deficiency in the growth medium of *Euglena gracilis* causes arrest of cell division (*5*, *6*): there is a drop in the protein RNA content of the cell, and growth is arrested at a stage where the DNA content of each cell is doubled (*5*); considerable work has been done recently on DNA-dependent RNA-polymerases that bind zinc, but these fascinating studies are as yet in their early stages (*6–11*).

We shall be concerned here with three zinc-containing enzymes that have been subjected to the most detailed study (carbonic anhydrase, liver and yeast alcohol dehydrogenases, and carboxypeptidase A) to see how chemical and physical techniques are employed to unravel some of the mysteries of, or at least to limit the number of alternatives possible for, the mechanism of action at the zinc center of these enzymes.

We shall see how nature uses unusual and distorted low coordination numbers of the metal ion to achieve catalytic activity and how the metal works in unison with delicately positioned groups on the ligand protein to optimize its catalytic efficiency: the metal ion itself is not sufficient, nor are the ligand groups alone.

To assist readers who may not be familiar with some of the biochemical terminology used, a glossary (Section VI) is included. Abbreviations used in this chapter are listed below.

Abbreviations

ADP	adenosine diphosphate
ADP-R·	adenosine-5′-diphosphate-4-(2,2,6,6-tetramethyl-4-phosphopiperidine-1-oxyl)
AP	alkaline phosphatase
BGGP	benzoylglycylglycyl-L-phenylalanine
BGP	benzoylglycyl-L-phenylalanine
BovCA, BCA	bovine carbonic anhydrase
BovCAB	bovine carbonic anhydrase, B isozyme
CA	carbonic anhydrase
CD	circular dichroism
CDTA	cyclohexenediaminetetraacetic acid
CGP	carbobenzoxyglycyl-L-phenylalanine
CPA	carboxypeptidase, isozyme A
dipy	2,2′-dipyridyl
DNA	deoxyribonucleic acid
ESR	electron spin resonance
FAD	flavine adenine dinucleotide
HCAB	human carbonic anhydrase, B isozyme
HCAC	human carbonic anhydrase, C isozyme
HPLA	benzoylglycyl-L-phenyllactate
LADH	liver alcohol dehydrogenase
MCA	metal-substituted carbonic anhydrase
MCD	magnetic circular dichroism
NADH, NAD^+	the β-isomer of nicotinamide adenine dinucleotide, reduced and oxidized forms, respectively
NMR	nuclear magnetic resonance
NTA	nitrilotriacetic acid
ORD	optical rotatory dispersion
RNA	ribonucleic acid
tren	2,2′,2″-triaminotriethylamine
tris	tris(hydroxymethyl)methylammonium
UV	ultraviolet
YADH	yeast alcohol dehydrogenase

Abbreviations for amino acids are to be found in Section VI.

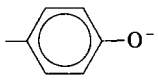
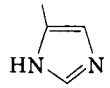
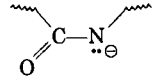
B. Zinc Coordination and Metalloenzyme Function

Amino acid side chains with donor groups are potential binding groups for zinc in metalloproteins, and the most likely candidates are shown in Table I.

Three amino acid side chains, those of His, Glu, and Cys-SH, are often concerned in zinc metalloenzymes in general and in the three we

TABLE I

DONOR GROUPS OF AMINO ACID SIDE CHAINS

	O-donors	N-donors	Heteroatom donors
Asp	$-CH_2COO^-$	Lys—$(CH_2)_4NH_2$	Cys —SH
Glu	$-(CH_2)_2COO^-$	Arg-$(CH_2)_3$—NH—C(NH_2)(NH_2^+)	
Tyr		His 	
		N-terminal α-amino acid —NH_2	
		Deprotonated peptide 	

consider here. Thus CA has three His imidazole ligands, ADH has two SH groups of Cys-SH ligands and one His ligand, and CPA has two His ligands and one carboxy from a Glu residue. In all three, a further coordination site is occupied by a water molecule, the acid dissociation of which, as we shall see, has a marked effect on the pH behavior of the system.

Studies of simpler complexes of ligands having the same donor groups and metal ion as those of the active center in metalloenzymes are numerous and can be helpful in revealing possible new chemistry that the complex has in common with the metal center of the enzyme. Such studies can lead to the discovery of novel reactions that mimic those found in the modeled enzyme, although these reactions usually differ in substrate specificity and mechanism from those of the enzyme (see, for example, *12–14*, and references therein). However, a key feature of metalloenzyme catalysis is the concerted action of a metal ion with other noncoordinating groups of the protein, and the synthesis of such a "pendant active group" model is usually difficult. Also, if the model is too simple it will tend to adopt a metal-controlled stereochemistry, whereas in the metalloenzyme it is now clear that the stereochemistry is dictated by the ligand geometry, a fact that can, for example, have profound effects on the affinity of the protein ligand for various metal ions. The bond lengths and angles at the multidentate metal coordination site in enzymes are dictated as much by the stereo-

chemistry of the relatively inflexible protein ligands as by the electron distribution of the metal ion (*15*).

Furthermore, models are often studied in aqueous solution, whereas the metal ion with its coordinating groups in an enzyme is usually in a cleft in the protein structure, the environment of which more closely resembles an oil drop than water; the cleft itself may also consist of regions of greater and lesser polarity. These are profoundly important features (*16*), where the substrate is attacked by a charged or partially charged nucleophile at the active site. It is significant that catalytically active metalloproteins have highly distorted and unusual coordination geometries; there has been much interesting discussion on the influence of such distortion on catalytic activity (*17*), but there seems to be little doubt that strain induced by distortion is intimately connected with activity.

Again, thermodynamic behavior, which appears unusual in terms of the behavior of simple complexes, is found with metalloenzymes: highly purified zinc metalloenzymes, for which the relative affinities of several first-transition series and group IIB metal ions for the metal binding site have been determined, show a remarkable preference for zinc compared with cobalt(II), nickel(II), and copper(II), although the sites are mostly made up of N,O ligands that in simple models have greater affinity for the other ions. Sulfur ligands also might be expected from models to have an especially high affinity for zinc (*18*, *19*) and to be a common ligand. Such is not the case, and His, for instance, is a more common ligand than Cys–SH.

It is natural to ask whether the metal induces in the protein during its assembly the particular configuration of groups necessary to give the unusual stereochemistry of the active site. If so, the metal ion would have to be present during the synthesis of the ligand, i.e., the enzyme apoprotein. This point has been investigated in some detail for *Escherichia coli* alkaline phosphatase (AP). The apoenzyme forms readily in a zinc-free medium: it has physicochemical characteristics identical with those of the apoenzyme formed by zinc removal from *E. coli* AP and is completely reactivated by the addition of zinc (*20*). This is firm evidence that the presence of the Zn(II) ion is not necessary for the synthesis of the apoprotein. An intriguing finding is that *E. coli* will synthesize AP containing Co(II), Cu(II), and Cd(II) if these metals are added to the growth medium, but Zn(II) will displace these ions, and it does so at relative concentrations well below those expected for competitive binding to analogous model complexes (*20*). Clearly the configuration of active site groups imparts a high and specific affinity for zinc. The reason for this is again the subject of speculation, but,

like all complex formation processes in solution, the free energy of formation will be composite and contain differences between large quantities, e.g., the hydration energies of the ions and the total binding energy of the metal ion to the active site ligands. It is noteworthy that entropy changes are important in the control of affinity and can be overwhelmingly so; thus, the binding of Zn^{2+}(aq) to the apoenzyme of CA is accompanied by an enthalpy *increase* that is more than offset by a very large entropy increase, the entropy increase dominating the affinity of the apoenzyme for Zn(II) (see below). In a very real sense mammalian respiration is allowed to occur by courtesy of entropy.

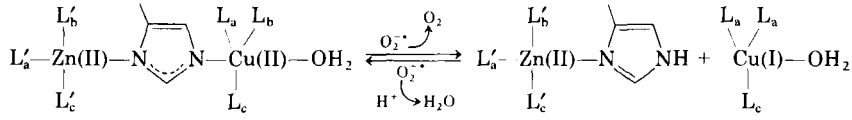
C. Characterization of Zinc(II) Metalloenzymes

Enzymes that require ions for activity are divided operationally into two classes: metal-activated and metalloenzymes, a division—somewhat arbitrary—determined by the tightness of binding of the metal ion to the protein (*21*, *22*). A zinc metalloenzyme is assigned to the latter class if it satisfies certain criteria first put forward by Vallee (*21*, *21a*). These criteria avoid ambiguities found with inhibition and activation studies and include the demonstrations of (a) an increase in the metal: protein ratio as the enzyme is purified and (b) a direct correlation between specific activity and metal content for the isolated homogeneous protein. If these requirements are met, then the metal ion is tightly bound to the protein. For several metal–protein complexes that may be classified as metalloenzymes the dissociation constant of the complex is found to be of the order of 10^{-10} M or less. As Chlebowski and Coleman (*15*) have pointed out, the definition means that the binding constant of the metal for the protein site is large enough to ensure that the metal is carried with the protein through the isolation procedures. Enzymes meeting the metalloenzyme criteria have been much easier to work with and have been the more intensively investigated. Since the stability of metal–protein complexes may be expected, like the stabilities of model coordination complexes, to cover a wide range, many important enzymes may be Zn(II) activated in that the metal is bound loosely enough to dissociate, at least partially, during isolation procedures. As well as analytical difficulties there is also the problem that, with such systems, activation may not be specific for a given metal ion; it may not, even in some Zn(II) metalloenzymes (see Sections II,C,1, III,C, and IV,A), and it may then be difficult to determine which, among the several divalent ions [Mg(II), Ca(II), Mn(II), Fe(II), Cu(II), and Zn(II)] occurring in significant concentration in biological systems, is the natural activator. Some forty or so enzymes have been

listed that are activated by Zn(II), or are believed to contain zinc, but have not yet had the rigorous metalloenzyme criteria applied to them [see, for example, Chlebowski and Coleman (15)].

D. Range of Reactions Catalyzed by Zinc(II) Metalloenzymes

Nine of the well-characterized Zn(II) metalloenzymes catalyze hydrolysis or hydration reactions. In these cases, acid–base catalysis is probably involved and the Zn(II) can be pictured as functioning in its capacity as a Lewis acid. Two of the zinc metalloenzymes catalyze oxidation reactions, alcohol dehydrogenase (see Section III), and superoxide dismutase. The function of the zinc ion in these proteins is, at first sight, different, and it may at first seem strange that zinc should have a catalytic role in a redox process: the chemistry of Zn(II) certainly does not suggest a role for it as an electron acceptor. The hydride (or 2-proton plus 2-electron) shift in the NAD^+-dependent dehydrogenases is to the C-4 position of the nicotinamide ring of the coenzyme (see Fig. 14), and we note that many NAD-dependent dehydrogenases are not Zn(II) enzymes. The Zn(II) may function by inducing the proper positioning or polarization of the substrate with respect to the coenzyme, although it is not necessary for coenzyme binding (see Section III,D). Superoxide dismutase* is also a copper metalloenzyme and catalyzes an apparently diffusion-controlled oxidation of the superoxide radical ion at the Cu(II) ion center of this enzyme (*23*). There is evidence that an imidazole ring of a histidine bridges the Zn(II) and Cu(II) centers (*23a*), and a rapid protonation and deprotonation of the bridging group takes place (*24*, *24a*) when it reacts with the O_2^- ion with change in oxidation state of the copper:



L_a , L_b, L_c, L'_a, L'_b = His; L'_a = Asp (24a–26)

Proton NMR studies show that 4–6 histidine imidazoles are ligands for the metals (*25*), and histidine modification studies suggest that the zinc function here is to organize the structure of the active site (*26*).

* This enzyme catalyzes the conversion of O_2^- into molecular oxygen and hydrogen peroxide. It is found in most, if not all, organisms that use oxygen.

Zinc metalloenzymes also catalyze group-transfer reactions. Mechanisms for these may be postulated in which Zn(II) withdraws electrons from a group on the substrate by forming a coordinate bond in a mixed enzyme–metal–substrate complex. An interesting case is that of yeast aldolase, in which Zn(II) polarizes the carbonyl (and phosphoryl group) of the substrate $CH_3 \cdot C(O) \cdot CH_2O(PO_3H_2)$, facilitating substrate deprotonation and enolization and thus substituting for the positive lysyl-NH_3^+ group involved in the *non*metal mammalian aldolases (*27*). On the other hand, the Zn(II) ion in aspartate transcarbamylase has proved not to be the substrate binding site, but is located in the regulatory subunits (*28*). It seems clear that zinc may have a role both as an acceptor (Lewis acid) at an active site and in maintaining structure as well.

We shall now look for other subtleties in the function of zinc as we consider the examples of three zinc metalloenzymes—carbonic anhydrase, the alcohol dehydrogenases LADH and YADH, and carboxypeptidase A. Carbonic anhydrase and its isozymes are considered in rather more detail than the others to illustrate broadly how physical techniques are used to gain insight into the metal function and its role in the overall picture of the enzyme mechanism. The other enzymes will be considered more briefly, with emphasis on metal coordination and its relation to function.

II. Carbonic Anhydrase

A. Introduction

Meldrum and Roughton in 1933 isolated from blood an enzyme that catalyzed the reversible hydration of carbon dioxide; they named it carbonic anhydrase (EC 4.2.1.1, carbonate hydro-lyase) (*29*). The enzyme is present in most organisms, and is found in many different tissues of plants and animals (*30*). The literature concerned with the chemical and physiological aspects of carbonic anhydrase has been reviewed up to 1971 (*31–34*), and comprehensive reviews dealing with more biochemical and kinetic detail than we shall consider here are available (*15*, *35*). The enzyme is involved in a variety of physiological functions, including photosynthesis, calcification, pH maintenance, ion transport, and CO_2 exchange, where its specific catalytic role is the interconversion of carbon dioxide and bicarbonate (*30*) at a rate which is one of the fastest known for enzymic catalysis.

The most widely studied carbonic anhydrases are those from human and bovine erythrocytes, the enzymes being the major protein component after hemoglobin. The mammalian enzymes consist of a single-

chain protein of molecular weight about 30,000, each chain coiling and forming a ligand binding a zinc ion that is essential for catalysis (*36*).

There are two isozymes of carbonic anhydrase, designated B (or I) and C (or II), in the erythrocytes of most mammalian species that have been studied (*37–40*). Reports have appeared of further distinct variants (*41–44*), but we shall be concerned here with the human carbonic anhydrases B and C, and with the bovine isozyme B (although labeled B, in its activity this isozyme belongs to class C).

The isozymes are antigenically distinct; compared with the B form, the C isozymes generally show higher specific activities, higher thermal stabilities (*45, 46*), different affinities for inhibitors (*44*), different chemical reactivity toward modifying agents (*43, 47–49*), a lower abundance in the red cell (*50*), different rates of biosynthesis and a difference in physical properties, some of which are shown in Table II. These differences indicate that each isozyme has a different physiological role, and this is discussed elsewhere (*33, 51*).

B. Structure

1. General Features

Studies of the complete primary structures of the human B (*53–56*) and C (*57–59*) isozymes and of the bovine B isozyme (*60*) show that each isozyme has a distinct amino acid composition. HCAB consists of 260 amino acid residues, and HCAC has 259 residues. It is interesting that the 60% sequence homology suggests that the two isoenzymes arose through gene duplication and subsequent independent evolution some 150 million years ago, before the divergence of marsupials, but after the divergence of birds, from the mammalian line (*30*). It is interesting also that carbonic anhydrase has evolved to a stage of maximum catalytic efficiency (see Section V).

2. Structure of the Isozymes and the Coordination of Zinc

The complete structures of the human isozymes B and C have been deduced from high-resolution X-ray studies on enzyme crystals obtained from 50 m*M* tris–sulfate buffer solutions, pH 8.5 (*61–65*). The tertiary structures of the two isozymes are very similar.

The enzymes are ellipsoids of dimensions 4.1 × 4.2 × 5.5 nm, and the zinc ion is near the center of the molecule at the bottom of a 1.2 nm-deep conical cavity, which is 2 nm wide at the mouth (*66*); the general

TABLE II

COMPARISON OF THE CARBONIC ANHYDRASE ISOZYMES FROM HUMAN AND BOVINE RED BLOOD CELLS

Source	Activity type	CO_2 turnover number (mol/min[a] $\times 10^6$)	Molecular weight[b]	Isoelectric point	Molar absorptivity$_{280}$ $\times 10^{-4}$ mol^{-1} cm^{-1}	Relative abundance
Human erythrocytes	B, low	1.2	28,800	5.85	4.69	7/1
	C, high	36	29,300	7.25	5.47	
Bovine erythrocytes	A, high	30	30,000	—	5.7	1/4
	B, high	30	30,000	5.65	5.7	

[a] pH 7, 25°C (*34*, *52*).

[b] The molecular weights for the human isozymes were calculated from the amino acid sequences.

features of this cavity have been confirmed by spin-label probe techniques (*66–68*). A large region of rigid β-structure extends through the center of the molecule, and the zinc ion is bound to this through the nitrogen atoms of three histidine imidazole ligands (His 94, 96, and 119); this is consistent with the results of both proton NMR (*69, 70*) and ESR (*71*) studies.

The zinc ion has distorted tetrahedral coordination (*72*), the fourth ligand being a water molecule or a hydroxide ion (Table III); the largest deviation from a tetrahedral angle is 20°.

TABLE III
ZINC LIGANDS IN HUMAN CARBONIC ANHYDRASE C (*72*)

Ligand	Zinc–ligand distance (Å)
His 94 (3'N)	2.4
His 96 (3'N)	2.0
His 119 (1'N)	2.0
H_2O/HO	1.9

The coordination by His 94 is unusual; of the three coordinating histidines, it has the largest electron density and the position of this imidazole ring varies between two extremes (cf. Section II,H), which differ by 0.07 nm (the shortest Zn–N distance is 0.24 nm) and is also the most exposed ligand—features that may be important in the reaction mechanism (*72*).

Details of the active site cavity are shown in Fig. 1. The cavity can be divided into hydrophobic and hydrophilic regions. Threonine 199 in HCAC is hydrogen-bonded to the metal-bound water and interacts with most inhibitors. Apart from the histidine ligands, other residues in the active site that have not changed during evolution are Thr 199, Pro 201, Pro 202, His 64, Gln 92, and those involved in a hydrogen-bonded sequence: His 119–Glu 117–His 107–Tyr 194–Ser 29–Trp 209 (Figs. 1 and 2); these residues may be important also in the catalytic mechanism; for example, it has been suggested that the above hydrogen-bonded sequence may be a charge (proton) relay system of the kind that has been suggested for chymotrypsin and the serine proteases (*73*).

There are 11 amino acid differences between HCAC and HCAB, at the active site (*66*); for example, Tyr 202 is oriented away from the cavity in HCAB serving to make the cavity more open than in HCAC, where the residue is Leu 202. However, the region close to zinc is more

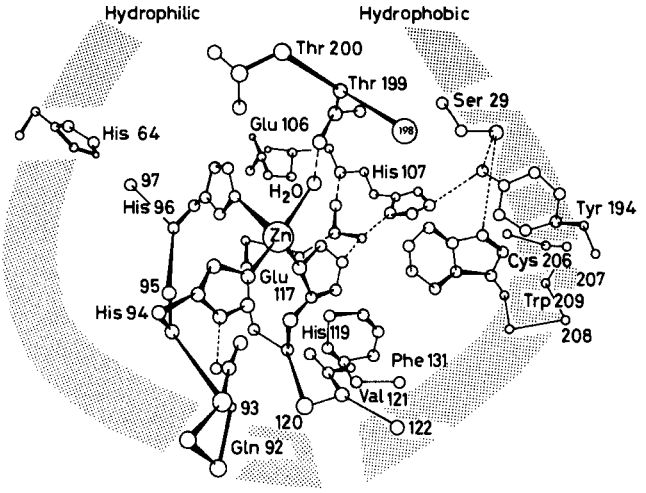


FIG. 1. Part of the active site of human carbonic anhydrase C (*35*).

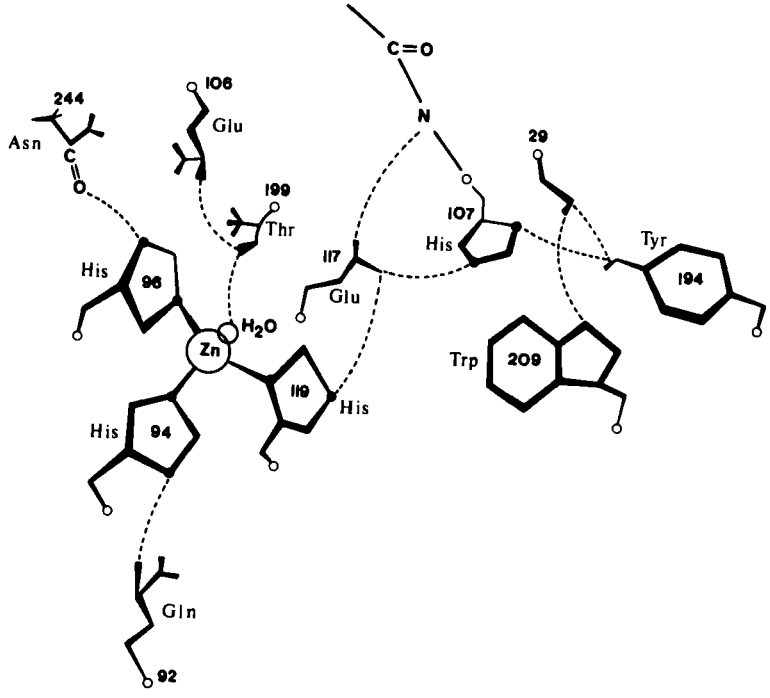


FIG. 2. The hydrogen bond network at the active site of human carbonic anhydrase B. [Redrawn from Wyeth and Prince (*35*) and Nostrand *et al.* (*63*)].

restricted in HCAB, owing to the substitution of Asn 67, Val 121, and Thr 200 in HCAC for His 67, Ala 121, and His 200 in HCAB; these differences may be connected with the lower catalytic efficiency of the B isozyme.

There are three pronounced aromatic clusters that may, inter alia, have a structural role, maintaining the stability of the active site cavity. The existence of a hydrophobic region is probably also important in determining catalytic activity. Although refinement of the crystal structure has led to the disappearance of what was once thought to be an icelike arrangement of water molecules within the active-site cavity (*62*), γ–γ correlation spectroscopy has recently indicated that, as might be expected, water at the protein–solution interface is highly ordered (*74*). It appears also that there remains sufficient space within the cavity for an icelike structure to exist; since proton transfer is more rapid through ice than through water, such a structure could provide a device for efficient proton transfer, a point to which we shall return later.

Although protein crystals differ from those familiar to inorganic chemists, such crystals are usually spongy and bathed in buffer, a cautionary note, not unfamiliar to inorganic chemists, must be sounded about the relevance of the structures of such crystals to the enzyme conformation in solution; X-ray resonance absorption measurements have indicated that a slow conformational change may occur upon dissolution (*75*) and a number of other cases are known where the behavior of the protein crystal is different from that of the protein in solution (*76*).

C. Metallocarbonic Anhydrases

1. Metal Substitution Studies

The zinc ion in carbonic anhydrase, although tightly bound (*77*), can be removed at low pH. The half-time for the metal dissociation is 80 hours for HCAC and 30 hours for HCAB at pH 5.3, 4°C, in the presence of 20 m*M* dipyridyl (*78*). While the apoenzyme undergoes no gross structural changes relative to the holoenzyme, it is catalytically inactive; the metal also confers conformational stability (*79*).

The apoenzyme can be reconstituted with other divalent metal ions, and these occupy the zinc site (*34*); significant restoration of catalytic activity is brought about only by Zn(II) and Co(II)* (*32*) (Table IV) (*80*);

* Recently the Cd derivative has been shown to have some activity (*79a*).

TABLE IV

RELATIVE ACTIVITIES OF SOME M(II)-SUBSTITUTED HUMAN B AND C CARBONIC ANHYDRASE DERIVATIVES[a]

Metal ion derivative	Relative CO_2 hydration activity	Relative rate of *p*-nitrophenylacetate hydrolysis
Apoenzyme	1.3	1.2
Mn(II)B	1.3	5
Co(II)B	20	120
Co(II)C	55	100
Ni(II)B	2	4.5
Cu(II)B	0.4	6.9
Zn(II)B	30	37
Zn(II)C	100	100
Cd(II)B	1.4	2.7
Hg(II)B	0.02	1.2

[a] Modified from Dunn (*80*).

despite this, a number of investigations have concerned the Mn(II)- and Cu(II)-substituted enzymes, and it should be stressed that data obtained from these experiments may not be relevant to the native enzyme. The relative inactivity of these metallocarbonic anhydrases may be due to several factors, including changes in coordination geometry, rates of ligand exchange, and coordination flexibility.

In all carbonic anhydrases, the catalytic activity and other processes related to the active site appear to be controlled by a group titrating around neutrality; the apparent pK_a of this group in the low-activity isozyme B is about 1 unit higher than that in the high-activity variant (*32*). The introduction of cobalt rather than zinc does not cause a large change in the pK_a of this group (*81–83*); this substitution also has a negligible effect on the UV optical rotatory dispersion curves of the bovine (*84*) and human B (*85, 86*) enzymes, and on the ESR spectra of spin-labeled sulfonamide inhibitors bound to HCAB (*34*). This suggests that Co(II) and Zn(II) coordination is similar. However, substitution with cobalt does appear to induce some changes at the active site of HCAB (*87*); e.g., marked changes in the pH-dependence of anion binding at low pH are observed.

Cobalt(II) is the most valuable of the substituting ions because it serves as an ESR, optical spectral, and nuclear resonance probe as well as a relaxation probe for nuclei in its environment. The visible absorption and MCD spectra of Co(II) BovCAB are shown in Fig. 3; the pH dependence of the spectra reflects the ionization of the activity-linked group. The MCD spectra are particularly useful since they are

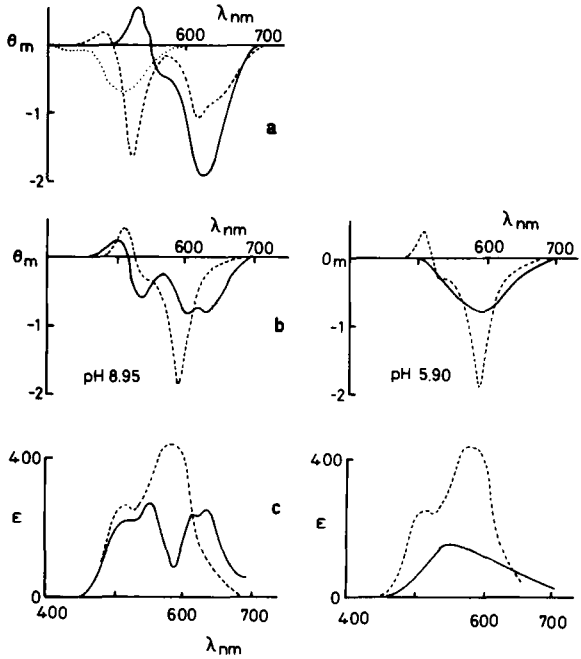


FIG. 3. (a) The magnetic circular dichroism (MCD) spectra of some Co(II) complexes of known geometry: ····, $Co(H_2O)_6^{2+}$ octahedral; ----, $Co(Me_6tren)Br_2$ pentacoordinate; ———, $Co(OH)_4^{2-}$ tetrahedral. (b) The MCD spectra and (c) the optical spectra of Co(II) BovCAB at pH 8.5, 5.9, and in the presence of 1.5 equivalents of the sulfonamide inhibitor acetazolamide (– – – –). Enzyme concentration = 2.5×10^{-4} *M*. MCD spectra were measured at 40 kG. [Reproduced from Wyeth and Prince (*35*), with permission.]

less sensitive to coordination distortion and give a clear indication of the cobalt coordination geometry. The metal is tetrahedrally coordinate at low pH and probably pentacoordinate at alkaline pH (*88*); the addition of the sulfonamide inhibitor acetazolamide again appears to induce tetrahedral coordination. Magnetic susceptibility data are in accord with this interpretation of the coordination geometry of high-spin Co(II) (*89*); however, the analysis appears to be at variance with X-ray crystallographic results for the zinc enzyme, which show a tetrahedrally coordinated zinc ion in enzymes crystallized at pH 8.5.

2. *Binding of Zinc to the Enzyme Protein*

The kinetics and thermodynamics of the binding of zinc to the carbonic anhydrase apoenzyme have been investigated (*91*, *92*), and Table V shows the second-order rate constants, activation parameters,

TABLE V

SOME RATE AND EQUILIBRIUM PARAMETERS FOR THE REACTION Zn^{2+} + LIGAND AND M^{2+} + apoCA → MCA[a]

	k (1 mol^{-1} sec^{-1})	ΔH^* (kcal/mol)	ΔS^* (e.u)	ΔH (kcal/mol)	ΔS (e.u.)
Zn typical small unidentate	10^{7-8}	—	—	—	—
Zn typical small bidentate (bipyridyl)	10^6	+6	−9	−10	+8
Zn typical small tridentate (terpyridyl)	10^6	+8	−4	—	—
Zn apoCA, pH 5.5	$<10^4$	+21	+28	+3.9	+61
Zn apoCA, pH 7.0	10^4	+21	+30	+9.8	+88
Co apoCa, pH 7.0	—	—	—	+9.4	—
Ni apoCA, pH 7.0	—	—	—	+3.2	—
Cu apoCA, pH 7.0	—	—	—	+3.4	—
Cd apoCA, pH 7.0	—	—	—	+4.4	—
Zn CDTA (cyclohexenediamine tetracetate)	—	—	—	+4.7	+82

[a] Data from Henkens and Sturtevant (*91*) and Henkens *et al.* (*92*).

and thermodynamic constants of binding. They are compared with those for smaller ligands giving complexes of various coordination numbers. Both rate process and equilibrium are entropy-dominated, in spite of a smaller opposing enthalpy. The entropy terms are substantial, and the only ligand comparable in this respect is CDTA, a formally hexadentate ligand. While it is most unlikely that apocarbonic anhydrase is a hexadentate ligand, the similarity between carbonic anhydrase and CDTA probably has the same origin: the expulsion of a large number of water molecules. The effect in the case of CDTA is due to charge neturalization, and in carbonic anhydrase the binding of the metal may also result in charge neutralization in the active site region.

Further evidence for this may be cited [see literature quoted in Henkens *et al.* (*92*)]: the difference UV spectrum of ZnCA minus apoCA is similar to the difference spectra produced when a tyrosine or a tryptophan derivative is transferred from water to a less polar solvent. This similarity suggests that one or more tyrosine and/or tryptophan groups (residues 194, 209 perhaps?) are in a more polar environment in the apoenzyme than in the holoenzyme. Other workers concluded on the basis of studies with fluorescent sulfonamides that the active site of the native enzyme is nonpolar. It is interesting to note that structural work on the holoenzyme shows the active site to possess a polar and nonpolar environment (cf. Fig. 1).

The positive binding enthalpy may well indicate a small coordination number, so that during the reaction more M—L bonds are broken than formed, as expected if the zinc is three or four coordinate (aquated zinc is hexacoordinate). It is interesting that the metals zinc and cobalt, whose binding gives an active enzyme, are those with the most endothermic binding to carbonic anhydrase. Dennard and Williams (*93*) have pointed out that a catalytically active metal must not bind too strongly to its apoenzyme or it will lose coordination flexibility.

D. Catalytic Properties of Carbonic Anhydrase

Carbonic anhydrase, *in vitro*, catalyzes a range of reactions in which nucleophilic attack of oxygen at an electrophilic center occurs; these include the hydration of carbon dioxide and some aldehydes and the hydrolysis of some carboxylic, sulfonic, and carbonic esters (*94–96*). The reaction of importance in biological systems is the reversible hydration of CO_2. One may expect that, if there exists among a variety of possible mechanisms for the catalysis of a given reaction one that is particularly effective, then an enzyme which also catalyzes that reaction may, in the course of evolution, have incorporated it. We shall therefore first consider some general aspects of the catalysis of the carbonyl hydration reaction to see what features are shown by an effective catalyst.

1. Some General Features of the Catalysis of Carbonyl Hydration

A number of studies have been made of the catalysis of CO_2 hydration (*93, 97, 98*), and several interesting features are revealed. The reaction has been shown not to be proton-catalyzed by studies of ^{18}O exchange between gaseous CO_2 and water in acidified solutions. Purely general base catalysis can be rejected because the Brønsted dependence on acid dissociation constant is not observed, e.g., $(CH_3CO)_2CH^-$ is not catalytically active whereas the ion $AsO_3H_2^-$ is active although they both have about the same pK values, 9.18 and 9.26, respectively. The anions germanate, arsenite, sulfite, selenite, tellurite, and tellurate are active whereas phosphite, phosphate, arsenate, sulfate, and selenate have very small or no catalytic activity. The essential feature appears to be that the most effective catalysts have a *dual* Lewis acid–proton-acceptor function, suggesting a mechanism of the kind shown in Fig. 4, where X—O is part of the catalyst structure. It is interesting in this context that hypohalites are very effective catalysts; e.g., Caplow (*99*) has found that HOBr is a powerful

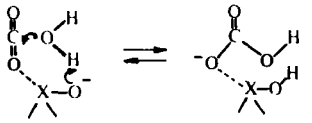


FIG. 4. Lewis acid-Brønsted base features of the most effective catalysts of CO_2 hydration.

catalyst of the dehydration reaction and concluded that a concerted electrophilic displacement occurred:

$$HO—Br + HO—CO_2^- \rightarrow HO^- + Br—OH + CO_2$$

The bromine atom is here acting as the Lewis acid, X.

Table VI summarizes data on the catalytic efficiency of various catalysts for CO_2 hydration (*99a*).

TABLE VI

EFFICIENCIES OF VARIOUS CATALYSTS IN CO_2 HYDRATION[a,b]

Catalyst	Efficiency (mol^{-1} sec^{-1})
$H_2AsO_3^-$	0.9
$HAsO_4^-$	0.0002
BrO^-	2
SeO_3^{2-}	1.2
SO_3^{2-}	0.2
Cu(glycylglycinate)OH	20
$ZnCROH^{+c}$	25
Carbonic anhydrase	~ 10,000

[a] From Woolley (*99a*).

[b] If the forward reaction has velocity constant k, then efficiency is defined as dk/d [catalyst] (*99a*). The anions NO_2^-, NO_3^-, CO_3^{2-}, SO_4^{2-}, and the complexes MX_n, where M = Mn, Fe, Co, Ni, Cu, Zn, and X = EDTA, NTA, dipy, Cys, mercaptoethylamine, and CN^- have zero efficiency.

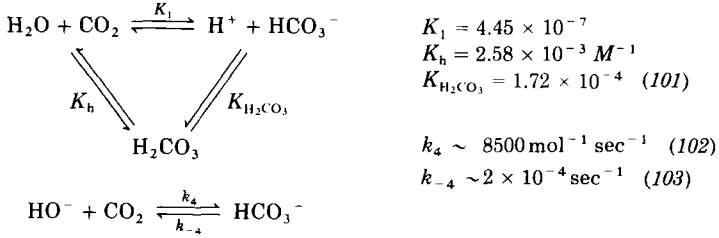
[c] The structure of CR is shown in Fig. 6.

Several studies have been made of carbonyl hydration reactions; of particular interest are the hydrations of pyridine 2-aldehyde and pyridine 4-aldehyde using Zn^{2+}aq and Co^{2+}aq solutions and the Zn^{2+}- and Co^{2+}-activated bovine CA (*124*, *125*). The enzyme is about 10^8 more effective than water for both 2- and 4-aldehydes; Zn^{2+} and Co^{2+} are about 10^7 times as effective as water in the case of the 2-aldehyde, but much less effective for the 4-aldehyde. This suggests that there is

no pyridine nitrogen-to-metal coordination in the enzyme, and the metal in the enzyme either directly polarizes the carbonyl group or does so through a bridging water molecule.

2. *The Enzyme-Catalyzed Reversible Hydration of CO_2*

In the absence of a catalyst the hydration proceeds at a relatively slow rate (0.037 sec^{-1}, 25°C) (*34*), and a catalyst is therefore necessary in the capillary circulation of mammals, where the rapid transfer of CO_2 is essential (*90*). In the absence of a catalyst the reversible hydration can be written as shown in Scheme 1 (*101–103*).



SCHEME 1

The enzyme accelerates CO_2 hydration by a factor of 10^7 at neutral pH. The carbonic anhydrases follow a simple Michaelis–Menten behavior with respect to both CO_2 hydration and HCO_3^- dehydration; the Haldane relationship is also obeyed. Some selected values of the Michaelis parameters are given in Table VII (*104–106*).

TABLE VII

MICHAELIS PARAMETERS FOR THE CARBONIC ANHYDRASE-CATALYZED REVERSIBLE HYDRATION OF CARBON DIOXIDE (*104–106*)

		CO_2 hydration		HCO_3^- dehydration	
Isozyme	pH	k_{cat} (sec^{-1})	K_m (m*M*)	k_{cat} (sec^{-1})	K_m (m*M*)
Zn(II) HCAC	7.05	6.2×10^5	14	3.7×10^5	68
Co(II) HCAC	7.05	2.6×10^5	5.9		
Zn(II) HCAB	7.05	1.5×10^4	2.6	2.3×10^4	32
Co(II) HCAB	7.05	9×10^3	1.8		
Zn(II) BovCA	7.05			8.9×10^{6a}	
Co(II) BovCA	7.05	1.7×10^5	2.4		

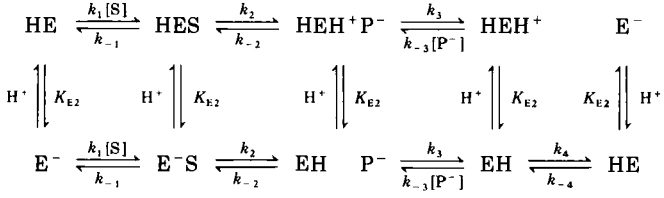
[a] k_{cat}/K_m mol^{-1} sec^{-1}.

We have seen that a group in the active site with a pK_a near neutrality controls the catalytic activity, so that hydration requires the basic form and the rate of hydration increases with pH and that of dehydration decreases. The pH–rate profile takes the form of a simple sigmoid titration curve in the case of HCAC (*35*). We may note that although BovCAB and HCAC are kinetically similar in some respects (*107–109*), for the B isozyme the situation is more complicated because the state of ionization of additional groups appears to influence the rate (*107*), and this has been confirmed by bromoacetate binding studies (*110, 111*).

These studies use the fact that $BrCH_2COOH$ carboxymethylates His 200, which is near the zinc ion. (The carboxymethylated HCAB has its own CO_2-hydration and esterase activity, 2.5–20% that of unmodified HCAB, depending upon substrate). When 90% ^{13}C-labeled bromoacetate is used, only one ^{13}C-NMR peak not found in HCAB or in unenriched carboxymethylated HCAB is found: therefore the ^{13}C label is functioning as a probe. The probe signal shifts with pH, and the shift-pH data can be fitted to a curve with two pK_a's of 6.0 and 9.2, but not to a curve with a single pK_a. The former value could be due to the imidazole side chain of His 200, and the latter to a zinc-bound water molecule.

It is interesting that modification of His 200 may also increase the rate of exchange of anions at zinc (*133*).

This is discussed in detail elsewhere (*35*) but, to summarize, a general mechanistic scheme (Scheme 2) (*78, 112, 113, 115*) assumes that two ionizing groups are present. One of these is the catalytically active group, and the enzyme with this group protonated is represented as EH; the other ionizing group is responsible for transferring protons between the solution and the catalytically active group: the enzyme with this group protonated is represented as HE.



SCHEME 2

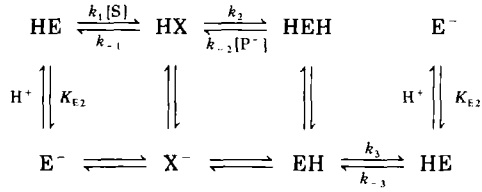
This scheme replaces an earlier one (*116*) which is unable to account satisfactorily for the pH dependence of the Michaelis parameters.

The scheme also assumes that the basic form of the catalytic group is required for the binding of CO_2, and the acidic form for the binding of HCO_3^-; however, product-inhibition studies indicate that CO_2 does bind weakly to the acidic form (*113*).

An important feature is that proton transport occurs in two stages; there is an intramolecular transfer between the two active-site groups, and the transfer to solvent, which is necessarily buffer mediated (*117–119*), occurs independently of the rest of the catalytic cycle (*112*). At sufficiently low buffer concentrations, this latter step limits the rate of the catalyzed reaction (*115, 120, 121*), and there is also some buffer dependence for $k_{cat\,max}$; this may indicate that the proton-transfer group represents a specifically bound buffer molecule rather than an amino acid residue. In this connection a specific interaction of imidazole with HCAB has been noted (*69*). Indeed, the X-ray analysis of the HCAB–imidazole complex has been determined: the imidazole lies in a hydrophobic pocket binding directly to the metal at a fifth coordination site (*122*).

The analysis has been applied to the interpretation of the kinetics of reaction in ^1H_2O and ^2H_2O (*112, 113, 115*), of product inhibition, and of the results of a ^{13}C-NMR study (*78*).

The rate constants in Scheme 2 cannot all be obtained from accessible kinetic data, but the scheme may be simplified to give Scheme 3, where



SCHEME 3

again EH represents the protonated catalytic group (K_{E1}) and HE represents the protonated transfer group (K_{E2}) in Scheme 2. ES^- and EHP^-, the transitory complexes, are symbolized by X^-. While this is an oversimplification, rate constants and pK_a values can now be found that are in accord with all the experimental data (Table VIII).

We note that K_{E1}, the dissociation constant of the protonated catalytic group, is related to that of the proton transfer group and is given by: $K_{E1} = k_3K_{E2}/k_{-3}$. An interesting feature of the data in Table VIII is the large isotope effect on k_3 and to a lesser extent on k_{-3} corresponding to the proton transfers between the catalytically active group and the proton transfer group of the enzyme. The intramolecular

TABLE VIII

RATE CONSTANTS (± 20%) FOR THE HCAC-CATALYZED INTERCONVERSION OF CO_2 AND HCO_3^- AT 25°C IN 50 m*M* TRIS-SULFATE BUFFER[a]

Constant[a]	Solvent	
	^1H_2O	^2H_2O
k_1 ($mol^{-1}\,sec^{-1}$)	3×10^8	3×10^8
k_{-1} (sec^{-1})	2.5×10^6	2.5×10^6
k_2 (sec^{-1})	1.5×10^6	1.5×10^6
k_{-2} ($mol^{-1}\,sec^{-1}$)	3×10^7	3×10^7
k_3 (sec^{-1})	3×10^6	0.3×10^6
k_{-3} (sec^{-1})	0.7×10^6	0.2×10^6
pK_{E1}	6.9	7.5
pK_{E2}	7.5	7.7

[a] From Scheme 3 (*113*).

proton transfer step is not quite rate-limiting in ^1H_2O but becomes so in ^2H_2O.

3. *Hydrolytic Reactions*

Carbonic anhydrase, as well as catalyzing the hydration of the carbonyl group (*123–125*), can also function as an esterase (*126*), and a well studied substrate, used in activity assay, is *p*-nitrophenyl acetate (Table IX) (*44*, *78*, *106*, *127*). The pH-rate profile of *p*-nitrophenyl acetate hydrolysis is sigmoidal for BovCAB, HCAB, and HCAC, and there appears to be a close correspondence with the pH-dependence of the CO_2 hydration reaction (*32*). The complex behavior of HCAB becomes more marked during anionic inhibition of the esterase

TABLE IX

MICHAELIS PARAMETERS FOR *p*-NITROPHENYL ACETATE HYDROLYSIS AT 25°C

Isozyme	pH	k_{cat} (min^{-1})	K_m (m*M*)	k_{cat}/K_m ($min^{-1}\,mmol^{-1}$)	Reference
Zn(II) HCAC	8.1	1300 ± 100	8.7 ± 0.2	150	(*127*)
Co(II) HCAC	8.1			130	(*78*)
Zn(II) HCAB	8.2	150 ± 10	4.5 ± 0.2	33	(*127*)
Co(II) HCAB	8.2	200	4	50	(*127*)
Zn(II) BovCAB	7.0	7.8	1.4	5.6	(*106*)
	9.0	66	8.6	7.6	(*44*)

activity; there are at least two independent ionizing groups (*87*), one with pK_a 7.3 and another with pK_a 6.1, and the latter group affects only the *p*-nitrophenyl acetate K_m value. Linear free-energy relationships indicate that the hydrolysis mechanism is similar for Zn(II) HCAC and Zn(II) HCAB and probably also for Co(II) HCAB (*127*).

A biphasic pH-activity profile is observed in the case of long-chain and branched alkyl esters of *p*-nitrophenol (*128, 129*) (Fig. 5).

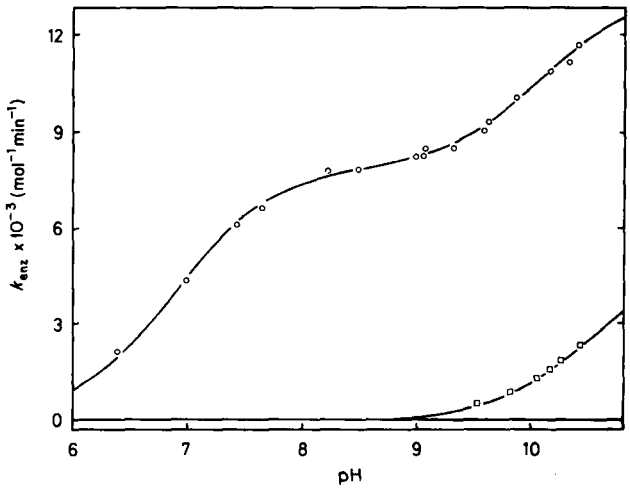


FIG. 5. pH dependence of k_{enz} for the enzymic hydrolysis of *p*-nitrophenyl propionate at 25°C. ○, BCAB; □, alkylated BCAB. k_{enz} is defined, for the ester hydrolysis in presence of buffer, B, by the equation

$$k_{obs} = k_o + k_{H_3O^+}[H_3O^+] + k_{OH^-}[OH^-] + k_{HB}[HB] + k_B \cdot [B^-] + k_{enz}[E]$$

Reproduced from Wells *et al.*, (*129*).

There are two points of inflection, one around neutrality, reflecting ionization of the active-site catalytic group(s), and the second above pH 10. A detailed analysis of this high-pH activity, which involved a study of acetazolamide-inhibited HCAB and mono- and dialkylated derivatives of HCAB, indicated that the second site, represented by the high-pH ionization, is outside the active site cavity (*130*). Other substrates hydrolyzed catalytically by carbonic anhydrase include methylpyridyl carbonates (*96*), *p*-substituted phenylesters (*131*), 3-pyridyl and nitro-3-pyridyl acetates (*132*) and mono- and disubstituted derivatives of carbonic acid (*96*).

E. The Activity-Linked Group at the Active Site

1. Possible Candidates

The results of modification studies have indicated that His 64, once implicated in the catalytic mechanism (*112*), is not the ionizable catalytic group since there is residual activity in HCAC carboxyketomethylated at His 64 (*134*). However, the group could be another active-site histidine (*135–137*). Another possibility is that the metal-bound water molecule ionizes to provide the necessary nucleophile in the form of zinc-bound hydroxide ion (*117, 119, 137, 138*) and recently a third candidate for the ionizable group was suggested, viz., the carboxyl group of Glu 106, which occurs in a hydrogen-bonded sequence: Glu 106–Thr 199–H_2O · Zn (*122*).

Detailed NMR studies on the protons of the histidine residues in zinc and cobalt HCAB (*69, 139, 140*) and HCAC (*70, 139, 140*) indicate that the catalytic group is Zn—OH_2 rather than an active-site histidine. However, His 64 may be the proton transfer group (*112*) so that in Schemes 2 and 3 EH could contain ≡ $ZnOH_2$ as the active group and HE could have His 64 as the proton transfer group. The pK_a derived from the NMR studies on HCAC is consistent with this (*70*), and the X-ray crystallographic results indicate that the residue is ideally set up for this role. In HCAB, however, the pK_a of His 64 is at least 2 units below that of the catalytic group, and this casts some doubt on its proposed role in proton transfer in this isozyme.

It has been suggested that the enzyme may employ two catalytic groups, depending upon the substrate; for example, the zinc-bound hydroxide could be active in CO_2 hydration, and an imidazole site could be active in ester hydrolysis (*141*).

2. Relaxation Measurements on Solvent Water

Observations of the increased relaxation rate of protons in the coordination sphere of a paramagnetic ion in a metallocarbonic anhydrase have allowed the determination of proton exchange rates and metal-to-proton distances (*142, 143*).

The results on the cobalt-substituted enzymes can be explained, approximately, in terms of an alkaline form of the enzyme, in which the cobalt binds a water molecule or a hydroxide ion, the protons of which have residence times $\ll$ 34 μsec or 17 μsec and distances of 0.285 nm and 0.25 nm from the metal, respectively; and in terms of an acid form of the enzyme in which, it was previously thought, the cobalt either has a bound water molecule, the protons of which have a residence time $>$0.2 msec (perhaps due to strong hydrogen bonding), or has no bound

group possessing exchangeable protons (*144*). Bertini and co-workers (*145*), however, have recently demonstrated the presence of these exchangeable protons. It is suggested that earlier studies may be in error owing to the presence of sulfate that interacts with the metal ion and reduces the proton exchange rate. More detailed observations also indicate that at least two ionizations with pK_a values between 6 and 8 are revealed in the relaxivity measurements on Co(II) BovCAB (*146*). The pH dependence up to pH 9 is similar to that of the enzymic activity. The data are inconsistent with the location of the exchangeable proton on a histidine ligand, but consistent with an ionizable metal-bound water molecule.

^{13}C and ^{18}O tracer studies of the carbon dioxide-bicarbonate interconversion and of the labeling of H_2O are consistent with NMR data (*120*); from the results of these experiments it was concluded that the metal-bound hydroxide has a long lifetime at low pH.

The sulfonamide inhibitors acetazolamide and ethoxazolamide (see Section F) abolish the proton relaxation enhancement, and this is also true for the monobromacetazolamide-alkylated Co(II)BovCAB. There is an increase in the relaxation enhancement at high pH (>9), which may imply a conformational change of the enzyme (*146*).

The results of the relaxation studies in the presence of Mn(II)-BovCAB can also be interpreted in terms of a metal-bound H_2O or OH^-. A peculiarity in these results is that while sulfonamides reduce the enhancement to the order of that observed at low pH values, the anions N_3^- and NO_3^-, which are also inhibitors of the native enzyme, have no effect. In this case the anions may enter a fifth coordination position, so that a metal-bound water molecule with exchangeable protons remains (*143*).

The protons of the water ligand could exchange by whole-molecule exchange, or by a cyclic proton-exchange mechanism. While the exchange rates of ^{17}O and ^1H of H_2O coordinated to simple transition-metal complexes are identical, indicating whole-molecule exchange (*147*, *148*), a complex possessing a bound water molecule with a low pK_a has not yet been studied, in this case the cyclic exchange mechanism may dominate.

3. An Ionizable Water Ligand

It is interesting that studies on model systems such as that shown in Fig. 6 demonstrate that the pK of the zinc-bound water may approach 7 if the coordination number of zinc in the complex is 4 or 5 (*99a*) (Table X) The metal-bound hydroxide has sufficient nucleophilic power to account for the enzyme's activity in acetaldehyde hydration, although

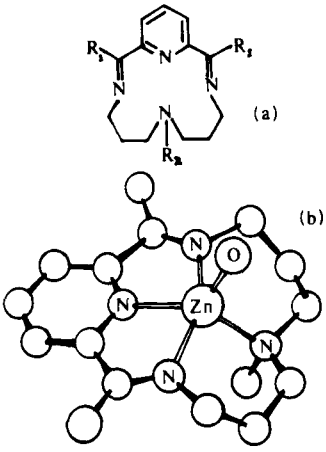


FIG. 6. Macrocyclic ligands based on 2,6-dicarbonyl pyridines. (a) The free ligand. (b) In the complex the four nitrogen atoms coordinate to a metal ion, Zn^{2+}, Cu^{2+}, Ni^{2+}, or Co^{2+}, which also binds a water molecule. The skeleton of the $Zn(N\text{-}MeCR)(H_2O)^{2+}$ complex is shown (H omitted; unlabeled atoms are C). CR: $R_1 = CH_3$, $R_2 = H$; N-MeCR: $R_1 = R_2 = CH_3$; *des*diMeCR: $R_1 = R_2 = H$ (*99a*).

not in carbon dioxide hydration *if* the zinc environment were the same in the enzyme as in CR complexes (*99a*, *149*); however, a poorly solvated nucleophile would, in a nonpolar environment, be more reactive (*141*), a point we shall return to later. Certain other inactive metallocarbonic anhydrases, e.g., those containing copper and vanadyl ions (*150*), show

TABLE X

IONIZATION OF WATER BOUND TO METAL IONS (M) IN MACROCYCLIC COMPLEXES[a]
pK_a's FOR THE PROCESS[b]: $M(ligand)OH_2^{2+} \rightleftharpoons M(ligand)OH^+ + H^+$

Ligand[c]	M	Temperature (°C)	pK_a	Approx. heat of ionization (kcal mol^{-1})
CR	Zn	25	8.69	7.1 ± 1
CR	Zn	0	9.17	
CR	Cu	25	11	
CR	Ni	25	11	
CR	Co	25	8[d]	
N—MeCR	Zn	25	8.12	8.3 ± 1
N—MeCR	Zn	0	8.68	
N—MeCR	Cu	25	11	
*des*diMeCR	Zn	25	8.13	6.0 ± 1
*des*diMeCR	Zn	0	8.53	
$(H_2O)_5$	Zn, Co	—	10	
Apocarbonic anhydrase	Zn, Co	—	7	

[a] From Woolley (*99a*).

[b] Aqueous solutions; ionic strengths in the range 0.002–0.04 *M*.

[c] Ligand nomenclature is given in Fig. 6.

[d] An approximate value only; cluster formation prevented a more accurate determination.

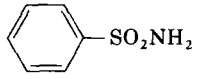
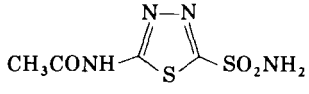
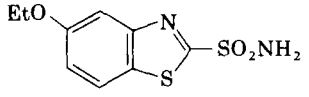
an ionization having a pK_a around neutrality and *provided that the active site structure of the two metalloenzymes does not differ significantly from that of the Zn holoenzyme* (which is not very likely), it must be shown that this does not represent a metal-bound water before the zinc-bound hydroxide nucleophile mechanism is accepted unequivocally (*99a*).

F. Inhibition by Sulfonamides

Sulfonamides are the most potent and selective inhibitors of animal and bacterial carbonic anhydrase (*31, 32, 151*), and inhibition by the sulfonamide acetazolamide (pK_I 7–8, Table XI) has become an accepted criterion for the active-site-directed nature of any process associated with the enzyme (*130*).

TABLE XI

Inhibition Constants of HCAC for Various Sulfonamides

Sulfonamide	K_I (M)	References
Benzene sulfonamide 	2×10^{-5}	(*153*)
p-NO_2 benzene sulfonamide	5×10^{-7}	(*154, 155*)
p-NH_2 benzene sulfonamide (sulfanilamide)	8×10^{-6}	(*154, 155*)
p-CH_3 benzene sulfonamide	8×10^{-6}	(*153*)
Acetazolamide 	6×10^{-8}	(*34*)
Ethoxzolamide 	10^{-9}	(*34*)

Coleman (*152*) has reviewed physical studies on the interaction of sulfonamides with carbonic anhydrase, and the structure-inhibition relationships have been reviewed by Maren (*31*); the most powerful inhibition is found when an aromatic ring is substituted with a free sulfonamide group (Table XI) (*34, 153–155*).

The strong binding of the sulfonamides has been used as the basis of a convenient method of determining the enzyme concentration: the

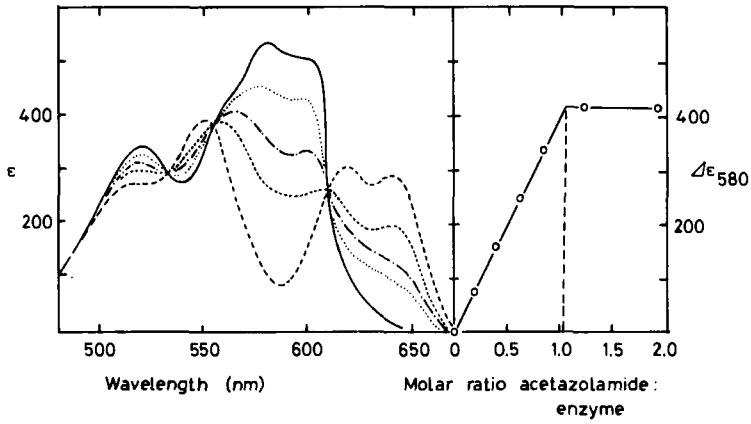


FIG. 7. The visible absorption spectrum of Co^{2+} carbonic anhydrase at different concentrations of acetazolamide, measured at 25°C, pH 8.0. The enzyme concentration was 6.9×10^{-5} *M*. The curves on the left represent the spectra at acetazolamide concentrations of 0 *M*, 2.8×10^{-5} *M*, 4.2×10^{-5} *M*, 5.6×10^{-5} *M*, respectively. The diagram on the right shows the increment in extinction coefficient at 580 nm ($\Delta\epsilon_{580}$) as a function of the molar ratio of inhibitor to enzyme. [Reproduced from Lindskog (*157*).]

activity of the carbonic anhydrase solution is titrated with acetazolamide or ethoxazolamide (*156*); the visible absorption spectrum of the cobalt enzyme can be titrated in a similar manner (Fig. 7).

Both Co(II) and Zn(II) BovCAB bind acetazolamide stoichiometrically; however, the affinity is reduced by at least two orders of magnitude in other metallocarbonic anhydrases and is comparable with binding to the apoenzyme (*157*, *158*).

Sulfonamide complexes of Zn(II) HCAC and Zn(II) HCAB have been studied by X-ray crystallography; the sulfonamide nitrogen or oxygen binds the zinc (*62*), and a second atom of the sulfonamide group may form an additional long bond with the metal (*159*). This rather irregular coordination geometry is consistent with recent magnetic susceptibility measurements (*160*). A distorted tetrahedral coordination is also suggested by absorption and MCD spectroscopic studies (*88*, *161*) and by ESR studies on the cobalt enzymes in the presence of various sulfonamides (*52*).

Further hydrogen bond and van der Waals's interactions with the hydrophobic part of the active site cavity (*159*) restrict sulfonamide motion (*162*, *163*) (Fig. 8).

The nonpolar nature of the sulfonamide binding site is consistent with the changes observed in the fluorescence and absorption spectra of certain sulfonamides (*34*, *156*); evidence for the involvement of tryptophan and tyrosine residues in the interaction is provided by UV

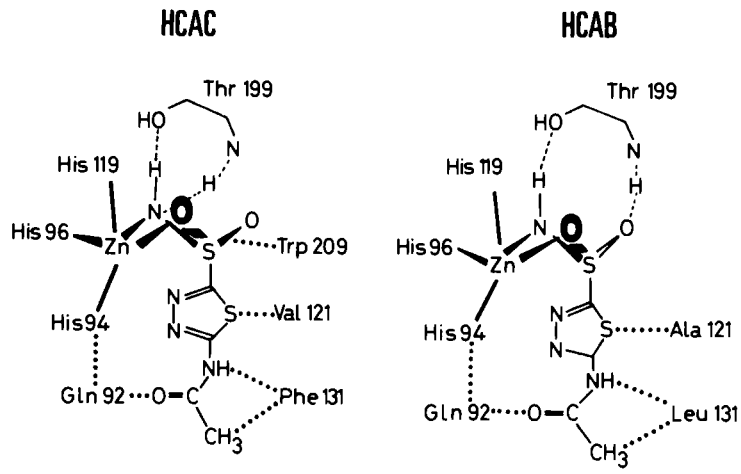
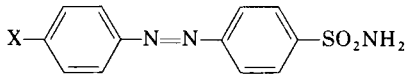


FIG. 8. Protein–sulfonamide interactions: the acetazolamide complexes of HCAC and HCAB. The sulfonamides are coordinated in the anionic form. ---, hydrogen bonds; · · · · · · , van der Waals contacts. [Details from Kannan *et al.* (*159*).]

difference spectroscopy experiments (*164*) and triplet–triplet energy transfer studies (*165*, *166*). The apoenzyme retains a significant affinity for the sulfonamides (K_1 acetazolamide = 10^{-4} *M*), which indicates that van der Waals and hydrogen bond interactions may play a large part in stabilizing the holoenzyme complexes—indeed it has been concluded from the results of NMR and fast-binding kinetic studies that sulfonamides initially bind to the enzyme without coordinating the metal (*153*, *167*) and that this initial complex then isomerizes into one in which the sulfonamide group is so coordinated; i.e., kinetic studies require a scheme of the type:

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EA_2$$

On the other hand, the kinetics may depend on the sulfonamide structure, thus, in the binding of the azosulfonamide (I) to bovCA the rapid kinetics of the induced CD and difference spectra proceed in parallel, indicating that a single binding process is being observed (*168*).

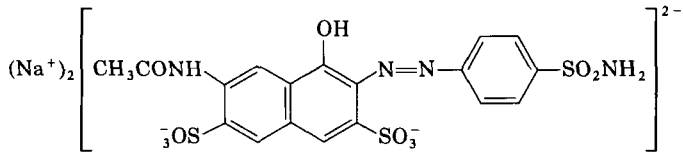


a: X = OH
b: X = NMe_2
c: X = NH_2

(I)

NMR studies on several sulfonamide complexes of human CA indicate that enzyme conformational change is involved. It is suggested that

inhibition of CA involves the following: stabilization of an appropriately oriented initial complex through the hydrophobic binding of the aromatic ring of the inhibitor to residues of the active site cavity; ionization of the $—SO_2NH_2$ group, facilitated by the proximity of the zinc ion; protonation of the proton-accepting ligand controlling catalytic activity; and the formation by the SO_2NH^- group of an ionic bond to zinc and a hydrogen bond to the hydroxy group of serine (in HCAB) or threonine (in HCAC) (*140*). Evidence has been obtained from resonance Raman experiments for the presence of the $—SO_2NH^-$ group in the complexes of I(a–c) with various isozymes of CA, and it seems that in these cases hydrophobic binding can be ruled out (*169*). The affinity of the enzymes for sulfonamides varies with pH (*82*); it is the second association reaction that is affected (*153*, *167*), the isomerization probably being selective for the ionized sulfonamide and the acid form of the catalytic group in the enzyme (*153*). A number of investigators have concluded that the sulfonamide is complexed as the anion (*155*, *156*, *164*, *171*), although the results of a recent resonance Raman spectroscopic study indicate that neoprontosil [disodium 2-(4′-sulfamyl phenylazo)-7-acetamido-1-hydroxy naphthalene 3,6-disulfonate (II)], binds in the neutral form (*172*). The lifetime of the complex



(II)

at pH < 10 is dominated by k_{-2}, there is little hydrophobic dependence, the stability of the complex depending mainly upon the coordination energy of the metal-sulfonamide bond; at pH > 10, however, the affinity decreases markedly, indicating dependence upon a group in the enzyme with $pK_a > 11$ (*153*). In an homologous series of sulfonamides, the increase in binding constant at low and intermediate pH is therefore due mainly to an increase in the association rate constant [k_{ass}(acetazolamide) = 10^5–10^7 $mol^{-1}\,sec^{-1}$].

Amino acid differences at the active sites result in different modes of sulfonamide interaction with HCAC and HCAB (Fig. 8), and this can result in marked differences in association and dissociation rates (Table XII), for example, benzene sulfonamides substituted at the ortho position show a relative decrease in affinity for HCAC compared to HCAB (*152*).

TABLE XII

ASSOCIATION (k_a) AND DISSOCIATION (k_d) RATES FOR DANSYLAMIDE BINDING TO THE HUMAN CARBONIC ANHYDRASE ISOZYMES[a]

	k_a ($mol^{-1}\ sec^{-1}$)	k_d (sec^{-1})
HCAC	2.4×10^5	0.390
HCAB	1.34×10^5	0.030

[a] From Coleman (*152*).

The interactions in the acetazolamide complexes are different from those in the sulfanilamide complexes; in particular, there is an additional interaction with Gln 92 on the hydrophobic side of the cavity (Fig. 8). Mutual inhibition experiments indicate that acetazolamide and HCO_3^- compete for a binding site which is the same as that for aldehyde hydrates but different from that used by esters (*173*). Since the dissociation from the complex is slow, preequilibration of acetazolamide with the enzyme results in pseudoirreversible inhibition (*82*) so that, for example, inhibition of CO_2 hydration is apparently noncompetitive.

G. INHIBITION BY ANIONS

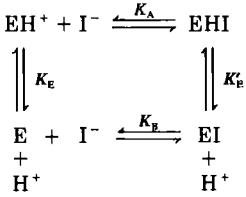
1. General Features

The manner in which anions bind to a metalloenzyme is a question of great interest to the inorganic chemist.

Monovalent anions are inhibitors of carbonic anhydrase (*174*), but divalent anions are not (*105, 157*)*; some apparent anion inhibition constants are given in Table XIII (*44, 82, 86, 153, 174a*).

The reversible inhibition of the CO_2 hydration activity of Co(II) and Zn(II) carbonic anhydrase by anions is noncompetitive (*44, 175*) whereas the dehydration reaction is inhibited competitively (*32*); the inhibition of the esterase activity is also noncompetitive (*82, 173*). The anionic inhibition is pH-dependent, being controlled predominantly by the ionization of the catalytic group; for the salts of relatively strong acids ($pK_a < 5$), the interaction with HCAC can be described approximately by Scheme 4, with $K_A \ll K_B$, that is, the interaction with the acid form of the enzyme is favored.

* Although, we have seen, there is evidence (*145*) that the SO_4^{2-} ion is not innocuous.



SCHEME 4

A more complex situation exists for H_2S and HCN, where HX also interacts with the alkaline form of the enzyme to yield EH · X (*176*). In the presence of anions, the pK_a of the activity-linked group is shifted to higher pH values, the stronger binding anions causing the greater shifts (*89*).

Studies of mutual inhibition (*81*, *110*, *173*, *175*, *176*), [^{3}H] acetazolamide binding (*158*, *177*), and $^{35}Cl^-$ NMR line broadening (*178*, *179*)

TABLE XIII

APPARENT ANION INHIBITION CONSTANTS FOR CARBONIC ANHYDRASE, MEASURED FROM THE INHIBITION OF THE CATALYZED HYDROLYSIS OF *p*-NITROPHENYL ACETATE (*35*)

Anion	K_I M		
	BovCA[a]	HCAC[b,c]	HCAB[b,c]
HS^-	1.9×10^{-6}	—	—
CN^-	3.2×10^{-6}	—	—
OCN^-	3.9×10^{-5}	—	—
NCS^-	5.9×10^{-4}	8×10^{-4}	7×10^{-4}
N_3^-	5.9×10^{-4}	—	—
I^-	8.7×10^{-3}	3.1×10^{-3}	2×10^{-3}
ClO_4^-	1.6×10^{-2}	1.5×10^{-3}	1.3×10^{-3}
HCO_3^-	2.6×10^{-2}	—	—
HSO_3^-	3.0×10^{-2}	—	—
NO_3^-	4.8×10^{-2}	1.8×10^{-2}	1.5×10^{-2}
Br^-	6.6×10^{-2}	2.7×10^{-2}	2.3×10^{-2}
CH_3COO^-	8.5×10^{-2}	3.4×10^{-2}	2.6×10^{-2}
Cl^-	1.9×10^{-1}	2.0×10^{-1}	3.0×10^{-3}
F^-	1.2	—	0.4

[a] pH7.55, 25°C. Data from King and Burgen (*153*) except for HS^-, CN^-, OCN^- (*82*); the values for HS^- and CN^- are calculated on the basis of total sulfide and cyanide ($HX + X^-$).

[b] 25°C. Data are from Verpoorte *et al.* (*44*) for HCAC, pH 6.8, and HCAB, pH 7.3, except for $K_I(Cl^-)$.

[c] Data for $K_I(Cl^-)$: from Whitney (*174a*) for HCAC, pH 6.5; from Whitney and Brandt (87) for HCAB $\lesssim 6$.

show that the anions compete with each other as well as with acetazolamide for the same or overlapping sites in the zinc and cobalt enzymes. The inhibition may not be so simple, however, for while the strength of inhibition appears to reflect a binding to the metal for the more potent anionic inhibitors, the weaker inhibitors ($K_I \gtrsim 10^{-3}$ *M*) conform to the Hofmeister lyotropic series (*180*), which reflects the effects that the anion has upon the water structure at the binding site (*181*); this could be taken as evidence of ion-pair formation.

The different modes of anion-metal interaction are also reflected in the visible absorption, ORD, and CD spectra of the Co(II) carbonic anhydrase–anion complexes (*32, 175, 182*) (Fig. 9).

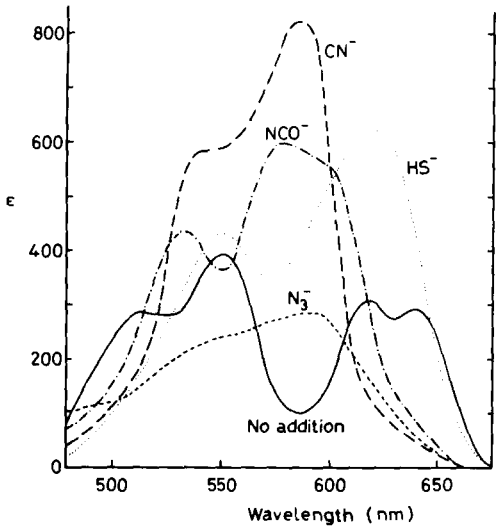


FIG. 9. The visible absorption spectra of Co^{2+}-bovine carbonic anhydrase saturated with different inhibitors, pH 8.0, 22°C. Enzyme: 3.5×10^{-4} *M* to 4.2×10^{-4} *M* giving absorbances of 0.1–0.29. Inhibitor: cyanide, 8.4×10^{-3} *M*; cyanate, 4.2×10^{-3} *M*; sulfide, 4.8×10^{-4} *M*; azide, 8.4 × 10⁻3 *M*. [From Lindskog (*157*).]

The metal in the cyanide complex is tetrahedrally coordinated, but the other complexes show distinct distortions from this geometry and may have pentacoordination. The changes in the absorption spectra parallel the inhibition of CO_2 hydration (*175, 183*) and ester hydrolysis (*110*) at low pH; however, unlike the inhibitory effects, the spectral changes of Co(II) BovCAB give no indication of a weak association between the anions and the basic form of the enzyme (*175*).

In some cases the anion is directly bound to the metal; this has been confirmed for N_3^- by infrared studies (*184*) and for CN^- by NMR (*176*)

and ESR (*71*, *185*) studies. The results of $^{35}Cl^-$ (*178*, *186*, *187*) and $^{81}Br^-$ (*133*) NMR relaxation enhancement studies in the presence of the zinc and cobalt carbonic anhydrases also indicate a metal-halide interaction. It has been suggested, however, that this interaction is more like that of a charge-transfer complex, since log K_I values of the Zn(II), Co(II), and Cu(II) BovCAB halides depend linearly on the redox potential of the X_2/X^- couple (*188*). Charge-transfer bands are indeed observed, in particular for the iodide complex, and the zinc-halide distances determined from X-ray crystallography (Br^-, 3.0 Å; I^-, 3.4 Å) (*72*) are consistent with this interpretation.

2. *The Question of Two-Anion Binding Sites*

The X-ray studies indicate that there are two anion binding sites within the active site cavity. The anion binding positions were derived exclusively by difference techniques from crystals of HCAC prepared in 20 m*M* Cl^-; since it has been reported that one Cl^- binds to the native enzyme in 5 m*M* KCl (*44*), the results of these investigations must be treated with some caution. However, any chloride binding interference would result only in the observation of fewer binding sites than are actually present, for those anions which bind less tightly than Cl^- or are about as electron dense as Cl^-; thus for the enzyme–iodide complex, both the binding sites may be observed.

The first site represents a direct interaction with the metal (Fig. 10) to give an inner sphere complex. In this site chloride is bound to zinc

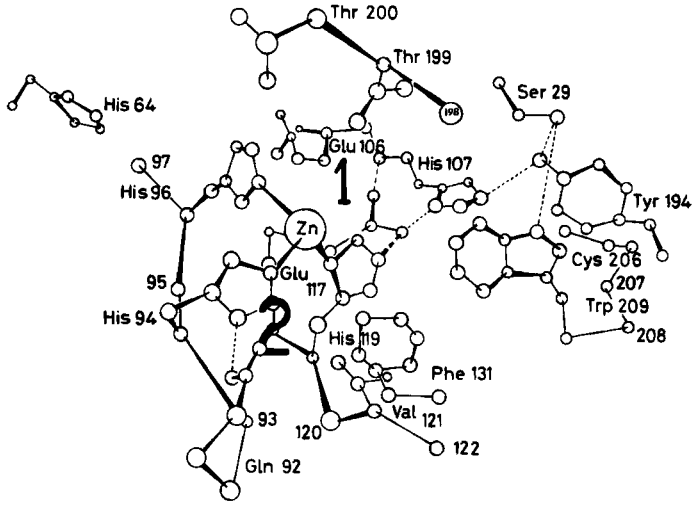


FIG. 10. The anion binding sites of HCAC, as reported by Vaara (*72*).

while the larger iodide interacts significantly with Thr 199; this hydrogen bond interaction may account for the marked stability of the anionic complexes and for the fact that *both* the holo- and apoenzymes bind the same numbers of anions at high salt concentrations (*44*).

The second site (Fig. 10) was demonstrated clearly for $Pt(CN)_4^{2-}$, $AuCl_4^-$, and $Au(CN)_2^-$ (Fig. 11). Iodide was also found at this site. The interaction appears to be with Gln 92, Phe 131, and with the zinc-bound water; i.e., it represents a metal outer-sphere site. The behavior of His 64 is also affected by occupancy of this site; in the presence of $Au(CN)_2^-$ this residue shows an increased reactivity toward bromopyruvate (*134*) and methylmercurithioglycolic acid (*72*), although there is no apparent rearrangement of the amino acid residues.

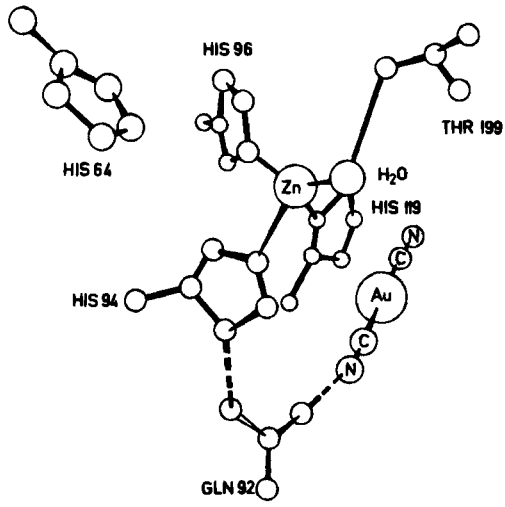


FIG. 11. The binding of $Au(CN)_2^-$ at site 2 of HCAC. [From Vaara (*72*).]

We may note that iodide binding to the human B isozyme is influenced by a group with a pK_a of 6.1, and this has been identified (*189*) as His 200. The results of X-ray crystallographic studies had indicated that a water molecule may separate the zinc and iodide ions in the enzyme–iodide complex, but X-ray absorption studies suggest that the iodide binds directly to the metal ion with a Zn–I distance of 2.65 ± 0.06 Å (*190*). However, both studies required solid samples, and the behavior in solution may be different; the different pH values of the solutions from which the solids were isolated may have some bearing on these results. Under appropriate conditions there is little doubt that the iodide ion may occupy either of the two anion binding sites.

A third anion binding site is present on the surface of the enzyme and involves the amino acid residues Lys 24, Arg 246, Pro 13, Pro 247, and Glu 14.

The results from the crystal structures and NMR dispersion experiments indicate that sulfonamides and CN^- enter site 1 and displace the metal-bound water. However, in other cases, for example the halides or carboxylate anions (*191–194*), the anion may enter as a fifth ligand; the formation of a dicyanide complex of Co(II) carbonic anhydrase at very low temperatures indicates that five coordinate complexes do form (*71, 185*). The associative nature of this interaction has been invoked to explain the apparently high rates of formation of the anion complexes (*195*), although, as discussed below, this may not be necessary.

The anion $Au(CN)_2^-$ reduces drastically the $^{35}Cl^-$ NMR line width induced by carbonic anhydrase (*196*); since the relaxation enhancement is due to binding at site 1, while $Au(CN)_2^-$ binds at site 2, there must be significant cross-site interactions. For smaller anions, however, it has been suggested that the two anion binding sites in the active site cavity can be occupied simultaneously (*197, 198*); the primary evidence for this comes from the relaxation enhancement of the methyl protons of acetate induced by Zn(II) and Mn(II) BovCAB (*197*).

With *p*-toluene sulfonamide bound (i.e., site 1 occupied) a relaxation enhancement of the acetate protons is still observed, but this can be titrated with N_3^-; azide on its own will titrate both sites independently

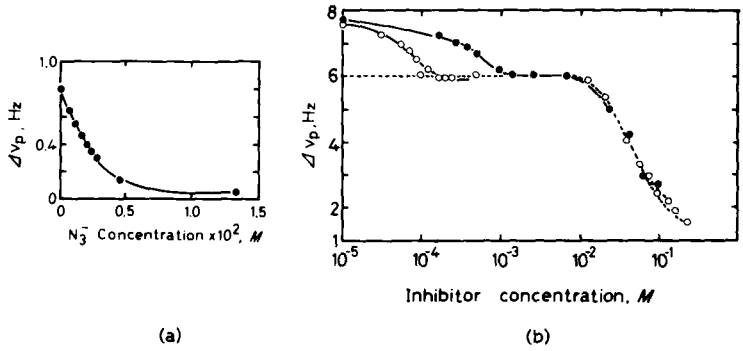


FIG. 12. (a) The net line broadening of acetate ion bound to bovine carbonic anhydrase B, upon titration with N_3^-. Enzyme concentration 0.98×10^{-3} *M*, and acetate 0.033 *M*. Tris–sulfate buffer in ^2H_2O, pH 7.0, 30°C. (b) Titration of 0.5 *M* acetate bound to 2.07×10^{-4} *M* Mn(II) bovine carbonic anhydrase B by: O——O, *p*-toluene sulfonamide; ●——●, N_3^-; □——□, N_3^- to solution of enzyme containing 6.5×10^{-4} *M* *p*-toluene sulfonamide. All the ^2H_2O solutions contained 2×10^{-4} *M* $MnCl_2$ in 0.01 *M* Tris–sulfate buffer, pH 7.5. [Reproduced from Lanir and Navon (*197*).]

(Fig. 12). Acetazolamide, which interacts significantly with Gln 92 at site 2 (Fig. 8) may be expected to inhibit the relaxation enhancement completely if this represents the second site detected by NMR. The conclusion, based on the NMR and esterase inhibition results, must be that there are two anion-binding sites and that the first represents a weak-binding inhibitory, metal inner-sphere site, and the second a stronger, but noninhibitory, interaction with the protein, not necessarily within the active-site cavity (Table XIV). However, the results are consistent with binding at sites 1 and 2.

TABLE XIV

INHIBITOR BINDING CONSTANT FOR BovCA (*197*)

	$K_i M^{-1}$				
	Inhibitory site			Noninhibitory site	
Metal	CH_3COO^-	N_3^-	*p*-CH_3-sulfonamide	CH_3COO^-	N_3^-
Zn	9	$\sim 1.2 \times 10^3$	2.1×10^6	35	$\sim 1.2 \times 10^3$
Mn	0.8	2.8×10^3	2.6×10^4	29	2.8×10^2

Although an earlier study of the interaction of acetate and mono-, di- and trifluoracetate with Co(II) HCAC, using ^1H and ^{19}F NMR (*190*), gave no evidence for two binding sites, this may not be at variance with the above conclusions since in this case control spectra were run with *p*-carboxybenzene sulfonamide; this sulfonamide may not displace acetate at the second site so that the difference between the control and observed spectra represent a specific site 1 effect. In the case of Co(II) HCAC the linewidth in the presence of the sulfonamide was comparable with that in the presence of the zinc enzyme; however, the linewidth broadening was only of the order of a few Hertz and the effect of binding at site 2 may not be observable within the experimental error. For Co(II) HCAB a complete return to the natural linewidth was not always observed, and this was attributed to the presence of paramagnetic impurities; however, it could arise from significant second-site contributions. A second acetate binding site, titratable with azide but not with *p*-toluene sulfonamide, has been found in Co(II) BovCAB (*194*).

While azide binds to the second site with an affinity similar to that for the metal-binding site, it is interesting to note that a radiolabeled cyanide binding study indicates that if a second site exists for CN^- the concomitant dissociation constant is >2 m*M*, three orders of magnitude higher than that for site 1 (*78*).

The affinity of acetate ions for the noninhibitory site of Zn(II) BovCAB is pH dependent, the related enzymic ionizations having a similar pK_a to that which controls activity (*197*), as expected if the second site was site 2 and there was a site–site interaction; the binding to the noninhibitory site is not nullified in the basic form of the enzyme. The pH-dependent $^{35}Cl^-$ NMR relaxation in the presence of bovine carbonic anhydrase (Fig. 13) is also consistent with the two-site hypothesis provided that the binding to site 2 is very weak ($K_1 \gg 0.5\ M$) in the basic form of the enzyme, or that binding in site 2 does not produce a significant effect on the line width. Both of these assumptions are reasonable. The titrations of the proton resonances of histidine with iodide (*69, 70*) also indicate that there are two anion binding sites in the cavity of Zn(II) HCAC and Zn(II) HCAB.

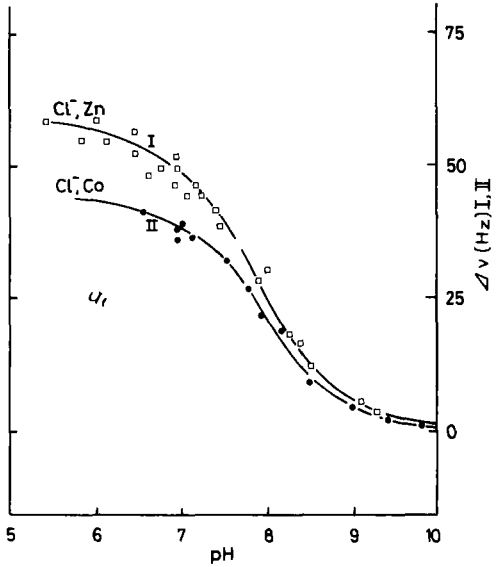


FIG. 13. Variation with pH of: (I) the increase in linewidth of the resonance of $^{35}Cl^-$ ions in a solution containing 0.5 *M* NaCl, upon addition of 23 μM native bovine carbonic anhydrase; (II) similar data for addition of 29 μM cobalt-substituted carbonic anhydrase. [Reproduced from Lindskog *et al.* (*32*); original data from Ward (*178*) and Ward and Fritz (*187*).]

Further evidence for the two-site hypothesis has been adduced from the observation of a binding constant, determined from $H^{13}CO_3^-$ NMR studies in the presence of Co(II) BovCAB, which is much less than that determined from esterase inhibition measurements (*198*); the deri-

vation of this binding constant is valid only if the $H^{13}CO_3^- - {}^{13}CO_2$ system is in slow exchange on the NMR time scale (T_2) or there is a relatively small exchange contribution to the line width. Although further experimental data were analyzed in terms of two sites (*198*), it being suggested that the second site was an outer sphere site (i.e., site 2), the data can be analyzed in terms of single-site occupation; in this case a dissociation constant of 100 m*M* for the Co(II) BovCA–HCO_3^- complex would yield a metal–$^{13}C(HCO_3^-)$ distance of 3.9 Å, consistent with inner-sphere coordination.

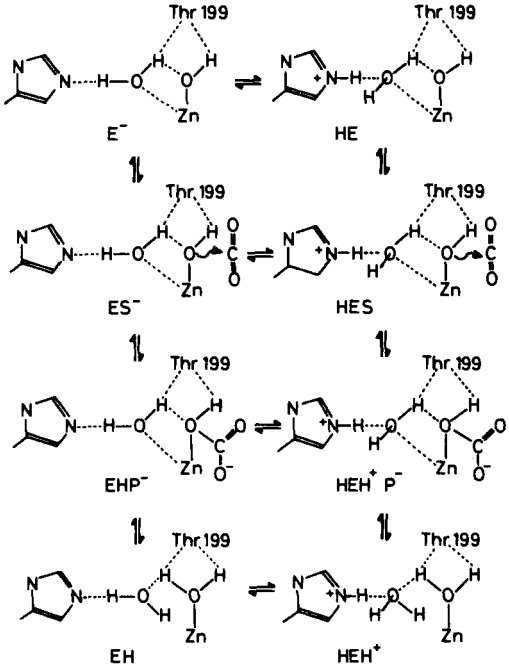
An investigation of the interaction of HCO_3^- with Co(II) HCAC, by ^{13}C NMR, has given no evidence for a second bicarbonate binding site (*78*), and proof of a second anion binding site on the enzyme, in solution. similar to that found for Co(II) BovCA (*194, 197*), is awaited. We see that from evidence at present available it appears that there may be two anion binding sites within the active-site cavity, a noninhibitory tight binding site and a weaker-binding inhibitory site. The anion interacts most strongly with the enzyme when the catalytic group is in its acidic form, but it also interacts, at both sites, with the alkaline form of the enzyme, but with reduced affinity. However, the question should probably not be regarded as closed; for example, since occupancy of the anion binding site affects the behavior of His 64, it would be expected that ionization of His 64 [i.e., ionization of the possible proton transfer group (*112*)] would affect anion binding; however, no evidence for this is found from the pH dependence of the anionic inhibition. For a deeper discussion of the kinetic aspects of anionic inhibition the reader is referred to Wyeth and Prince (*35*).

H. Mechanism of Action

Various physical techniques have shown us so far that the active site contains a proton-transfer group that may be an amino acid residue, possibly His 64, or a specifically bound buffer molecule, as well as a catalytic group that is probably an ionizable zinc-bound water molecule. The metal is four-coordinate at low pH and probably five-coordinate when the bound water molecule is ionized; this change in coordination may result in more mobile histidine ligands [the histidine proton resonances sharpen when the pH is raised (*69, 70*)] consistent with a small zinc displacement, possibly required to achieve the higher coordination number. This could explain the anomalous X-ray crystallography results for His 94. The relaxation spectra of complexes of the human carbonic anhydrases with aromatic sulfonamides are consistent with the view that the catalytic activity may be associated with the

capacity of the metal to undergo fast changes in coordination geometry (*199*).

The dehydration of ^{18}O-labeled HCO_3^- *leaves an* ^{18}O *atom in the active site* (*120*), and with this vital piece of experimental evidence in mind a plausible mechanism (Scheme 5) for the reversible hydration of



SCHEME 5

CO_2 may be proposed (*46*), in which the imidazole ring of His 64 is drawn as the proton-transfer group. Note that we may regard the active nucleophile not simply as an OH^- ligand bound to zinc, but as a coordinated $H_3O_2^-$ ligand hydrogen-bonded to a proton acceptor group, His 64.

The bicarbonate ion does not displace the metal-bound water. However, more strongly binding anions, for example, CN^- and N_3^-, will do so, resulting in tetrahedral coordination about zinc. Significant hydrogen bond interactions with Thr 199 must be invoked, at low pH, to account for the results of the solvent proton relaxation studies.

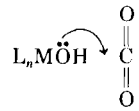
The results of the labeling studies indicate that the metal-bound hydroxide has a long lifetime at low pH, but that exchange is rapid at high pH (*120*). The high-pH form is E^- but the protonated form HE

also has the metal-bound hydroxide ion. His 64 (or the specifically bound buffer molecule) can assist the hydroxide displacement in E^-, or it may reduce the hydroxide dissociation rate in HE by virtue of the adjacent positive charge.

An increase in buffer concentration does increase the hydroxide-exchange rate (*120*). This scheme could also account for the fluctuations observed in the magnetic axis of cobalt in Co(II) HCAC (*70*), but fluctuating coordination of His 94 could also account for this.

Since hydroxide displacement from E^- is faster than from HE it might be expected that the rate for $ES^- \rightarrow EHP^-$ would be faster than $HES \rightarrow HEH^+P^-$, i.e., Scheme 2 is a poor description of the real system.

It is interesting that the nucleophilic power of a metal-coordinated hydroxide ion is considerably larger than would be expected from the pK_a of the corresponding aquo complex in inert d^3 and d^6 complexes (*149*), an effect reminiscent of the α-effect in general mechanistic chemistry (and possibly having a similar origin but with filled, or partially filled, *d* orbitals of T_2 symmetry playing the role of filled *p* orbitals). However, the zinc-bound hydroxide–nucleophile mechanism (*99a*, *149*), in which the essential process is



is unlikely to account for the full catalytic activity of the enzyme in CO_2 hydration (*149*), but an assisted attack with a hydrogen-bonded $H_3O_2^-$ ligand may do so. Also, the zinc ion may act as a Lewis acid, polarizing the CO_2 molecule; and the CO_2 molecule could be distorted toward the transition state conformation in its binding site: we have already seen (Section II,D1) that a Lewis acid–proton acceptor combination is particularly effective in the catalysis of CO_2 hydration. The infrared studies of Riepe and Wang (*184*), while indicating that CO_2 binds near the metal, give no indication of a conformational or polarization change. The difficulty is that these studies were performed at low pH and may represent CO_2 bound to HEH^+ (Scheme 2) which is *inactive* in CO_2 hydration; there may be subtle changes in the binding site as the pH is raised that result in a distortion of the CO_2 molecule. This illustrates the difficulty in determining the mode of CO_2 binding in the *active* form of the enzyme. It has been suggested, from the results of a ^{13}C NMR study, that the CO_2 binding site in HCAB is about 3.4 Å from the metal (*200*), although this result requires certain assumptions and could again represent binding to HEH^+.

III. Zinc-Based Alcohol Dehydrogenases

A. Introduction

Alchohol dehydrogenases from various sources catalyze the reaction:

$$R—CHO + NADH + H^+ \rightleftharpoons R—CH_2OH + NAD^+$$

where NAD is nicotinamide adenine dinucleotide.

Reports of the oxidation of alcohols by different animal tissues appeared toward the end of the 19th century, and since that time the dehydrogenases, the cofactors NADH and NAD^+, their analogs, and the various substrates have been the subject of an extensive literature. This survey is therefore confined to the metal ion involvement in the enzyme reaction, together with a brief resumé of the relevant background. More comprehensive reviews, particularly on the biochemical and structural aspects, are available. The dehydrogenases, such as LADH and YADH, illustrate again how knowledge of the involvement of the metal ion in an enzymic reaction may be sought: the effect of varying metal ion or metal ion content; the effect of metal-chelating agents; variation of cofactor, substrate, and inhibitors, together with X-ray, spectrophotometric, kinetic and equilibrium studies are all possible tools. There is again the added possibility of observing analogous nonenzymic reactions of simpler complexes, which may throw light on the possibilities open to the metal ion as it functions in the enzyme.

B. Enzymes

Alcohol dehydrogenase activity occurs widely in natural systems (*201*, *201a*) for example, in the livers of humans, fishes, horses, and rats, in plant tissues, in microorganisms, and in yeast. The enzymes do not possess pronounced substrate specificity and can react with a large number of normal and branched-chain aliphatic and aromatic primary and secondary alcohols and carbonyl compounds. The transfer of the formal hydride is direct between coenzyme and substrate and is stereospecific.

The first successful crystallization of a pure alcohol dehydrogenase was that from yeast (YADH) in 1937 (*202*), and that of LADH was reported in 1948 (*203*). Of all the reported dehydrogenases, LADH and YADH have been the most widely studied (the former somewhat more

than the latter) and are the two with which we are concerned here. LADH has a molecular weight now thought to be about 80,000 (*204*). Initial investigations, based on an incorrect molecular weight of ~73,000 (*205*), gave the number of gram atoms of zinc per mole of enzyme as 2 (*206*, *207*). More recent studies, using purified enzyme, have shown the true value to be 4 (*208*).

The enzyme itself is a dimer (*209*) consisting of two identical subunits (*210*). Each subunit binds two zinc ions (*210*) and has one site at which to bind the coenzyme NADH in reductions, or NAD^+ in oxidations. The structure of the coenzyme is shown in Fig. 14.

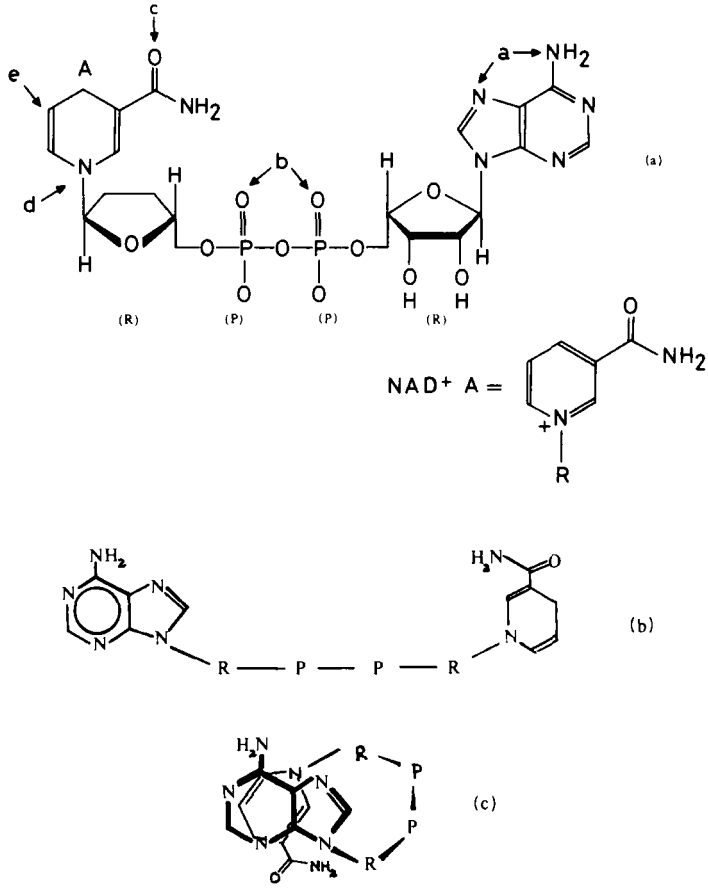


FIG. 14. (a) NADH, the coenzyme used by LADH and YADH; loss of a formal H^- from the 4-position of the nicotinamide ring gives NAD^+ the coenzyme for dehydrogenations. (b) The extended form of the coenzyme. (c) The coiled form of the coenzyme.

Improvements (*211a*) in crystallization techniques (*211b*) were needed for X-ray studies.

The primary sequence of 374 amino acids in each residue has been described (*210*) and X-ray structural studies of the whole enzyme (*212*) at 2.9 Å resolution and of the active site at 2.4 Å (*213*) have confirmed the dimeric natures and the presence of two coenzyme binding sites and have identified the positions of the four zinc ions. The X-ray studies are discussed further below: the gross features of LADH are that it is a dimer, with four zinc ions and two active sites.

YADH, on the other hand, is a tetramer (*214*) of molecular weight ~140,000, which contains four zinc ions (*215*). Initial work indicated that the enzyme bound 4 mol of cofactor per mole of enzyme (*216*), but more recent work by Dickinson (*217*) has suggested that the number is more probably 2.

C. Metal Ion–Reactivity Relationships

1. LADH

The evidence for zinc ion involvement in both a catalytic and a structural role in LADH has been reviewed (*204*, *217a*). The enzyme is inhibited by metal-chelating agents, such as 1,10-phenanthroline and 2,2′-dipyridyl; the metal can be removed from the enzyme by dialysis against such metal-chelating agents, and the resulting apo- or "half-apo"-enzyme, depending on the system (*204*, and see below), is inactive. The activity can be regenerated by replacement of the metal ion using either zinc itself or cobalt.

The evidence for both a structural and a catalytic role for the metal ion comes from several sources. In phosphate buffer, for example, all the zinc ions will exchange with $^{65}Zn^{2+}$ within 24 hours (*204*), whereas in acetate buffer only two of the four do so, which indicates two different "types" of zinc. Inhibition by 1,10-phenanthroline is total at a ratio of 2 mol of 1,10-phenanthroline per mole of enzyme, and 1,10-phenanthroline binds to the "catalytic" zinc (*212*). Dialysis of the enzyme against EDTA can give an enzyme containing only two zinc ions, and this "half-apo" enzyme is inactive, 1,10-phenanthroline does not interact with the "buried" zinc (i.e., noncatalytic or not easily dialyzable) (*212*), which presumably plays a part in maintaining the enzyme

structure (*204*, *212*). It is the detailed function of the catalytic zinc that is of interest here.

2. YADH

The inhibition of YADH by 1,10-phenanthroline differs from that of LADH in that the metal-free apoenzyme cannot be prepared by dialysis: rather the tetramer dissociates into monomer when 1,10-phenanthroline is present in large excess. The dissociation, which is irreversible, may be represented as

$$[(\text{YADH})_4\text{Zn}_4] + 12\ 1,10\text{-phenanthroline} \rightarrow 4(\text{YADH}) + 4(\text{Zn}(1,10\text{-phenanthroline})_3$$

Progressive inhibition by nonexcess amounts of 1,10-phenanthroline (or 8-hydroxyquinoline-5-sulfonic acid) produces a linear dependence of the activity and structural integrity of the enzyme on inhibitor concentration (*215*).

Inhibition by these reagents results in solutions having UV spectra very similar to those of the ligand and free zinc salts in aqueous solution (*219*). An interesting feature is that the inhibition is prevented by the coenzyme, NADH or NAD^+ (*215*). Thus, both ligands are competitive inhibitors, and both bind at or near the metal site.

The foregoing is a very brief resumé of the evidence for metal ion involvement in the enzyme. More detailed reviews may be found in Sund and Theorell (*201*, *201a*), Brändén *et al.* (*219*), Ulmer and Vallee (*204*), and Scrutton (*220*). The specific point of interest in this instance is whether, when catalysis occurs, the metal ion binds the coenzyme, the substrate, both simultaneously, or neither. Figure 15 shows the possibilities schematically. We shall see in Section III,D how this problem can be approached.

(M)[S, CoE]	(M, S)[CoE]	(M, CoE)[S]	(M, CoE, S)[–]
(I)	(II)	(III)	(IV)

FIG. 15. A schematic representation of the possible modes of interaction of an alcohol dehydrogenase enzyme with its coenzyme (CoE) and substrate (S): ( ) represents a catalytic metal binding site, and [ ] an alternative site or sites capable of binding coenzyme, or substrate, or both. If the coenzyme can bind in either a coiled or extended conformation, the number of possibilities is doubled. There is also the possibility that CoE and CoE^+ may select different binding sites.

D. Coenzyme–Enzyme Interactions

There are two considerations in the binding of the coenzymes to the two dehydrogenases: where the coenzyme binds the enzyme, and where the enzyme binds the coenzyme. The possible metal binding sites on NADH are illustrated in Fig. 14a. The metal ion may bind at the adenine (a), the pyrophosphate (b), the nicotinamide carbonyl group (c), the pyridine ring nitrogen (d), or the nicotinamide C-5 position (e). In NAD^+, only a, b and c are possible candidates.

The five possibilities for NADH have been reviewed by Mildvan (*221*), who pointed out that a and b are unlikely, since 1,10-phenanthroline and ADP-ribose (Fig. 16) (both NADH-competitive inhibitors) are not mutually competitive and, furthermore ADP-R' (a paramagnetic analog of NADH; see Fig. 24 and Section III,E below) has been found to bind with the same stoichiometry to both native and apo-LADH (*222*). (This latter point must be treated with caution, however. As discussed below, that there is ligand–enzyme and ligand–apoenzyme interaction does not necessarily reveal anything about metal-ligand binding.)

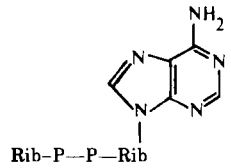


Fig. 16. ADP-R, an NADH-competitive inhibitor.

Mildvan (*221*) also pointed out that it is "not apparent how coordination at 'c' and 'e' would facilitate hydride transfer, although coordination at 'd' remains a possibility." In terms of simultaneously coordinated substrate, however, there would seem to be no reason why coordination at c should not facilitate reaction [and there is evidence that this may occur in model systems (*222a*)]; coordination at e (besides being intuitively unlikely) might be seen to raise certain steric difficulties. If a simultaneously coordinated substrate is not proposed, then it might be thought that each of the possibilities a to e is equally unlikely.

Within this context the UV spectrum of NADH is of interest. NADH in aqueous solution has $\lambda_{max} = 340$ nm, $\epsilon = 6.2 \times 10^3\ cm^2\ mol^{-1}$ (*223*). The transition has been visualized as shown in Fig. 17. On binding to LADH, the maximum is shifted to 325 nm. Kosower (*224*) has shown

that such a shift may be explained by the interaction of a positively charged nitrogen (in the form of an ammonium ion) situated ~3 Å from the increasingly positive nitrogen of the pyridine ring.

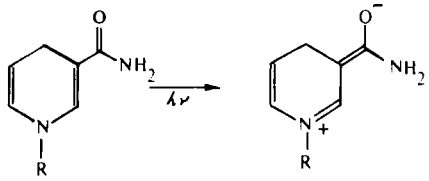


FIG. 17. Electronic transition of β-NADH. [From *224.*]

We may note here that coordination of a metal ion to the amide carbonyl would be expected to promote the transition and lower the energy, leading to a shift to longer wavelengths. Kosower, however, included in his assumptions that the zinc ion bound the NADH via the pyrophosphate group and so could not constitute the positive charge responsible for the shift in absorbance. The zinc ion does not so coordinate (see above), and it may be argued that the zinc ion–dihydropyridine interaction [i.e., Mildvan's (*221*) "d"] is therefore possible. That this may not be the case is shown by differences and similarities between LADH and YADH. There is no shift in NADH absorbances on binding to YADH, and yet the metal ion function in both cases is very similar (Section III,F), indicating that in the LADH active site there may well be a positive nitrogen moiety that is absent in YADH.

There is a further consideration regarding the conformation of NADH on binding. Several investigators utilizing NMR (^{1}H, ^{13}C, and ^{31}P) spectroscopy (*224a–d*) have examined the conformations of NADH, NAD^+ and related dinucleotides and have shown, *inter alia*, that NADH exists in a stacked conformation in aqueous solution at pH 4.0 and 30°C. The stacking is such that there is interaction between the nicotinamide and the adenine (Fig. 14c). Barrio *et al.* (*225*), working on the related dinucleotide, ϵ-FAD, have pointed out that such a stacked or folded conformation results in a quenching of the nucleotide fluorescence. The binding of NADH to LADH or YADH results in an enhancement of the coenzyme fluorescence (*226*), indicating that the NADH is binding in an "open" conformation. This is confirmed by the X-ray structural study on the enzyme-ADP-ribose complex (*212*) and by NMR (*227*).

In the detailed X-ray study of the active site (*213*), the binding of the coenzyme analog is revealed to be primarily a result of an interaction of the adenine with a hydrophobic pocket on the enzyme. The adenine NH_2 points away from the enzyme (*213*), and the O_2 hydroxyl of the adenine ribose is hydrogen-bonded to Asp 223. The situation is represented in Fig. 18 (*213, 228*), where it is seen that there is also an ionic interaction between the guanidinium group of Arg 47 and the pyrophosphate group. This illustrates the rich variety of interactions to be found in the biological systems, for here we have a hydrophobic interaction, a hydrogen bond, and an electrostatic attraction acting in concert to bind a reactant, viz., the coenzyme.

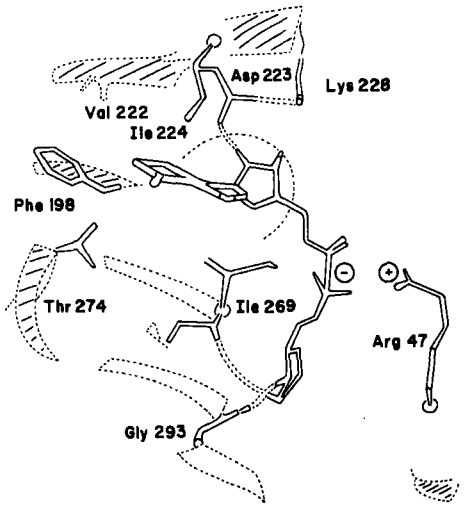


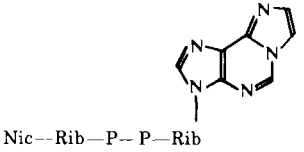
FIG. 18. Schematic representation of the interaction of LADH with ADP-ribose. [From Eklund *et al.* (*213*) and Hughes and Prince (*228*).]

An assumed position for the nicotinamide moiety can be deduced from the given structure (*212, 213*), and the C-4 atom of the nicotinamide ring is ~ 4.5 Å from the catalytic zinc. It is also of interest that in all the dehydrogenase structures reported, the residue Asp is invariant in the coenzyme binding site (i.e., in lactate dehydrogenase, LADH, and glyceraldehyde-3-phosphate dehydrogenase), indicating that the ribose oxygen–Asp hydrogen bond is an important feature of coenzyme

binding (*213*, and references therein). This then narrows the number of possibilities to (I) and (II) of Fig. 15.

That such a situation exists in the solid is, of course, no guarantee that the same is true in solution [see, for example, Lipscomb (*76*)] since conformational changes may occur that alter the enzyme–coenzyme interaction. Luisi *et al.* (*229*) have reported, however, that the binding of the NAD^+ analog nicotinamide 1-N^6-ethenoadenine dinucleotide (ϵ-NAD^+) (Fig. 19), to various dehydrogenases results in an enhancement of the coenzyme fluorescence by a factor of 10–13 [indicating unfolding (*225*)] and in a blue shift of the fluorescence maximum, which the authors (*229*) interpret as the adenine "sensing" a hydrophobic region. This indicates that the solution enzyme–coenzyme interaction may well be the same as in the solid. [Indeed, the involvement of the adenine moiety in coenzyme binding may be inferred from the relative K_m's and V_{max}'s exhibited for several NADH analogs with modified adenine substituents (*228*, *228a*).] It is clear from such studies that adenine–metal ion interaction is precluded.

FIG. 19. The structure of ϵ-$NAD.^+$



The X-ray work puts the C-4 of the nicotinamide 4.5 Å from the zinc, a distance that may well be changed in solution, so that direct nicotinamide–metal ion interaction is possible. Shore and Santiago (*230*) recently reported coenzyme interactions with totally cobalt-substituted LADH. Binding of NADH causes a shift in the 655-nm peak of the heteroenzyme to 670 nm, and similar perturbations were observed on binding pyrazole and trifluoroethanol with NAD^+, but not with NAD^+ alone. The authors interpreted this as indicating metal ion involvement in binding alcohol and NADH, but not NAD^+.

On the other hand, Weiner *et al.* (*231*) and Iweibo and Weiner (*232*) have shown by the techniques of fluorescence enhancement, quenching, and polarization that the native zinc enzyme may bind as many as 6 or 8 mol of NADH per mole; that, of these, two are tightly bound; that apo-LADH binds 2 mol of NADH with the same affinity as the 2 that are tightly bound to the native enzyme, and that the apoenzyme also binds substrate with the same affinity as the native enzyme (*232*). The work confirms earlier studies involving a spin-labeled analog of the NAD

skeleton (Fig. 24), which was found to bind to both the native and apo-LADH with the same stoichiometry as that reported above (i.e., 8 to the native enzyme and 2 to the apoenzyme), although the binding of the analog to apo-LADH was slightly weaker than that to the native enzyme. Thus, there is an indication that zinc is not involved in interaction with either substrate or coenzyme.

There is, however, a further point to consider, in that it is not necessarily the case that the binding of the coenzyme (or the substrate) to apo-LADH occurs in the same way as to LADH. Weiner *et al.* (*231*) reported that they were prompted to use fluorescence polarization to search for additional binding sites for NADH on LADH since such binding sites may not enhance the coenzyme fluorescence, as was evidenced *by the binding of NADH to the apoenzyme.* Since (as was discussed above) the coenzyme binds to the enzyme in an open as opposed to a folded conformation, and the opening of the coenzyme enhances the fluorescence (*225*, *226*, *229*), the coenzyme cannot be binding to the apoenzyme in the same way as to the active enzyme. A comparison of binding strengths between native and apoenzyme therefore becomes invalid in considerations of metal ion involvement; rather, the mode of binding is of importance, and this is seen to be different. One possibility is that, in both cases, NADH binds via the adenine, but the metal promotes opening of the coenzyme (and fluorescence enhancement), either by conformational changes in the enzyme or by nicotinamide–metal interaction.

The use of ^{35}Cl NMR line-broadening experiments has yielded valuable information with regard to zinc ion function in the enzymes alkaline phosphatase (AP) (*233*) and carbonic anhydrase (CA) (Section II,G) (*234*). The addition of LADH to KCl solutions causes the ^{35}Cl resonance to broaden (*235*), and the subsequent addition of NADH decreases the broadening, which is consistent with the displacement of bound Cl^- (*233-235*). The metal ion in a metalloenzyme may be regarded as a possible chloride binding site [in AP and CA it is the actual site (*233*, *234*)], but the addition of 1,10-phenanthroline, which binds to the zinc in LADH (*212*), cyanide, or 8-hydroxyquinoline derivatives, has no effect on the ^{35}Cl line width of an LADH/KCl solution (*235*), indicating that such ligands do not bind at the same place as NADH.

On the other hand, an analogous study involving ^{81}Br linewidths (*236*) showed that binding of bromide to LADH was insensitive to the addition of NAD^+ or NADH. The most likely explanation for this is a stronger enzyme–bromide interaction or the bromide ion binds at a different place from that of the chloride. Unfortunately, the effect of chelating agents, which might have indicated whether the bromide was bound to the zinc ion, was not studied. It is noteworthy that

chelated zinc, e.g., in the ligand CR (Fig. 6) is able to bind bromide ion strongly (*237*).

In a study of the effects of chloride on coenzyme binding, Coleman and Weiner (*238*, *239*) established coenzyme-competitive inhibition at saturating substrate levels. With coenzyme saturating, however, the anion was found to be a noncompetitive inhibitor against either acetaldehyde reduction or isobutyramide binding (*238*). The authors interpreted this as evidence for the existence of at least two specific anion binding sites on the enzyme, since they observed the formation of an enzyme–NADH–aldehyde–chloride complex (*239*). They also reported that chloride ion diminishes the fluorescence of enzyme-bound NADH (*238*), other spectroscopic techniques (UV, ORD) showing the coenzyme to be still bound.

The latter observation suggests that the addition of chloride is similar in effect to the removal of metal ion (see above) in that both cause a different mode of binding of coenzyme. Whether the chloride ion effects mimic those of metal ion removal by blocking a positive charge on the zinc ion is a matter of conjecture; the ^{35}Cl-NMR results (*235*) militate against the suggestion, but the indication of multisite anion binding (*238*, *239*), supported by the ^{81}Br-NMR results, suggests that there is some uncertainty about the mode of chloride binding.

The absence of any coenzyme–metal interaction has been proposed by Takahashi and Harvey (*240*) as a result of measurements of energy transfer between bound NADH, thionicotinamide NADH, or Rose Bengal and cobalt in a hybrid Co(II)Zn(II)-LADH. Measurements based on the fact that fluorescence enhancement is less with the hybrid than with the native enzyme result in a Co(II)-nicotinamide ring distance of 19 ± 2 Å (*240*). This is in accord with the observation (*241*) that binding of azide, 1,10-phenanthroline, or pyrazole does not affect the absorption spectrum of a similar hybrid enzyme. Such hybrid enzymes are prepared by utilizing the known (*204*) (see above) rapid exchange of two of the four zinc ions in acetate buffer. Ulmer and Vallee (*204*, and references contained therein) originally equated the two rapidly exchanging zincs with catalytic activity, while the other two were equated with maintenance of structure. Therefore, the above observations indicate that the catalytic metal ion serves no substrate or coenzyme binding function.

There is, however, another explanation (*242*). If the two structural zincs, in fact, are rapidly exchanging ones, then their removal must necessarily affect the enzyme structure and hence the activity.

A recent report by Sytkowski and Vallee (*217a*) is of particular interest in this connection: using the ^{65}Zn and Co hybrid enzymes, they showed that the catalytic atoms are those which are reactive to

1,10-phenanthroline, while the noncatalytic pair are not affected by this reagent. But metal–metal exchange studies shows the converse to be true: the chemical reactivity of the noncatalytic atoms is much higher, and they exchange more rapidly.

The X-ray studies of Brändén *et al.* (*212*) have shown the two different zincs to be ~20 Å apart. There is one on the enzyme surface (*212*) and one at the bottom of the cleft that is the active site (*213*); 1,10-phenanthroline binds to this second, catalytic zinc (*212*), and 1,10-phenanthroline binding affects the absorption spectrum of the *totally* cobalt-substituted enzyme (*230*). The surface zinc might possibly exchange more rapidly than the zinc at the bottom of the cleft. One would therefore expect no evidence of any coenzyme–cobalt (or substrate–cobalt) interaction in the hybrid enzyme (*240*) and no indication of a metal-inhibition interaction with 1,10-phenanthroline (*241*), and a distance of ~20 Å (*240*) between the nicotinamide ring and the cobalt ion is to be expected. It is also significant in this context that ESR studies on the interaction of ADP-R' (see Fig. 24) with totally cobalt-substituted LADH (*243*) show the spin label to be ~6 Å away from the catalytic cobalt, although the NMR results of Mildvan *et al.* (*244*) on the same system (see below) indicate that this is the case in the hybrid enzyme, which does not agree with the work of Takahashi and Harvey (*240*) or the UV results on inhibitor binding (*241*). Thus, the work of Mildvan *et al.* (*244*) indicates that the two rapidly exchanging zincs are those at the bottom of the cleft, not the structural ones as argued above, although this offers no explanation of the other observations discussed above (*230*, *240*, *241*).

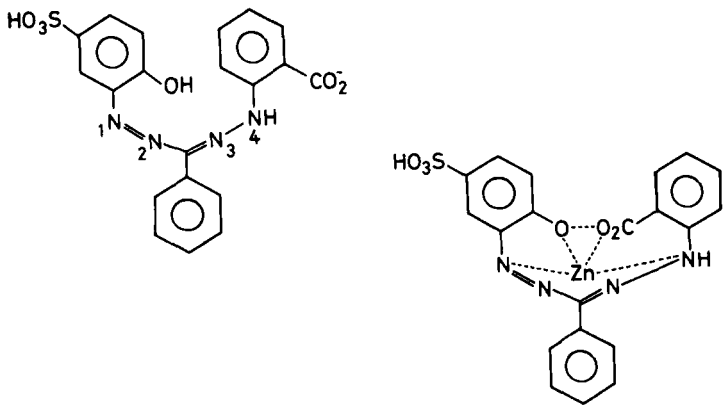


FIG. 20. The mode of binding of Zincon to zinc. [From McFarland *et al.* (*246*).]

Some further evidence for a direct metal ion/NADH interaction comes from pH effects on 1,10-phenanthroline binding (*245*) and resonance Raman investigations of binary complexation between 2-carboxy-2-hydroxy-5′-sulfoformazyl benzene (Zincon) (Fig. 20) (*246*) and LADH or zinc.

1,10-phenanthroline binds weakly to LADH at alkaline pH, and Reynolds *et al.* interpret this as resulting from displacement of zinc-bound hydroxide as opposed to more readily displaced zinc-bound water at pH < 7; i.e., the 1,10-phenanthroline binds directly to the metal ion. Since 1,10-phenanthroline is competitive with NADH, there is an intimation that this may also be one mode of NADH–enzyme interaction.

Zincon is also strictly competitive with NADH (*246*) and binds zinc as shown. The resonance Raman study shows that in the enzyme the binding at pH 8.75 is to the zinc, but not via the carboxylate and N-4, only via the phenolate and N-1. Thus, there is a further indication of coenzyme–metal ion interaction.

To summarize, on the basis of the evidence available no definite conclusion can be drawn regarding *direct* coenzyme–metal ion interaction: The inhibition results indicate an interaction, since chelating agents (*204*, *215*, *217a*, *246*) are competitive; the X-ray data indicate the opposite (*212*, *213*); spectroscopic evidence is contradictory (*214*, *230*, *240*, *247*); NMR and the effect of anions are inconclusive (*235*, *236*, *238*, *239*) (and see above) while the results of resonance Raman experiments are more positive (*246*). Although doubt remains it seems that (III) and (IV) (Fig. 15) can be eliminated.

It is interesting that the 3-amido group of the nicotinamide ring in NAD^+ appears to be necessary for the correct positioning of the coenzyme molecule in the active site (*248*), but activity is not confined to the 3-amido derivative: 3-halogeno (Cl, Br, I) analogs are active as well (*249*), but the unsubstituted pyridine derivative is not. However, in a structural study of the adducts of 3-iodo and the unsubstituted pyridine adenine dinucleotides with equine LADH, Samama and coworkers (*248*), have found that, although the binding of the adenosine part of the molecule is very similar to that of NADH or its analog ADP-ribose (Fig. 16), the conformations of the remaining parts of the analog are quite different (Fig. 21).

The striking fact is that, mainly owing to a difference in the orientation and position of the pyrophosphate group, the pyridine rings of the 3-substituted analog are at the surface of the molecule, well removed from the zinc ion at the active site. Moreover, a conformational change in LADH is induced by coenzyme binding (*248*, *249*), and the $CONH_2$

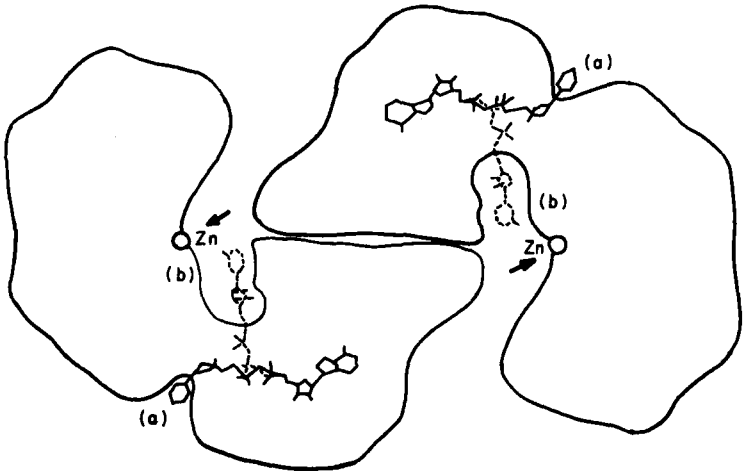


FIG. 21. Schematic diagram of a section through the alcohol dehydrogenase molecule with bound nucleotides projected onto this section. [after Samama *et al.* (*248*).] The pyridine and 3-halogeno pyridine adenine dinucleotides take up position (a); the nicotinamide adenine dinucleotide, position (b). The swing about the pyrophosphate group is clearly indicated. Substrate can take up the arrowed positions [cf. also Brändén (*100*).]

group appears to be essential for this change to occur (e.g., ADP-ribose does not induce it). The 3-iodo and pyridine analogs also do not induce the change.

The question then arises how it is possible for the 3-iodo analog to be an active coenzyme: if the mechanism remains essentially the same as with NAD^+ the iodopyridine ring must swing through a considerable distance, using the pyrophosphate group as a hinge to position itself near the zinc ion in the active site. (We have not met such a large group movement with CA, but we shall come across a rather similar and remarkable movement of a phenolic side chain with CPA).

It is likely that there are two different conformations of the 3-iodo derivative in solution; one of them is active, and the other has the inactive binding mode described. However, if such a contortion is possible for the 3-iodo compound, it should also be possible for the (inactive) unsubstituted pyridine derivative. The origin of the activity difference is at present unknown.

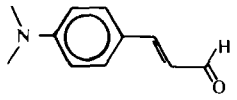
E. SUBSTRATE–ENZYME INTERACTIONS

The suggestion that the function of the zinc ion in alcohol dehydrogenases was to act as a Lewis acid in polarizing the carbonyl group

of coordinated aldehydes was first made by Abeles *et al.* (*247*) after studies on model reactions with NADH analogs and thioketones. Supporting evidence based on the stereospecificity of the transferred hydrogen and the deuterium isotope effect on the YADH-catalyzed reaction led Sund and Theorell (*201*, and references contained therein) to suggest a mechanism in which both coenzyme and substrate were bound to zinc, the coenzyme binding through adenine and the substrate through oxygen. The nicotinamide ring was pictured as interacting both with groups in the active site cavity wall and with the substrate ligand on the side remote from zinc.

A number of recent investigations have supported the suggestion of a direct carbonyl–zinc ion interaction. Jacobs *et al.* (*250*) compared the rates of reduction of several benzaldehydes under both enzymic and nonenzymic conditions. The ratio of *p*-chloro to *p*-methoxy benzaldehyde was 100 for reduction by sodium borohydride, whereas for reduction by NADH with LADH as catalyst the ratio was 2. This lack of substituent effect on the enzymic reaction was thought (*250*) to be a result of polarization of the carbonyl bond by zinc coordination. Dunn and Hutchinson, in a kinetic study of the stable intermediate formed between *trans*-4-*N*,*N*-dimethylamino cinnamaldehyde (Fig. 22), NADH, and LADH at pH $\geqslant$ 9.0, concluded that the zinc ion in the enzyme played a Lewis acid role by binding through the carbonyl oxygen. The YADH-catalyzed reduction of substituted benzaldehydes has been shown to involve a general acid in the active site (*264*), and, in subsequent work on the pH dependence of the reaction, Klinman (*265*) concluded that the ionizing group (which has a pK of $\sim$8.6) could be either an imidazole, a cysteine, a lysine, or a zinc-bound water molecule. In similar studies on the reduction of acetaldehyde and butyraldehyde (*251*) and the oxidation of butan-1-ol and propan-2-ol (*252*), Dickenson and Dickinson (*251*, *252*) reached the same conclusion.

FIG. 22. *trans*-4-*N*,*N*-Dimethylamino cinnamaldehyde.



Shore *et al.* (*253*), in a study of proton liberation during the steady-state turnover of LADH, concluded that there was on the enzyme a group X with a pK of 9.6 and that this pK was perturbed by the binding of NAD^+ to 7.6 (*253*). The scheme is depicted in Fig. 23.

The group plays a significant part in the binding of the substrate and was thought to be either an amino group or a zinc-bound water molecule (*253*).

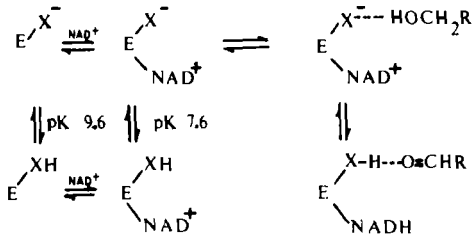


FIG. 23. Schematic representation of the mechanism of alcohol dehydrogenase catalysis (*253*).

McFarland and Chu (*254*), however, have shown that in the LADH-catalyzed reduction of an aromatic aldehyde (β-naphthaldehyde or benzaldehyde) to an alcohol, or oxidation of alcohol to aldehyde, the only pH-dependent parameters are the kinetic binding constant for ternary complex formation and the rate of dissociation for alcohols. The other parameters (catalytic step and isotope effect) are pH independent. This is consistent only with *direct* carbonyl–zinc ion coordination. The same direct coordination has been deduced (*213*) from X-ray studies.

Some of the most detailed solution studies on the nature of the metal ion–substrate interactions have been performed by Mildvan and Weiner (*255*, *256*) and Mildvan *et al.* (*244*) using NMR techniques involving spin-labeled ligands. The NAD^+ analog adenosine-5′-diphosphate-4-(2,2,6,6-tetramethyl-4-phosphopiperidine-1-oxyl) (ADP-R•)

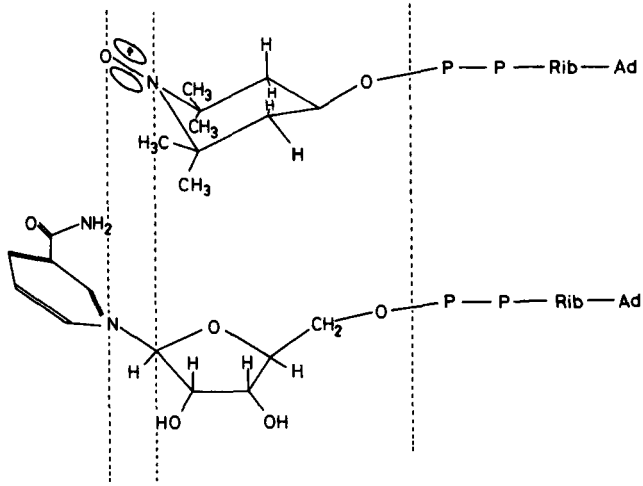


FIG. 24. The equivalence of ADP-R• and NAD. [From Mildvan and Weiner (*255*).]

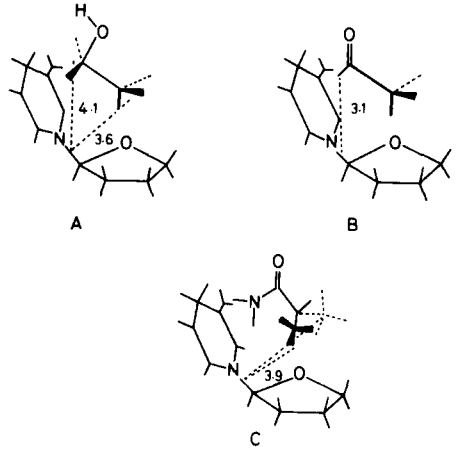


FIG. 25. Coenzyme-substrate (or inhibitor) distances in LADH: (A) ethanol; (B) acetaldehyde; (C)(isobutyramide).

Figure 24, first prepared by Weiner (*222*), has its unpaired electron located in a position equivalent to the nicotinamide nitrogen–ribose carbon bond in NAD^+ (Fig. 24) (*255*). The binding of ADP-R$^\bullet$ to LADH is found to enhance the effect of the unpaired electron on the proton relaxation rate of water (*255*). The addition of ethanol to the binary ADP-R$^\bullet$–enzyme complex reduces the enhancement, leading to the suggestion that the ethanol binds to the solvent side of the bound coenzyme and lies over the ribosidic bond to pyridine (*255*). The relative positions of the appropriate coenzymes and ethanol, acetaldehyde, and isobutyramide (a substrate-competitive inhibitor) as calculated from the enhancement of proton relaxation rates are shown in Fig. 25 (*256*).

A suggestion regarding the involvement of the metal ion is shown (*221*, *244*) in Fig. 26. The use of a spin-labeled iodoacetamide analog

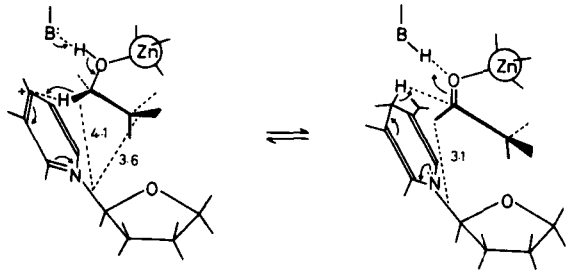


FIG. 26. Mechanism of LADH reaction based on calculated distances between the enzyme-bound substrates and enzyme-bound ADP-R$^\bullet$. [From Mildvan *et al.* (*244*).]

[4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidino oxyl] (Z) in similar studies on the yeast enzyme (*256*) revealed the close similarities between zinc ion function in LADH and in YADH through comparison with NMR (*244*) and X-ray (*212*, *213*) data. The calculated distances between the spin label, NADH, and isobutyramide (*i*-b) in the ternary complex EZ(*i*-b) are shown in Fig. 27. [The locations of zinc and Cys 43 are taken from Eklund *et al.* (*213*) and the YADH primary amino acid sequence (*257*) which is discussed below.] The substrate is thought to be *directly* coordinated to the zinc (*258*); i.e., we have an interaction approaching (II) in Fig. 15 with the coenzyme in an extended conformation.

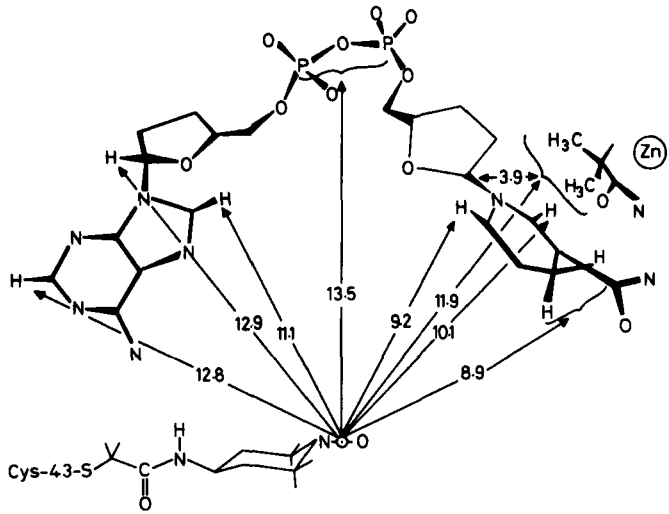


FIG. 27. Metal ion–substrate interaction in YADH. [From Sloan and Mildvan (*258*).]

In similar studies using the hybrid Co(II)Zn(II)-LADH enzyme and the totally cobalt-substituted enzyme, Sloan *et al.* (*227*) have found that binding of isobutyramide occurs with the methyl protons 9.1 Å from the "catalytic" Co(II). The distance shortens to 6.9 Å on binding NADH, and the metal–methyne distance is 6.6 Å (*227*). The construction of the active site that may be reached by consideration of Mildvan *et al.* (*227*, *244*, *257*, *258*) is shown in Fig. 28.

Again we see that a metal-bound water molecule is involved. Eklund *et al.* (*213*), concluded, however, that the water molecule bound to the catalytic zinc was displaced by substrate (see below). This seeming discrepancy may possibly be an effect of substituting Co(II) for Zn(II),

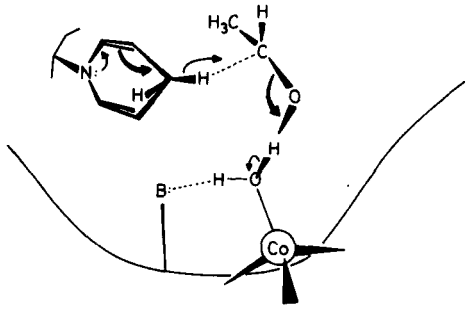
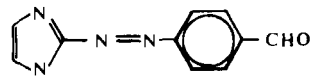


FIG. 28. Metal ion function in Co(II)-LADH. [From Sloan *et al.* (*227*).]

which involves a contraction in metal ion radius of some 0.16 Å and may cause some conformational changes. When 1,10-phenanthroline binds, the water molecule is displaced (*219*).

That there is *no* metal ion–substrate interaction has been suggested recently by Iweibo and Weiner (*232*, and references contained therein) based on studies of apoenzyme–substrate interactions, although there is, of course, no certainty that substrate binding to the apoenzyme occurs at the same site as with the zinc enzyme. In contrast, using spectrophotometric studies on the totally cobalt-substituted enzyme, Shore and Santiago (*230*) suggested direct metal–substrate interaction.

The binding of 4-(2′-imidazolylazo)benzaldehyde (Fig. 29) to LADH results in an unproductive binary complex (*259*, *260*) which has a UV spectrum very similar to that of the zinc(II)-azoaldehyde complexes (*260*).

FIG. 29. 4-(2′-Imidazolylazo)benzaldehyde. 

Both substrate- and coenzyme-competitive inhibitors displaced the aldehyde, indicating that it overlaps both binding sites. If the azoaldehyde is added to an enzyme–NADH mixture, then reaction occurs (*259*, *260*), indicating that, under these circumstances, a productive ternary complex is produced that involves a different interaction with the azoaldehyde. It is not impossible that the initial unproductive binding is via the imidazole to the zinc, whereas in the productive complex the interaction is via the aldehyde. There are also indications that pyrazole, a substrate-competitive inhibitor (*261*), acts by binding directly to the zinc (*262*).

Thus, as with coenzyme binding, there is uncertainty about the mode of metal ion–substrate interaction: NMR indicates an involvement (*227*, *244*, *255–258*); some kinetics suggest direct coordination (*201*, *201a*, *219*, *250*, *254*, *262*, *263*), and others suggest that there may be an alternative ionizable active site group involved in substrate binding (*251*, *252*, *264*, *265*); fluorescence studies and the apoenzyme–substrate interaction suggest (*232*) no involvement, while spectrophotometric (*230*) and X-ray (*212*, *213*) studies indicate the opposite.

F. LADH and YADH: Metal Ion–Enzyme Interaction

Although there are certain differences between YADH and LADH [specifically, subunit structure, the effect of metal ion removal, and activity toward certain substrates (Table XV) (see Section III,C)], the metal ion catalytic function has been treated here as virtually identical in the two enzymes. The basis for this is the following: Sloan and Mildvan (*258*) have established that the metal ion location and function in LADH are the same as in YADH; Jörnvall (*257*) has shown that, in the active site region, the principal amino acid residues are the same in YADH as in LADH. It is of interest here to speculate that it is a difference in amino acid residues that causes the difference in spectral shifts of NADH on binding to the two enzymes. As was discussed in Section III,D, Kosower (*224*) has shown that a shift from 340 to 325 nm on NADH binding by LADH may result from an ammonium ion–pyridine nitrogen interaction; Fig. 18 shows (*213*) an interaction between the ammonium group of Arg 47 and the pyrophosphate of ADP ribose in LADH. In YADH, the residue equivalent to Arg 47 is a histidine (*257*). Furthermore, the kinetic descriptions of the reactions of the two enzymes is the same [ordered bi-bi in both

TABLE XV

Some Differences in Substrate Specificity between LADH and YADH[a]

LADH (relative rate)	Substrate	YADH (relative rate)
100	Ethanol	100
0	Methanol	0.8
108	*n*-Propanol	65
159	*n*-Butanol	60
125	*n*-Hexanol	30
135	*n*-Octanol	40

[a] Data from Sund and Theorell (*201*).

cases (*266*, *267*); the sequence is NADH first and then aldehyde; alcohol leaves first and then NAD^+, and the inhibition patterns are identical, Section III,C].

In LADH, the metal ion is coordinated by two cysteine sulfurs and a histidine nitrogen (Cys 46, Cys 174, and His 67) with a water molecule or hydroxyl ion completing a distorted tetrahedron (*213*). In YADH, the equivalent residues in the active site are identical (*257*). It is felt, therefore, that the basic assumption of a similar catalytic function of the metal ion is justified.

It is of interest that the visible spectrum of cobalt–LADH has been interpreted as possibly representing an "entatic" state (*268*); i.e., the metal may be regarded as being in a distorted or "strained" configuration. The spectrum is represented in Fig. 30. Such phenomena are common in cobalt-substituted zinc metalloenzymes as well as in others and have been reviewed by Williams (*269*). The question of whether such a strained configuration enhances catalysis has been the subject of debate (*268–270*), but surely is evidence of some distortion from the more stable octahedral or tetrahedral environment, and such distortion is commonly associated with catalytic activity.

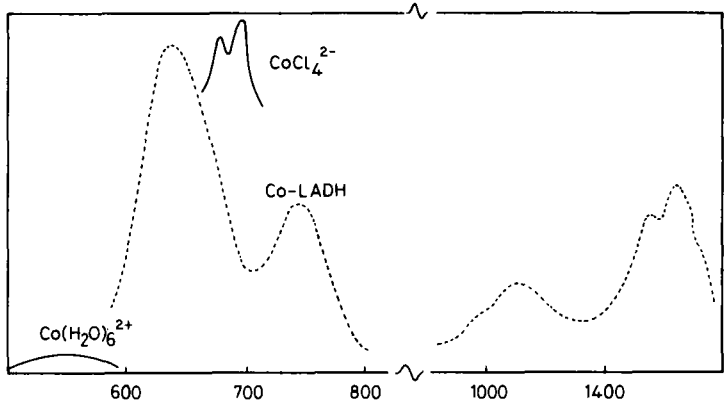


FIG. 30. The visible spectrum of Co(II)-LADH compared with those of the $Co(OH_2)_6^{2+}$ and $CoCl_4^{2-}$ ions (*268*).

IV. Carboxypeptidase A

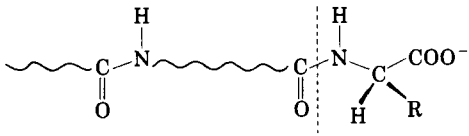
A. Introduction

Carboxypeptidase A, discovered in 1931 by Waldschmidt-Leitz (*271*) has been isolated from the pancreas of several animal species and shown to contain one zinc ion per molecule. It catalyzes the hydrolysis

of a variety of substrates ranging from *N*-acyl amino acids and dipeptides to large proteins. Its molecular weight is 30,000–35,000 depending upon the source and the form of the CPA from a given source. There are four forms of bovine CPA formed by enzymic release from the pancreatic CPA precursor, procarboxypeptidase (*272*). All have comparable specific enzymic activity; the three important forms are designated CPA_α, CPA_γ, and CPA_δ (CPA_β is a contaminant). CPA_α has different crystal cell constants from those of CPA_γ; CPA_δ has the same constants as CPA_α, but it has seven fewer amino acid residues and a different N-terminal residue (Asn instead of Ala). Conformational differences probably exist between CPA_γ and CPA_δ, both of which have 300 residues. These two forms differ in solubility and in the reversible removal of zinc: the γ form is more readily reactivated than the δ. The γ and δ forms have the same sedimentation and electrophoretic mobility.

The main features of the solution chemistry are as follows (*272*, *273*):

i. The substrate must have a free COO^- group at the C-terminal end (III),



L-configuration

(III)

ii. The C-terminal residue must have the L-configuration (but the penultimate residue does not have strict stereospecificity).

iii. The rate of hydrolysis is increased if R (see III) is an aromatic or a branched aliphatic group.

iv. The rate decreases if the NH group is alkylated.

v. At least five residues influence K_m and are therefore probably at a binding site about 18 A long.

vi. CPA shows esterase as well as peptidase activity, but the sequence of metal ions replacing zinc to give an active enzyme is not the same: the order for peptidase activity is Co > Zn = Ni > Mn > Cu(zero), whereas the order for esterase activity is Cd > Mn > Co > Zn = Ni > Hg > Cu(zero). This is illustrated quantitatively in Table XVI (*272*, *273a*). A Co(III) derivative of CPA can be prepared; although there is controversy concerning its catalytic activity (*274*), it appears that the activity of the derivative depends upon the method of metal

TABLE XVI

STABILITY AND KINETIC CONSTANTS FOR METALLOCARBOXYPEPTIDASES[a]

Metal ion	Log K^b	Substrate (a) BGGP K_m	(a) BGGP k_{cat}	(b) BGP K_m	(b) BGP k_{cat}	(c) CGP K_m	(c) CGP k_{cat}	(d) HPLA K_m	(d) HPLA k_{cat}
Zn	10.5	8.0	1.2	8.1	5.6	19.5	5.5	0.76	28.6
Ni	8.2	7.4	1.1	—	—	—	—	2.1	27.6
Co	7.0	6.0	5.9	4.8	7.4	11.7	12.3	0.98	37.7
Mn	5.6	2.9	0.23	1.1	0.45	22.9	2.3	3.2	56.8
Cd	10.8	—	—	—	—	—	—	5.5	61.5
Hg[e]	21.0	—	—	—	—	—	—	—	—
Cu	10.6	—	—	—	—	—	—	—	—

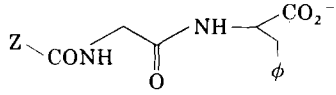
[a] From Quiocho and Lipscomb (*272*).

[b] K is corrected for competition by 1 M Cl^- and 0.05 M Tris buffer, pH 8.

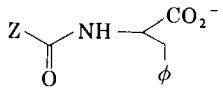
[c] K_m values are $M \times 10^4$; k_{cat} values are $min^{-1} \times 10^{-3}$.

[d] In the following designation of substrates (a)-(d), Z = $\phi CONH\text{-}CH_2$- and W = $\phi CH_2OCONH\text{-}CH_2$-:

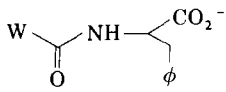
(a) benzoylglycylglycyl-L-phenylalanine



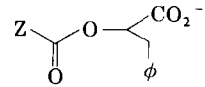
(b) benzoylglycyl-L-phenylalanine



(c) carbobenzoxyglycyl-L-phenylalanine



(d) benzoylglycyl-L-phenyllactate (hippuryl-L-phenyllactate)



[e] Some peptidase activity in crystals of CPA_γ has been reported (*273a*).

substitution (*275*), direct metal exchange and subsequent oxidation of Co(II) to Co(III) yielding a Co(III)-PCA, which has esterase activity.

vii. Peptide substrates bind to the apoenzyme and then prevent access of $^{65}Zn^{2+}$, suggesting that the substrate binding site is close to the metal ion.

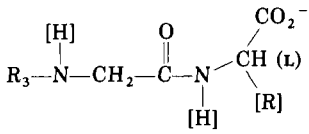
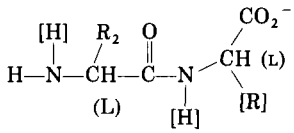
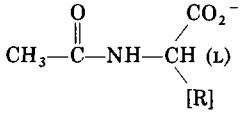
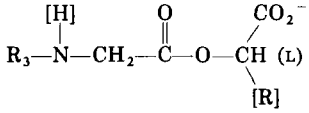
viii. The binding of various substrates depends upon whether or not the zinc ion is present. Table XVII summarizes the experimental findings (*273*). It is interesting that substrates 3, 4, and 5 will bind if metal ion is present, and then metal exchange is prevented. This suggests that

binding to the apoenzyme is cooperative and a C-terminal side chain in the L configuration, a free NH of the C-terminal residue, and a free NH of the penultimate residue are all required. Metal substitution gives derivatives that bind the substrates in Table XVII, but as the metal at the active site is changed it is found that the substrate binding tendency does not correlate with metal activity (e.g., Cu(II) induces substrate binding but the Cu(II)–ES complex is inactive). This is a good illustration of the distinction between binding and activity.

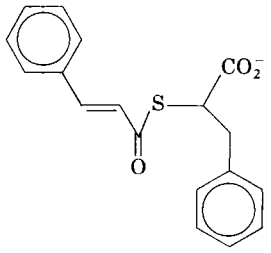
Again, caution must be exercised before one states that a metal-substituted metalloenzyme is inactive because the copper derivative,

TABLE XVII

BINDING OF SUBSTRATES TO CARBOXYPEPTIDASE A HOLO- AND APOENZYME

Substrate	Binding to holoenzyme	Binding to apoenzyme
1. Acylated dipeptide 	Bound	Bound
2. Dipeptide 	Bound	Bound
3. Same as 2, but configurations are (D)-(L)	Bound	Not bound
4. *N*-Acyl amino acid 	Bound	Not bound
5. Ester analog of acylated dipeptide 	Bound	Not bound

although inactive for the hydrolysis of the normal peptide and carboxylic ester substrates, will catalyze the hydrolysis of the thiol ester IV.



(IV)

The Cu(II)–PCA is about 45% as active as the zinc enzyme, but there is an intriguing stereospecificity difference between the zinc and copper enzymes: the former catalyzes the hydrolysis of both the D and L forms, the latter that of the L form only (*276*).

ix. Organic groups on the protein ligand are also involved in the catalytic function; thus acetylation with *N*-acetylimidazole causes peptidase activity to decrease and esterase activity to increase. The spectrum of the acylated enzyme shows that two tyrosine residues have formed *O*-acetyl derivatives. One of the two tyrosines can be selectively nitrated with $C(NO_2)_4$, and peptidase activity is then destroyed.

x. Esters and peptides inhibit each other competitively; therefore the peptidase and esterase sites must at least overlap, even if they are not the same.

The structure and function of carboxypeptidase A have been the subject of a number of reviews (*272–273a*), and we focus briefly here on some recent developments and on those aspects concerned with the role of the zinc ion.

B. Structure of Carboxypeptidase A; Coordination of Zinc and Interaction with Substrate

The structure of bovine CPA has been determined at a resolution of 2 Å (*277, 278*). It is an ellipsoidal molecule of approximate dimensions 50 × 42 × 38 Å. About 48% of the protein ligand has a random-coil structure that undergoes conformational changes on substrate binding.

The zinc ion is coordinated to the protein by two imidazole side chains and a carboxyl group of the three residues: His 69, Glu 72,

and His 196. A water molecule completes distorted tetrahedral coordination of the zinc ion, which lies in a groove and pocket in the enzyme surface.

Ideas on the possible mechanism of action in terms of the structure were aided by the determination of the structure of a Gly-L-Tyr complex with the native enzyme (*277*). The dipeptide is hydrolyzed 10^3 times more slowly than the best substrates and more slowly still if the crystals are cross-linked with glutaraldehyde (*279, 280*). In this way a sufficiently durable binding of the Gly-L-Tyr at the active site was obtained to enable the structure of the complex to be determined (*277*).

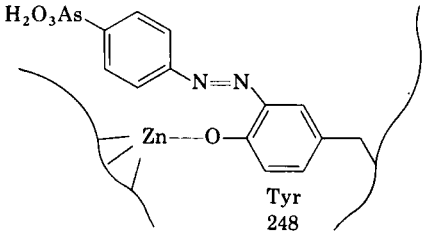
The substrate lies in the groove containing the zinc ion. The aromatic side chain of the substrate falls in a pocket that contains no specific binding groups and is large enough to accommodate a tryptophan side chain. This feature accounts for the observed specificity for the side chain of the C-terminal amino acid of the substrate, since the best substrates for carboxypeptidase A are those that have an aromatic or branched aliphatic side chain at the C-terminal residue (*281*). The most commonly used peptide substrate is carbobenzoxy-Gly-L-Phe (CGP) (cf. Table XVI). The free carboxyl group interacts with the positively charged guanidinium group of Arg 145. The carbonyl oxygen of the —CONH bond undergoing attack replaces the water molecule coordinated to the zinc ion. The carboxyl group of Glu 270 binds through a water molecule to the free amino group, an interaction possible only with dipeptide substrates with a free N-terminal amino group. The hydroxyl group of Tyr 248 is ~2.7 Å away from the nitrogen of the attacked peptide bond and ~3.5 Å from the α-amino group.

Figure 31A depicts the substrate (heavy bonds) interacting with the zinc ion and the active groups of the protein ligand. This quite complex interaction is brought about by some interesting conformational changes in the molecule induced by the binding of Gly-L-Tyr (see Fig. 31B) (*278*). Thus, the guanidinium group of Arg 145 moves 2 Å owing to rotation about the C_β—C_γ bond of the side chain, and the carboxylate of Glu 270 also moves by about 2 Å owing to rotations about both the C_α—C_β and C_β—C_γ bonds. Most spectacular of all, the phenolic group of Tyr 248 swings through 12 Å, mainly by rotation about the C_α—C_β bond to bring its —OH to bear on the peptide nitrogen. There are, also, some related movements of the peptide backbone and a system of hydrogen bonds in the region between Arg 145 and Tyr 248. These conformational changes provide an example of Koshland's "induced fit" theory (*282, 283*): this proposes that substrate interaction leads to conformational changes in the protein that are related to the electronic changes in the enzyme–substrate complex needed to effect catalysis. Binding of the C-terminal side chain

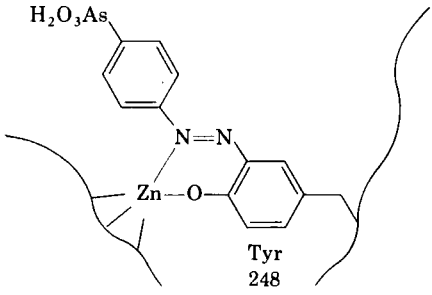
of the substrate in the pocket at the active site ejects the H_2O from this cavity, and the movement of Tyr-248 effectively closes off the active site from the solvent.

From the viewpoint of zinc coordination the question arises whether or not the hydroxyl group of Tyr 248 is coordinated to the zinc ion in the resting enzyme and has to be displaced when the substrate binds. Information on this is obtained in an interesting way by labeling the phenolic side chain of this residue with the arsanilazo group: arsanilazophenols show a marked color change on coordination to a metal ion, and coordination of the phenolic group may be monitored by observing such changes. The color change observed with CPA crystals depends upon the crystal modification: A_α, A_γ, and A_δ. In solution, the arsanilazo derivatives of all three are red, and the visible spectrum is similar to that of metal complexes of the azoaromatic ligand (*284*). When the A_γ arsanilazo derivative is crystallized, the crystals are yellow, suggesting that the modified Tyr 248 no longer coordinates zinc at the active site (*285*). On the other hand, the crystals of arsanilazocarboxypeptidase A_α of the type used for the crystal structure determination remain red (*286*). Crystals of carboxypeptidase A_α, elongated along the *a* axis, were those on which the crystal structure was done, so that the crystal packing in the A_γ derivative must prevent the orientation of the modified residue for coordination to the metal ion.

In solution then, arsanilazotyrosine-248 carboxypeptidase A forms a complex with the Zn(II) at the active site probably by coordination of the free phenolic hydroxyl group of the modified tyrosine as shown in structures (V) or (VI) (*284*, *287–289*).



(V)



(VI)

The absorption characteristic of the zinc(II)–arsanilazotyrosine complex is observed only with the zinc(II) enzyme, and it is stable only near neutral pH (*284*). Its formation and dissociation have two apparent pK_a values, 7.7 and 9.5 (*284*). The curve leading up to the

lower pK_a may be regarded as a formation curve for the complex, as the proton is displaced from the azophenolic hydroxyl group to form the zinc complex. The upper pK_a corresponds to dissociation of the anionic form from the zinc(II) ion: possibly hydroxide ion competes for the zinc(II) coordination site (*284*). The arsanilazo enzyme is still catalytically active, and addition of the dipeptide Gly-L-Tyr causes dissociation of the complex of zinc(II) with the arsanilazotyrosine group (*284*), the substrate displacing the coordinated phenolic hydroxyl group. The solution studies indicate that the phenolic OH is in a position to coordinate the zinc ion, and it may do this at least some of the time in the native enzyme (*284*), although the electron density map suggests that the side chain is mobile and free to rotate in solution. However, it becomes fixed near the peptide bond in the Gly-L-Tyr complex, and the phenolic hydroxyl group of the arsanilazotyrosine 248 may also behave similarly.

Resonance Raman studies (*290*) of arsanilazocarboxypeptidase A have shown that azophenol coordination can occur as shown in (VI), a type of coordination well established for azophenol ligands (*291*, *292*). Resonance Raman spectroscopy is a particularly useful tool since it is applicable to both solids and solutions; its application to both states of carboxypeptidases A (and also B) will be particularly interesting since the kinetic properties of the enzymes are significantly altered upon crystallization (*293*, *294*).

C. Role of Zinc and Enzyme Side Chain–Substrate Interactions in the Catalytic Mechanism

1. Structural Implications for the Mechanism

We have seen that the coordinated Zn(II) ion, the side chains of Glu 270, and Tyr 248 are close enough to the substrate to suggest direct assistance in catalysis. Zinc can clearly function as a Lewis acid and polarize the carbonyl of the peptide bond. Although the complex is unipositive (a carboxylate and two neutral donors as ligands), the single formal net positive charge (which, of course, says nothing of the actual site charge distribution, which may be more or less diffuse) may exert a powerful polarizing effect by finding itself in a relatively nonpolar environment when water is ejected from the side-chain binding cavity and the Tyr 248 side chain partially closes the cavity (*278*). The interaction between the free carboxylate of the substrate and Arg 145 may assist in locating the phenolic hydroxyl of Tyr 248 in the vicinity of the peptide bond. This could clearly explain the absolute necessity for a free carboxyl group if the hydroxyl of

Tyr 248 near the peptide bond is necessary for peptide hydrolysis (*278*, *295*).

The carboxyl group of Glu 270 may engage in nucleophilic attack on the peptide carbonyl carbon so forming a (labile) anhydride, or may function as a general base catalyst by accepting a proton from a water molecule poised near the carbonyl group. Tyr 248 may then, by a proton shift in a hydrogen bond, donate a proton to the incipient amino group as the peptide bond breaks. With dipeptide substrates another hydrogen bond may be made between the tyrosyl oxygen and the terminal NH_2 (or NH in substituted dipeptides) and such hydrogen bonds could help to produce strain at the substrate peptide bond. These postulated mechanistic features are summarized in Fig. 31. Of the two possible mechanisms, general base catalysis (Fig. 31C) and the specific nucleophilic attack by Glu 270 with anhydride formation (Fig. 31D), the latter has recently been favored (*278*, *296*).

The features shown in Fig. 31 are based on structural information from a relatively nonproductive complex, and, although the key structural features in an active enzyme–substrate complex may closely resemble those shown, the prediction of mechanism from structural information alone is hazardous. It is necessary to examine how far the extensive physicochemical and kinetic data on CPA are consistent with the mechanistic implications of Fig. 31, which we have just discussed.

2. *Comparison of Chemical Properties with the Structural Features*

The zinc ion has been shown to be essential for both peptidase and esterase activity (*295*, *297–299*). Substrate-binding studies showed that the metal ion was not, however, necessary for the *binding* of dipeptide or N-substituted dipeptide substrates: both form stable complexes with the apoenzyme and then prevent binding of the metal ion (*300*, *301*). A series of comparative binding studies with a variety of substrates, (see, for example, Section IV,A) established that the groups on the substrate necessary for binding to the apoenzyme were the side chain on the C-terminal residue (aromatic or branched aliphatic), the NH of the susceptible peptide bond, and the NH or NH_2 of the penultimate residue. That these three substrate groups have specific interactions with the protein is clear from Fig. 31A. However, the analogous ester substrates or N-acylamino acids do require a metal ion for binding, presumably because the former lack the NH on the C-terminal residue and the latter the penultimate NH or NH_2. Thus, the metal may have a substrate orientation effect besides polarizing the substrate carbonyl group. Such an effect may also occur with peptide substrates, although

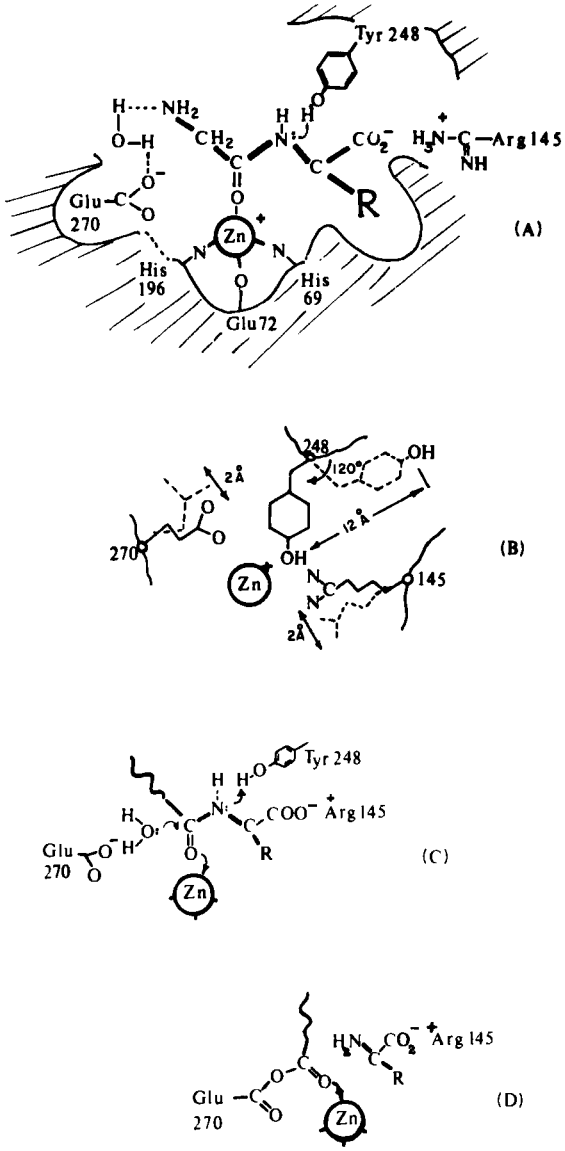


FIG. 31. The active site of carboxypeptidase A. (A) Schematic diagram showing side-chain interactions with substrate, the bonds of which are shown with heavy lines. (B) Side-chain movements on substrate binding. (C) General base-catalyzed attack on the peptide bond. (D) Nucleophilic attack by the carboxyl group of Glu 270 giving a labile anhydride.

the stronger interaction of the latter with the protein side groups provides for complex formation with the apoenzyme. The free C-terminal carboxyl is not absolutely necessary for binding, since amides will bind, but it is an absolute requirement for catalysis, possibly because its interaction with Arg-145 triggers conformational changes necessary for catalysis (*278*).

Chemical modifications, particularly those involving Tyr 248 (and at least one other tyrosyl residue) have been illuminating. Modifications involving Glu 270 and Arg 145 unfortunately have given less definite results (*302*). The chemical modifications and their effects on peptidase and esterase activity are extensive and are summarized by Chlebowski and Coleman (*15*). Briefly, most acylations appear to acylate the hydroxyl groups of tyrosyl residues (*302*, *303*). When acetylimidazole and acetic anhydride are the acylating agents, the O-acetylation of two tyrosyl residues is indicated from the spectroscopic changes (*303*, *304*). One of these residues is probably Tyr 248, although in only two of the modifications, iodination and reaction with diazotized arsanilic acid, has reaction with Tyr 248 been directly identified by the key experiment of isolation of the peptide containing the modified residue (*305*, *306*).

The most significant result of the tyrosyl modifications is the loss of peptidase activity and remarkable enhancement of esterase activity accompanying the apparent modification of Tyr 248. Thus, Tyr 248 would appear to be required for peptide hydrolysis, but not for the hydrolysis of the substrate ester used in these studies, hippuryl-β-phenyllactate (see Table XVI). The modification appears to decrease the affinity of peptide binding to the apoenzyme (*307*), as might be expected from the loss of the hydrogen bond interaction of Tyr 248 with the C-terminal NH (Fig. 31A).

The nitration of one tyrosyl residue with tetranitromethane abolishes most of the peptidase activity, so that residue 248 is probably nitrated (*308*).

3. *Comparison of Kinetic Behavior with the Structural Features*

Carboxypeptidase A kinetics are not simple, and substrate inhibition and product inhibition and activation have caused difficulties (*309–311*). However the pH–rate profile for CPA-catalyzed peptide hydrolysis is bell-shaped with an apparent pK_a on the acid side of ~ 6.5 and one on the alkaline side near 9.4, the optimum occurring at about pH 7.5 (*311*). Although the pH–rate profile for the ester substrate, hippuryl-β-phenyllactate, is not bell-shaped, a series of esters, including *O*-acetyl-L-mandelate and *O*-(transcinnamoyl)-L-β-phenyllactate, show

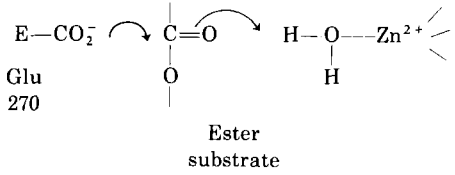
bell-shaped pH–rate profiles. The apparent pK_a values of ionizations on the free enzyme that affect activity are one of 6.5 ± 0.4 and another that varies from 9.4 to 7.5 (*296*). The pK_a of 6.5 could be attributed to Glu-270, required in its carboxylate form. This pK_a is rather different from those usually observed for β- or γ-carboxylic acid groups, but may be shifted by the environment in the protein. The observed shifts in the value of the alkaline pK_a, 9.4–7.5, and the fact that acetylation of Tyr-248 stops peptide hydrolysis and the hydrolysis of certain esters (e.g., *O*-acetyl-L-mandelate), but not others (e.g., the β-phenyllactate esters), make it less certain that the alkaline pK_a is that of Tyr 248 (*296*). It has recently been suggested that the alkaline pK_a is that of a Zn-coordinated H_2O molecule (*296*) and the observed pK_a is then a composite value reflecting competition between the substrate carbonyl and OH^- for zinc coordination.

As we have seen, there are marked differences between peptide and ester hydrolysis, and it is likely that at least some features of the mechanism differ in each case. For CPA-catalyzed hydrolysis of peptides, for example, there is no kinetic solvent isotope effect on the value of $k_{cat}/K_{m\,app}$, and the effect on k_{cat} is small ($k_{cat}H_2O/k_{cat}D_2O$ = 1.33 ± 0.15) compared with that expected for a reaction in which proton transfer occurs in a rate-determining step (*296*). In contrast, the $k_{cat}H_2O/k_{cat}D_2O$ ratio for the reaction of the ester is about 2, suggesting that a catalytic step involving proton transfer becomes important in the enzyme-catalyzed hydrolysis of esters. Comparison of the CPA-catalyzed hydrolysis rates of a series of esters and peptides showing simple kinetic behavior reveals that esters are about 5×10^3 times more reactive than the corresponding peptides (*296*).

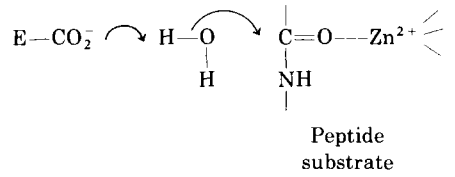
The lack of a solvent isotope effect in peptide hydrolysis and the existence of one in ester hydrolysis may be accommodated in terms of the mechanism involving anhydride formation with the carboxyl of Glu 270 (Fig. 31,D). It has been suggested that a tetrahedral intermediate (analogous to that observed in model ester hydrolyses) breaks down to give an anhydride species and the α-amino or α-hydroxy acid product (*296*). The lack of a solvent isotope effect in peptide hydrolysis suggests that anhydride *formation* may be rate-controlling, in which case an isotope effect would not be expected, whereas the solvent isotope effect on ester hydrolysis could be explained if *hydrolytic breakdown* of an anhydride intermediate were rate-controlling.

In this connection an experiment (*312*) on the esterase activity of CPA using the ester *o*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate as substrate is particularly interesting. Reduced-temperature kinetics were used to demonstrate the formation of an acyl-enzyme intermediate that is sufficiently stable at −60°C for spectral characterization and

accounts for the biphasic kinetics of the hydrolysis. These results and knowledge of the active-site groups led to the conclusion that a mixed anhydride intermediate forms at Glu 270 during the reaction:



Again, a zinc-bound water molecule is implicated in the reaction.* It has been suggested that such is not the case in peptidase action, a different alignment existing at the active site (*313*):



Calculations on the electrostatic environment of the scissile peptide bond suggest that catalysis is enhanced by stabilization of the products rather than by an induced polarization by the Zn(II) ion (*314*).

It is interesting that extensive studies of the kinetics of CPA-catalyzed hydrolysis indicate the existence of deeper subtleties; e.g., more than one substrate or inhibitor binding site and productive and nonproductive binding in one site have been proposed (*309*, *310*, *315*, *316*), and the decay of an E · S complex followed fluorimetrically have indicated that, at least in some cases of noncompetitive inhibition, two sites must be involved (*317*). Thus, structural information enables us to glimpse some of the broad features of the catalytic mechanism, but much remains tantalizingly concealed.

V. Conclusions

For carbonic anhydrase we have seen how modern kinetic and physical techniques have revealed much about the mechanism of the CO_2 hydration reaction without revealing all the secrets of the process.

* The Co(III)-enzyme could readily retain esterase activity in an analogous way, since no Co(III)-ligand fission (likely to be a slow process) is required.

The specificity for zinc and cobalt emerges clearly. The question here is: how does the zinc (or cobalt) work? Evidence is not firm that CO_2 coordinates during reaction, but the possibility has not been disproved. Indeed, it has recently been proposed that CO_2 binds weakly to the fifth coordination site of the zinc ion in the hydrophobic region of the active site; this suggestion is based on the results of a crystal structure study of the HCAB-imidazole complex (*122*), previous studies having indicated that imidazole is a competetive inhibitor of CO_2 hydration (*107*). The general chemistry of CO_2 hydration catalysis shows the interesting feature that a combined Lewis acid–proton acceptor is the most effective catalyst. Again, there is some evidence that a zinc-bound water molecule dissociates to give the effective nucleophile, but it is reasonably clear that a hydroxo complex, M—OH, *by itself*, is inadequate for the high enzyme reactivity. However, coupled with a proton-transfer group or groups at the active site an $(His)_xM$—OH complex bears a strong resemblence to the effective inorganic catalysts of CO_2 hydration.

The high reactivity of the carbonic anhydrases raises the intriguing question whether, in the course of evolution, maximum reactivity has been achieved. In contrast to the other two enzymes we have been considering, it is not difficult to answer this question for carbonic anhydrase. It can be shown [see, for example, Fersht (*318*)] that where the organism requires maximum rate [although this is true for most enzymes, there are cases where the rate is subordinate to control (*318*)] a criterion of the state of progress in evolution has two parts: (a) that k_{cat}/K_m is maximized, and (b) that K_m is greater than $[S]$.

The maximum value of k_{cat}/K_m is 10^8–10^9 sec^{-1} mol^{-1}, and a perfectly evolved enzyme should have a value for this ratio in this range and the additional feature that $K_m > [S]$. For carbonic anhydrase criterion (a) is met, and under physiological conditions the concentrations of CO_2 and HCO_3^- are such that the enzyme is only 6% saturated with each substrate and the K_m for CO_2 is too large to be measured. By these criteria then, carbonic anhydrase has already reached the stage of evolution of maximum rate.

The evidence on the role of zinc in the dehydrogenases, LADH and YADH, indicates that the coenzyme has an adenine binding site well removed from the catalytic zinc ion: NADH and NAD^+ bind in an extended conformation with the nicotinamide ring projecting near to the catalytic zinc but not coordinated to it, although as we have seen there is conflicting evidence on the latter point. The substrate is probably able to coordinate to the zinc in the enzyme–coenzyme complex, but again there is uncertainty about the precise mode of enzyme–coenzyme–substrate interaction.

Carboxypeptidase A, the first metalloenzyme to have its structure determined, shows remarkable conformational changes of large magnitude on substrate binding, and there is no better illustration of the key factor in the functioning of a metalloenzyme, viz., a cooperative process involving the metal ion and groups on the ligand protein functioning in concert to attack the substrate at several points. It seems likely that a zinc-bound water molecule plays a role in the esterase activity of CPA, but probably not in its peptidase activity.

VI. Glossary

Amino acids and protein structure: The essentials (*318a*, *318b*) are indicated in Charts 1 and 2.

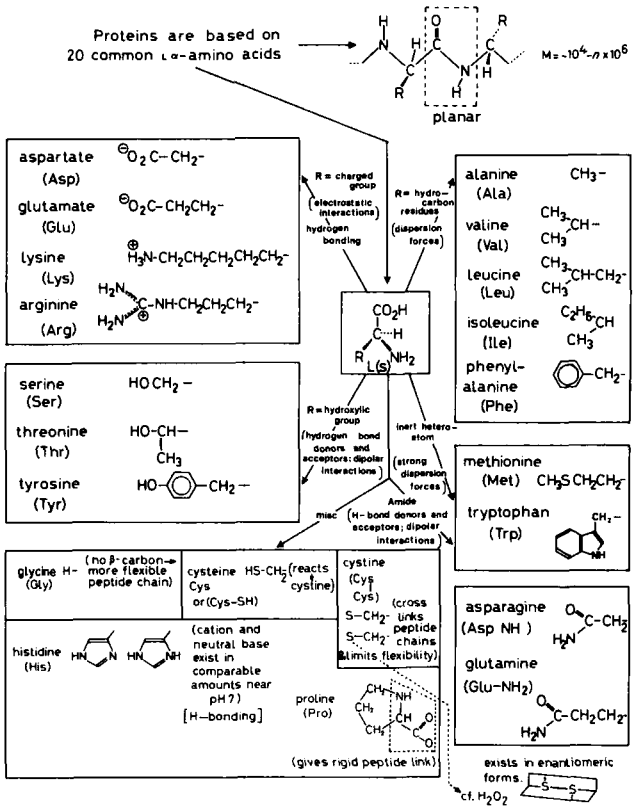


CHART 1. Amino acids. In writing a polypeptide, for instance, Gly-Glu-Arg—etc.—Ala, hyphens mean that the sequence is known (if it is not, commas are used). The free amino group is at the left (on Gly) and the free carboxyl at the right (on Ala).

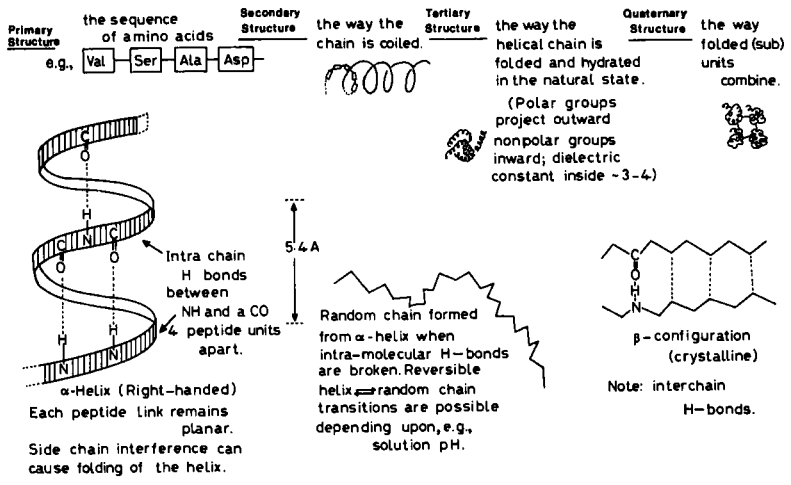


CHART 2. Protein structure. The properties of a protein are governed by its amino acid sequence, which is determined genetically, and by its structure.

Apoenzyme and holoenzyme: The apoenzyme is the protein part of the enzyme, e.g., a metalloenzyme from which the metal ion has been removed; more generally the term is used for an enzyme–coenzyme system without the coenzyme. The term holoenzyme refers to the enzyme complete with its metal ion or coenzyme. Some confusion is possible; e.g., in literature on dehydrogenases which use NAD^+ or the phosphate derivative, $NADP^+$, as coenzyme, the complex of the enzyme and coenzyme is called the holoenzyme and the free enzyme is called the apoenzyme, although with YADH and LADH it would contain zinc ions. The context usually makes clear the meaning intended.

Conformational change: A conformational change is a change in the average positions of the atomic nuclei, but does not include covalent bond changes. Thus, unfolding or rotation about the bonds of a protein is a conformational change, but the dissociation of a proton is not, although it may accompany a conformational change. Polarizations of electrons without change in the positions of the atomic nuclei are not conformational changes.

Enzyme kinetics (318c), a resumé: Experimentally, the concentration of enzyme, E, at least in most *in vitro* experiments, is negligible compared with that of the substrate, S, and as the concentration of the substrate is increased a saturation effect on the initial reaction velocity, v, is observed (Fig. 32a). Analysis of the experimental data shows that the variation of initial rate with substrate concentration is

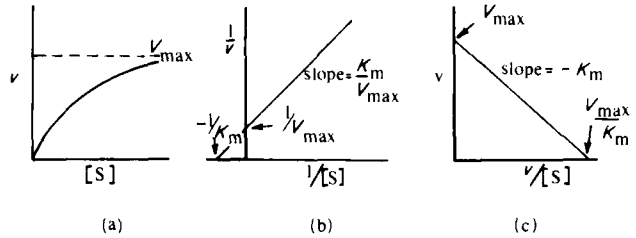


FIG. 32. The typical behavior of an enzyme-catalyzed reaction: (a) initial velocity, v, is plotted against substrate concentration [S]; (b) Lineweaver–Burk plot; (c) Eadie–Hofstee plot.

given by

$$v = \frac{V_{max}[S]}{([S] + K_m)} \tag{1}$$

and $V_{max} = k_0 E_0$. Equation (1) is called the Michaelis-Menten or Henri equation. K_m is called the Michaelis constant; k_0, which is V_{max}/E_0, is called the *turnover number* of the enzyme, the number of moles of substrate converted per mole of enzyme per unit time. Thus, the turnover number (sec^{-1}) of carboxypeptidase A is about 10^2, that of carbonic anhydrase 10^4–10^5, depending on the isozyme. Most turnover numbers are in the region of 10^2–10^4, although that of catalase is exceptionally high at 10^7. The rate observations are sometimes plotted in reciprocal form as a Lineweaver–Burk (Fig. 32b) or Eadie–Hofstee plot (Fig. 32c).

Michaelis and Menten, developing earlier ideas, proposed a mechanism in which E and S were in thermodynamic equilibrium with an enzyme–substrate complex, ES:

$$E + S \underset{K_s}{\rightleftharpoons} ES \xrightarrow[k_{cat}]{} EP$$

This scheme leads simply to the equation

$$v = \frac{[E_0][S]k_{cat}}{([S] + K_s)} \tag{2}$$

where $K_s = [E][S]/[ES]$. K_m is clearly the equilibrium constant in this scheme, i.e., the dissociation constant of the ES complex; the larger the K_m, the lower the stability of the ES complex.

The *mechanism* is only appropriate when k_{cat} is very much less than the rate of dissociation of ES. The Eq. (1) holds for many mechanisms,

but the particular mechanism of Michaelis and Menten is not always a correct description of the processes actually occurring in the system. Equation (1) can also be derived from a steady-state approach in which $d[ES]/dt = 0$, the Briggs–Haldane treatment; writing the mechanism as

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

it is readily shown that

$$v = \frac{[E_0][S]k_2}{[S] + \dfrac{(k_2 + k_{-1})}{k_1}}$$

which is identical with Eq. (1), where $K_m = (k_2 + k_{-1})/k_1$. Clearly if $k_{-1} \gg k_2$, $K_m = K_s$.

A mechanism using any number of intermediates, a two-step consecutive reaction ($k_{-1} \ll k_2$) and one involving a nonproductive ES complex, all lead to rate equations of the form of (1). K_m and k_0 are then composite. This question is discussed more fully in reference *318*, but we may note two points. First, although $K_m = K_s$ only for the Michaelis–Menten mechanism, K_m may be treated for some purposes as an apparent dissociation constant. For instance, the free enzyme concentration may be calculated from the relation

$$\frac{[E][S]}{\sum[ES]} = K_m$$

where $\sum ES$ is the sum of all the bound enzyme species (*318*). Care is needed in interpreting the effects of temperature, pH, etc., on an apparent K_m because the rate constants contained in this composite term are also affected. Similar remarks also apply to k_{cat}, which again is a composite function of rate constants of individual steps in mechanisms more complex than that corresponding to the Briggs–Haldane scheme. As Fersht has pointed out (*318*), there are examples in the literature where breaks in the temperature dependence of k_{cat} have been interpreted as conformational changes in the enzyme when they are really due to a different temperature dependence of the individual rate constants comprising k_{cat}. Second, although it would appear that little mechanistic information can be obtained by kinetic measurements per se, a diagnostic test of the Briggs–Haldane mechanism is possible using rapid reaction techniques to measure k_1, the rate of association between E and S. It can be shown (*318*) that association rate constants

should be of the order of 10^8 mol^{-1} sec^{-1}. Where $k_2 \gg k_1$ then $k_1 = k_{cat}/K_m$; if the latter ratio is $\sim 10^7$–10^8, then the Briggs–Haldane mechanism operates. This is true for a variety of enzymes, including, for instance, carbonic anhydrases (*318*).

From a purely mechanistic viewpoint it is unlikely that a simple scheme, such as the Michaelis–Menten or Briggs–Haldane scheme, will work without modification since each has basically three deficiencies. These are: (i) enzyme reactions are reversible so the step $E + P \rightarrow ES$ must be considered (but in steady-state conditions $[S] \gg [P]$, so that this is not serious); (ii) there should be at least one other step in which EP, product bound to enzyme, is considered; (iii) if there is a conformational change, e.g., when S combines with E, it is not usually possible to measure this kinetically (except when [E] is stoichiometric, not catalytic).

The most useful general picture is probably to regard an enzyme-catalyzed reaction as being made up of three elementary steps:

$$E + S \overset{1}{\rightleftharpoons} ES \overset{2}{\rightleftharpoons} E'S \overset{3}{\rightleftharpoons} EP \overset{1}{\rightleftharpoons} E + P$$

Step 1 is the formation and dissociation of an enzyme/reactant complex; step 2 is a conformational change in the complex; and step 3 is the chemical transformation, bond making or rupture, or electron or proton transfer.

As we have seen, combination rates for step 1 are often close to 10^8 and most fall in the range 10^6–10^8; however, dissociation rates of ES vary over many orders of magnitude. There is a parallel here with metal complex formation (rates of complex formation for a given metal with various ligands show relatively small variation whereas rates of dissociation vary widely and control stability), and the dissociation constants of acids ($HA \underset{b}{\overset{a}{\rightleftharpoons}} H^+aq + A^-aq$ – k_a varies widely, k_b relatively little).

For a description of the issue of the pH dependence of K_m and k_{cat}, the reader is referred to reference *318*.

Enzyme inhibition: Enzymes may be irreversibly inactivated by heat or chemical reagents; active-site-directed irreversible inhibitors or *affinity labels* are important in identifying catalytically important residues (*318*, *319*), and hundreds of them have been synthesized. They involve covalent bonding to active residues in the protein, and some of them are important in chemotherapy. The inhibitor may, however, bind reversibly and noncovalently: there are four main types of such inhibition.

1. Competitive inhibition: If the inhibitor, I, binds reversibly at the active site of the enzyme and prevents S binding and vice versa, I is

said to be a competitive inhibitor. For enzymes conforming to Eq. (1) this appears on a Lineweaver–Burk or Eadie–Hofstee plot as shown in Fig. 33a (*318, 320*). V_{max} is unaffected but K_m is increased. Results are often expressed in terms of an inhibition constant, K_I (see, for example, Tables XI and XIII) where K_I is [E][I]/[EI], i.e., the dissociation constant of the EI complex.

2. Noncompetitive inhibition: If the inhibitor binds at a site other than the active site, but such binding decreases the reactivity of the ES complex, the inhibitor is said to be noncompetitive because there is no competition with substrate for the active site. This behavior gives rise to the plots shown in Fig. 33b. Here V_{max} is decreased, but K_m is unaffected. Provided that the mechanism of the catalysis is two step, noncompetitive inhibition can indicate the existence of an equilibrium between S, E, and ES ($k_{-1} \gg k_2$): the observation of noncompetitive inhibition (k_0 varies, but K_m remains constant) shows that k_2 must be much less than k_{-1} for K_m to be constant because $K_m = (k_{-1} + k_2)/k_1$. The hydrogen ion is a typical noncompetitive inhibitor.

3. Mixed inhibition: For a Michaelis–Menten mechanism, it is readily shown that noncompetitive inhibition is observed when the

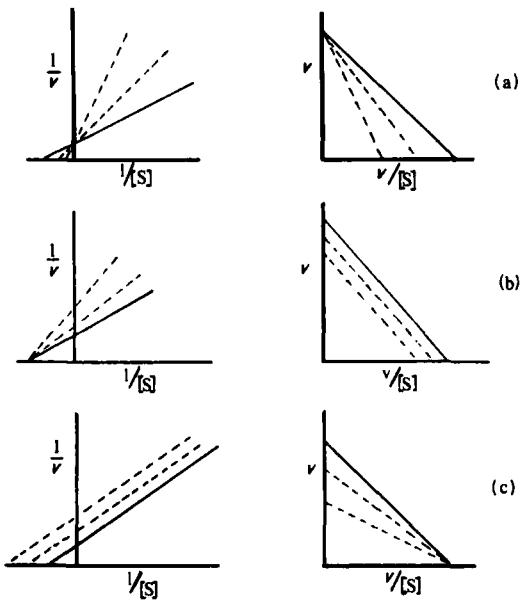


FIG. 33. Types of enzyme inhibition and their effects on Lineweaver–Burk (left) and Eadie–Hofstee (right) plots. The solid line is the plot with zero inhibitor concentration; the dashed lines are plots with inhibitor present at successively larger concentrations. (a) Competitive inhibition. (b) Noncompetitive inhibition. (c) Uncompetitive inhibition.

inhibitor has the same affinity for E as for ES (*318*); where this affinity differs, both K_m and k_{cat} are altered and the inhibition is described as mixed, with obvious consequences for the Lineweaver–Burk and Eadie–Hofstee plots.

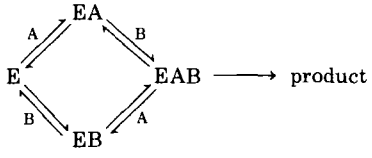
4. Uncompetitive inhibition: Here the inhibitor binds to ES, but not to E, and gives the behavior shown in Fig. 33c. This behavior can also arise (*321*) in the case where K_m approaches k_2/k_1; i.e., k_{-1} is small and the inhibitor has an effect on k_2, so producing proportional changes in V_{max} and K_m.

For the effects of pH on enzyme inhibition the reader is referred to Fersht (*318*).

Enzyme–coenzyme kinetics: Dehydrogenases such as LADH, YADH, etc., have two substrates: one is NAD^+ the coenzyme; the other is the alcohol, the oxidation of which is to be catalyzed. In fact, in the study of isolated enzyme reactions the distinction between coenzymes and substrates is not meaningful (*322*). The general solutions of the steady-state equations for multisubstrate systems are involved, and the reader is referred to Fromm and other authors (*323–326*) and to shorter summaries (*327, 327a, 328*) for further information. The key experimental feature is the fact that most reactions obey Michaelis–Menten kinetics when the concentration of one substrate is held constant and the other is varied. It also appears that only a limited range of mechanisms is observed in practice. The terms used are as follows.

Reactions in which all the substrates bind to the enzyme before the first product is formed are called *sequential*. Reactions in which one or more products are released before all the substrates are added are called *Ping-Pong*. In an *ordered* sequential mechanism, the substrates combine with the enzyme and the products dissociate in an obligatory order. A *random* mechanism implies no obligatory order of combination or release.

If the two substrates are A and B, then EAB is a ternary complex and we then have the following cases.



1. RANDOM SEQUENTIAL MECHANISM

$$E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \longrightarrow \text{product}$$

2. ORDERED MECHANISM

The ordered mechanism is the type observed with NAD^+-linked dehydrogenases. A special case of this, the Theorell–Chance mechanism, is found with LADH; the ternary complex does not accumulate.

$$E \overset{A}{\rightleftharpoons} EA \overset{B \quad P}{\longrightarrow} EQ$$

Here A is NAD^+, B is EtOH, P is CH_3CHO, Q is NADH.

3. Ping-Pong mechanism: This is of the kind

$$E + A \rightleftharpoons EA \rightleftharpoons E\text{–}P + Q$$

$$E\text{–}P + B \rightleftharpoons E\text{–}P.B \longrightarrow E + P\text{–}B$$

It is found with phosphate-transferring enzymes; A might be a phosphate ester, E–P a phosphorylated enzyme, B is a second alcohol, and P–B the phosphate ester of the second alcohol.

Sequence homologies, mutations, and isozymes: There are three basic postulates of chemical palaeontology (*329–331*). (i) Polypeptide chains in present-day organisms have arisen by evolutionary divergence from similar polypeptide chains that existed in the past. The present and past chains would be similar in that many of their amino acid residues along the chain match; such chains are said to be homologous or to show sequence homology. (ii) A gene existing at some past epoch can occasionally be duplicated so that it appears at two or more sites in the total stock of genetic information of descendant organisms. Thus, a contemporary organism can have two or more homologous genes giving rise to homologous polypeptide chains *that have mutated independently* and are therefore no longer identical in structure. (iii) Mutational events that are most commonly retained through natural selection are those that lead to a replacement of a single amino acid residue in a polypeptide chain. Human carbonic anhydrases B and C contain a 60% sequence homology (Section II,B,1); i.e., 60% of the sequence of amino acids match in the B and C forms.

It may be wondered how one can assume that the chains of the B and C forms have common ancestry when there are so many differences. The justification is that it is most improbable that two different and unrelated polypeptide chains could evolve in such a way as to have qualitatively the same function, the same conformation, and a substantial number of amino acid residues at corresponding molecular sites. The marked difference in sequence is then taken as evidence that a long time has elapsed since the B and C forms diverged from a common ancestor. These molecules, such as HCAC and HCAB, which have been produced by gene duplication and mutation, are called isozymes. They differ quantitatively in reactivity as we have seen for the carbonic

anhydrases, and, since kinetic measurements should be made on a single characterized species, it is important at an early stage to see what isozymes of a metalloenzyme exist (e.g., by chromatographic and/or electrophoretic methods) and to use a pure isozyme. The use of mixtures of isozymes has led to difficulties of interpretation, for example, with carbonic anhydrase, before the discovery of its constituent isozymes and in other cases (*332*).

References

1. Tam, S. W., Wilber, K. E., and Wagner, F. W., *Biochem. Biophys. Res. Commun.* **72**, 302 (1976).
2. Raulin, J., *Ann. Sci. Nat., Bot. Biol. Veg.* **11**, 93 (1869).
3. Keilin, D., and Mann, T., *Biochem. J.* **34**, 1163 (1940).
4. Eichhorn, G. L., *Inorg. Biochem.* **2**, 1191 and 1210 (1973). Cf. also, Auld, D. S., Kawaguchi, H., Livingston, D. M., and Vallee, B. L., *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 2091 (1974).
5. Wacker, W. E. C., *Biochemistry* **1**, 859 (1962).
6. Falchuk, K. H., Mazus, B., Ulpino, L., and Vallee, B. L., *Biochemistry* **15**, 4468 (1976).
7. Wandzilak, T. M., and Benson, R. W., *Biochem. Biophys. Res. Commun.* **76**, 247 (1977).
8. Sabbioni, E., *FEBS Lett.* **71**, 233 (1976).
9. Springate, C. F., Mildvan, A. S., Abramson, R., Engle, J. L., and Loeb, L. A., *J. Biol. Chem.* **248**, 5987 (1973).
10. Scrutton, M. C., Wu, C. W., and Goldthwait, T. A., *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2497 (1971).
11. Valenzuela, P., Morris, R. W., Faras, A., Levinson, W., and Rutter, J., *Biochem. Biophys. Res. Commun.* **53**, 1036 (1973).
12. Hughes, M. H., and Prince, R. H., *J. Inorg. Nucl. Chem., Bio-Inorg. Sect.* **40**, 703 (1978).
13. Hughes, M. H., and Prince, R. H., *J. Inorg. Nucl. Chem., Bio-Inorg. Sect.* **40**, 713 (1978).
14. Hughes, M. H., and Prince, R. H., *J. Inorg. Nucl. Chem., Bio-Inorg. Sect.* **40**, 719 (1978).
15. Chlebowski, J. F., and Coleman, J. E., *Met. Ions Biol. Syst.* **6**, p. 1 (1976).
16. Clark, I. D., and Wain, R. P., *Compr. Chem. Kinet.* **2**, 318, 329, and 334 (1969); cf. also Hix, J. E., and Jones, M. M., *Inorg. Biochem.* **1**, 361 (1973).
17. Vallee, B. L., and Williams, R. J. P., *Proc. Natl. Acad. Sci. U.S.A.* **59**, 498 (1968); Williams, R. J. P., *Inorg. Chim. Acta Rev.* **5**, 137 (1971).
18. Coleman, J. E., *Prog. Bioorg. Chem.* **1**, 159 (1971).
19. Coleman, J. E., *in* "Reactivities of Functional Groups of Proteins" (H. Gutfreund, ed.), MTP Int. Rev. Sci., p. 185. Med. Tech. Publ., Oxford, 1974.
20. Harris, M. I., and Coleman, J. E., *J. Biol. Chem.* **243**, 5063 (1968).
21. Vallee, B. L., *Adv. Protein Chem.* **16**, 401 (1961).

21a. Vallee, B. L., *Adv. Protein Chem.*, **10**, 317 (1955).

22. Malmstrøm, B. G., and Rosenberg, A., *Adv. Enzymol.* **21**, 131 (1959).
23. Rotilio, G. R., Morpurgo, L., Giovagnoli, C., Calabrese, L., and Mondovi, B., *Biochemistry* **11**, 2187 (1972).

23a. Richardson, J. S., Thomas, K. A., Rubin, B. H., and Richardson, D. C., *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1349 (1975).
24. Beem, K. M., Richardson, D. C., and Rajagopalan, K. V., *Biochemistry* **16**, 1930 (1977).
24a. McAdam, M. E., Fielden, E. M., Lavelle, P., Calabrese, L., Cocco, D., and Rotilio, G., *Biochem. J.* **167**, 271 (1977).
25. Cass, A. E. G., Hill, H. A. O., Smith, B. E., Bannister, J. V., and Bannister, W. H., *Biochemistry* **16**, 3061 (1977).
26. Lippard, S. J., Burger, A. R., Ugurbil, K., Pantoliano, M. W., and Valentine, J. S., *Biochemistry* **16**, 1136 (1977).
27. Mildvan, A. S., Kobes, R. D., and Rutter, W. J. *Biochemistry* **10**, 1191 (1971).
28. Nelbach, M. E., Pigiet, V. P., Gerhardt, J. C., and Schachman, H. K., *Biochemistry* **11**, 315 (1972).
29. Meldrum, N. U., and Roughton, F. J. W., *J. Physiol. (London)* **75**, 15 (1932).
30. Tashian, R. E., Goodman, M., Tanis, R. J., Ferre, R. E., and Osborne, W. R. A., *in* "The Isozymes" (C. L. Markert, ed.), Vol. 4, p. 207 et seq. Academic Press, New York, 1975.
31. Maren, T., *Physiol. Rev.* **47**, 595 (1967).
32. Lindskog, S., Henderson, L. E., Kannan, K. K., Liljas, A., Nyman, P. O., and Strandberg, B., *in.* "The Enzymes" (P. D. Boyer, ed.) 3rd ed., Vol. 5, p. 587 et seq. Academic Press, New York, 1971.
33. Carter, M. J., *Biol. Rev. Cambridge Philos. Soc.* **47**, 465 (1972).
34. Coleman, J. E., *Inorg. Biochem.* **1**, 488 (1973).
35. Wyeth, P., and Prince, R. H., *Inorg. Perspect. Biol. Med.* **1**, 37 (1977).
36. Keilin, D., and Mann, R., *Nature (London)* **144**, 442 (1939); cf. also Keilin and Mann (*3*).
37. Tashian, R. E., Tanis, R. J., and Ferrell, R. E., *Oxygen Affinity Haemoglobin Red Cell Acid Base Status, Proc. 4th Alfred Benson Symp., 1971* Vol. 4, p. 353 et seq. (1972).
38. Lindskog, S., *Biochim. Biophys. Acta* **39**, 218 (1960).
39. Nielsen, S. A., and Frieden, E., *Comp. Biochem. Physiol. B* **41**, 461 (1972).
40. Sciaky, M., and Laurent, G., *FEBS Lett.* **63**, 141 (1976).
41. Nyman, P. O., *Biochim. Biophys. Acta* **52**, 1 (1961).
42. Nassi, L., Poggini, G., Borselli, L., and Galvan, P., *Quad. Sclavo. Diagn. Clin. Lab.* **11**, 594 (1975).
43. Whitney, P. L., Nyman, P. O., and Malmström B. G., *J. Biol. Chem.* **242**, 4212 (1967).
44. Verpoorte, J. A., Mehta, S., and Edsall, J. T., *J. Biol. Chem.* **242**, 4221 (1967).
45. Osborne, W. R. A., and Tashian, R. E., *Biochem. J.* **141**, 219 (1974).
46. Osborne, W. R. A., and Tashian, R. E., *Isozyme Bull.* **7**, 40 (1974).
47. Bradbury, S. L., *J. Biol. Chem.* **244**, 2002 and 2010 (1969).
48. Kandel, M., Gornall, A. G., Wong, S.-C. C., and Kandel, S. I., *J. Biol. Chem.* **245**, 2444 (1970).
49. Whitney, P. L., Fölsch, G., Nyman, P. O., and Malmström, B. G., *J. Biol. Chem.* **242**, 4206 (1967).
50. Tashian, R. E., *Adv. Exp. Med. Biol.* **28**, 167–168 (1972).
51. Maren, T. H., Rayburn, C. S., and Liddell, N. E., *Science* **191**, 469 (1976).
52. Cockle, S. A., Lindskog, S., and Grell, E., *Biochem. J.* **143**, 703 (1974).
53. Anderson, B., Nyman, P. O., and Strid, L., *Biochem. Biophys. Res. Commun.* **48**, 670 (1972).
54. Lin, K.-T. D., and Deutsch, H. F., *J. Biol. Chem.* **248**, 1885 (1973).
55. Marriq, C., Sciaky, G., Kiraud, N., Foveau, D., and Laurent-Tabusse, G., *Biochimie* **55**, 1361 (1973).

56. Giraud, N., Marriq, C., and Laurent-Tabusse, G., *Biochimie* **56**, 1031 (1974).
57. Henderson, L. E., Henriksson, D., and Nyman, P. O., *Biochem. Biophys. Res. Commun.* **52**, 1388 (1973).
58. Henderson, L. E., Henriksson, D., and Nyman, P. O., *J. Biol. Chem.* **251**, 5457 (1976).
59. Lin, K.-T. D., and Deutsch, H. F., *J. Biol. Chem.* **249**, 2329 (1974).
60. Sciaky, M., Limozin, N., Fillipi-Foveau, D., Gulian, M. J.-M., Dalmasso, C., and Laurent, G., *C.R. Habd. Scances Acad. Sci., Ser. D* **279**, 1217 (1974).
61. Kannan, K. K., Liljas, A., Vaara, I., Bergstén, P.-C., Lövgren, S., Strandberg, B., Bengtson, U., Carlbom, U., Fridborg, K., Järup, L., and Petef, M., *Cold Spring Harbor Symp. Quant. Biol.* **36**, 221 (1971).
62. Liljas, A., Kannan, K. K., Bergstén, P.-C., Vaara, I., Fridborg, K., Strandberg, B., Carlbom, U., Järup, L., Lövgren, S., and Petef, M., *Nature (London), New Biol.* **235**, 131 (1972).
63. Nostrand, B., Vaara, I., and Kannan, K. K., *in* "The Isozymes" (C.L. Markert, ed.), Vol. 1, p. 575 et seq. Academic Press, New York, 1975.
64. Kannan, K. K., Nostrand, B., Fridborg, K., Lövgren, S., Ohlsson, A., and Petef, M., *Proc. Natl. Acad. Sci. U.S.A.* **72**, 51 (1975).
65. Vaara, I., Lövgren, S., Liljas, A., Kannan, K. K., and Bergstén P.-C., *Adv. Exp. Med. Biol.* **28**, 169 (1972).
66. Wee, V. T., Feldmann, R. J., Tanis, R. J., and Chignell, C. F., *Mol. Pharmacol.* **12**, 832 (1976).
67. Erlich, R. H., Starkweather, D. K., and Chignell, C. F., *Mol. Pharmacol.* **9**, 61 (1973).
68. Hower, J. F., Henkens, R. W., and Chesnut, D. B., *J. Am. Chem. Soc.* **93**, 6665 (1971).
69. Campbell, I. D., Lindskog, S., and White, A. I., *J. Mol. Biol.* **90**, 469 (1974).
70. Campbell, I. D., Lindskog, S., and White, A. I., *J. Mol. Biol.* **98**, 597 (1975).
71. Cockle, S. A., *Biochem. J.* **137**, 587 (1974).
72. Vaara, I., Inaugural Dissertation, University of Uppsala, Sweden (1974).
73. Blow, D. M., Birktoft, J. J., and Hartley, B. S., *Nature (London)* **221**, 337 (1970).
74. Glass, J. C., and Graf, G., *U.S. N.T.I.S. PB Rep.* **PB-252 190** (1975); *Chem. Abstr.* **85**, 139365w (1976).
75. Diehn, B., Halpern, A., and Stöcklin, G., *J. Am. Chem. Soc.* **98**, 1077 (1976).
76. Lipscomb, W. N., *Chem. Soc. Rev.* **1**, 319 (1972).
77. Tupper, R., Watts, R. W. E., and Wormall, A., *Biochem. J.* **50**, 429 (1952).
78. Wyeth, P., Ph.D. Thesis, University of Cambridge, England (1976).
79. Závodsky, P., Johansen, J. T., and Hvidt, A., *Eur. J. Biochem.* **56**, 67 (1975).
79a. Bauer, R., Limkilde, P., and Johansen, J. T., *Biochemistry* **15**, 334 (1976).
80. Dunn, M. F., *Struct. Bonding (Berlin)* **23**, 61 (1975).
81. Thorsland, A., and Lindskog, S., *Eur. J. Biochem.* **3**, 117 (1967).
82. Lindskog, S., and Thorsland, A., *Eur. J. Biochem.* **3**, 453 (1968).
83. Taylor, P. W., King, R. W., and Burgen, A. S. V., *Biochemistry* **9**, 3894 (1970).
84. Lindskog, S., *Biochim. Biophys. Acta* **122**, 537 (1966).
85. Myers, D. V., and Edsall, J. T., *Proc. Natl. Acad. Sci. U.S.A.* **53**, 169 (1965).
86. Coleman, J. E., *Biochemistry* 4, 2644 (1965).
87. Whitney, P. L., and Brandt, H., *J. Biol. Chem.* **251**, 3862 (1975).
88. Holmquist, B., Kaden, T. A., and Vallee, B. L., *Biochemistry* **14**, 1454 (1975).
89. Lindskog, S., and Ehrenberg, A., *J. Mol. Biol.* **24**, 133 (1967).
90. Edsall, J. T., *Harvey Lect.* **62**, 191 (1968).
91. Henkens, R. W., and Sturtevant, J. M., *J. Am. Chem. Soc.* **90**, 2669 (1968).
92. Henkens, R. W., Watt, G. D., and Sturtevant, J. M., *Biochemistry* **8**, 1874 (1969).
93. Dennard, A. E., and Williams, R. J. P., *Transition Met. Chem.* **2**, 116 (1966).
94. Pocker, Y., and Stone, J. T., *Biochemistry* **7**, 4139 (1968).

95. Lo, K., and Kaiser, E. T., *J. Am. Chem. Soc.* **91**, 4912 (1969).
96. Pocker, Y., and Guilbert, L. J., *Biochemistry* **13**, 4912 (1969).
97. Sharma, M. M., and Danckwerts, P. V., *Trans. Faraday Soc.* **59**, 386 (1963).
98. Dennard, A. E., and Williams, R. J. P., *J. Chem. Soc.* p. 812 (1966).
99. Caplow, M., *J. Am. Chem. Soc.* **93**, 230 (1971).
99a. Woolley, P. R., *Nature (London)* **258**, 677 (1975): *J. Chem. Soc., Perkin II*, 318, (1977).
100. Brändén, C.-I., *Biochem. Soc. Trans. 568th Meet., 1977* p. 612 (1977).
101. Edsall, J. T., and Wyman, J., "Biophysical Chemistry," Vol. 1, Chap. 10. Academic Press, New York, 1958.
102. Sirs, J. A., *Trans. Faraday Soc.* **54**, 201 (1957).
103. Gibbons, B. H., and Edsall, J. T., *J. Biol. Chem.* **238**, 3502 (1963).
104. Gibbons, B. H., and Edsall, J. T., *J. Biol. Chem.* **239**, 2539 (1964).
105. Kernohan, J. C., *Biochim. Biophys. Acta* **96**, 304 (1965).
106. Woolley, P. R., Ph.D. Thesis, University of Cambridge, England (1973).
107. Kalifah, R. G., *J. Biol. Chem.* **246**, 2561 (1971).
108. Magid, E., *Biochim. Biophys. Acta* **151**, 236 (1968).
109. Christiansen, E., and Magid, E., *Biochim. Biophys. Acta* **220**, 630 (1970).
110. Whitney, P. L., *Eur. J. Biochem.* **16**, 126 (1970).
111. Strader, D. G., and Khalifah, R. G., *J. Am. Chem. Soc.* **98**, 5043 (1976).
112. Steiner, H., Jonsson, B.-H., and Lindskog, S., *Eur. J. Biochem.* **59**, 253 (1975).
113. Steiner, H., Jonsson, B.-H., and Lindskog, S., *FEBS Lett.* **62**, 16 (1976).
114. Cleland, W. W., *Biochim. Biophys. Acta* **67**, 104 (1963).
115. Jonsson, B.-H., Steiner, H., and Lindskog, S., *FEBS Lett.* **64**, 310 (1976).
116. De Voe, H., and Kistiakowsky, G. B., *J. Am. Chem. Soc.* **83**, 274 (1961).
117. Lindskog, S., and Coleman, J. E., *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2505 (1973).
118. Khalifah, R. G., *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1986 (1973).
119. Prince, R. H., and Woolley, P. R., *Bioorg. Chem.* **2**, 337 (1973).
120. Silverman, D. N., and Tu, C. K., *J. Am. Chem. Soc.* **97**, 2263 (1975).
121. Tu, C. K., and Silverman, D. N., *J. Am. Chem. Soc.* **97**, 5935 (1975).
122. Kannan, K. K., Petef, M., Cid-Dresdner, H., and Lövgren, S., *FEBS Lett.* **73**, 115 (1977).
123. Pocker, Y., Meany, J. E., and Davis, B. C., *Biochemistry* **13**, 1411 (1974).
124. Pocker, Y., and Meany, J. E., *Biochemistry* **4**, 2535 (1965).
125. Pocker, Y., Meany, J. E., Dickerson, D. G., and Stone, J. T., *Science* **150**, 382 (1965).
126. Schneider, F., and Lieflander, M., *Hoppe-Seyler's Z. Physiol. Chem.* **334**, 279 (1963).
127. Lee, W. K., and Prince, R. H., unpublished data; Lee, W. K., Ph.D. Thesis, University of Cambridge, England (1977).
128. Pocker, Y., and Storm, D. R., *Biochemistry* **7**, 1202 (1968).
129. Wells, J. W., Kandel, S. I., Kandel, M., and Gornall, A. G., *J. Biol. Chem.* **250**, 3522 (1975).
130. Wells, J. W., Ph.D. Thesis, University of Toronto, Canada (1975).
131. Pocker, Y., and Beug, M. W., *Biochemistry* **11**, 698 (1972).
132. Pocker, Y., and Watamori, N., *Biochemistry* **12**, 2475 (1973).
133. Ward, R. L., and Whitney, P. L., *Biochem. Biophys. Res. Commun.* **51**, 343 (1973).
134. Göthe, P. O., and Nyman, P. O., *FEBS Lett.* **21**, 159 (1972).
135. Appleton, D. W., and Sarkar, B., *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1686 (1974).
136. Gupta, R. K., and Pesando, J. M., *J. Biol. Chem.* **250**, 2630 (1975).
137. Davis, R. P., *in* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd ed., Vol. 5, p. 545 et seq. Academic Press, New York, 1961.
138. Werber, M. M., *J. Theor. Biol.* **60**, 51 (1976).
139. Pesando, J. M., *Biochemistry* **14**, 675, 681 (1975).

140. Pesando, J. M., and Grollman, A. P., *Biochemistry* **14**, 689 (1975).
141. Harrowfield, J. M., Norris, V., and Sargeson, A. M., *J. Am. Chem. Soc.* **98**, 7282 (1976).
142. Fabry, M. E., Koenig, S. H., and Schillinger, W. E., *J. Biol. Chem.* **245**, 4256 (1970).
143. Lanir, A., Gradsztajn, S., and Navon, G., *FEBS Lett.* **30**, 351 (1973).
144. Koenig, S. H., and Brown, R. D., *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2422 (1972).
145. Bertini, I., Canti, G., Luchinat, C., and Scozzafava, A., *Biochem. Biophys. Res. Commun.* **78**, 158 (1977).
146. Wells, J. W., personal communication; cf. also Wyeth and Prince (*35*).
147. Pearson, R. G., Palmer, J., Anderson, M. M., and Allred, A. L., *Z. Elektrochem.* **64**, 110 (1960).
148. Swift, T. J., and Connick, R. E., *J. Chem. Phys.* **37**, 307 (1962).
149. Martin, R. B., *J. Inorg. Nucl. Chem.* **38**, 511 (1976).
150. Fitzgerald, J. J., and Chasteen, N. D., *Biochemistry* **13**, 4338 (1974).
151. Mann, T., and Keilin, D., *Nature (London)* **146**, 164 (1940).
152. Coleman, J. E., *Annu. Rev. Pharmacol.* **15**, 221 (1975).
153. King, R. W., and Burgen, A. S. V., *Proc. R. Soc. London, Ser. B* **193**, 107 (1976).
154. Olander, J., Bosen, S. F., and Kaiser, E. T., *J. Am. Chem. Soc.* **95**, 1616 (1973).
155. Kakeya, N., Aoki, M., Kamada, A., and Yata, N., *Chem. Pharm. Bull.* **17**, 1010, (1969).
156. Chen, R. F., and Kernohan, J. C., *J. Biol. Chem.* **242**, 5813 (1967).
157. Lindskog, S., *J. Biol. Chem.* **238**, 945 (1963).
158. Coleman, J. E., *Nature (London)* **214**, 193 (1967).
159. Kannan, K. K., Vaara, I., Nostrand, B., Borell, A., Fridborg, K., and Petef, M., *in* "Proceedings, on Drug Action at the Molecular Level" (G. C. K. Roberts, ed.), p. 73. Macmillan, New York, 1976.
160. Aasa, R., Hanson, M., and Lindskog, S., *Biochim. Biophys. Acta* **453**, 211 (1976).
161. Coleman, J. E., and Coleman, R. V., *J. Biol. Chem.* **247**, 4718 (1972).
162. Taylor, J. S., Mushak, P., and Coleman, J. E., *Proc. Natl. Acad. Sci. U.S.A.* **67**, 1410 (1970).
163. Lanir, A., and Navon, G., *Biochemistry* **10**, 1024 (1971).
164. King, R. W., and Burgen, A. S. V., *Biochim. Biophys. Acta* **207**, 278 (1970).
165. Galley, W. C., and Stryer, M., *Proc. Natl. Acad. Sci. U.S.A.* **60**, 108 (1968).
166. Galley, W. C., and Strambini, G. B., *Nature (London)* **261**, 521 (1976).
167. Lanir, A., and Navon, G., *Biochemistry* **11**, 3536 (1972).
168. Kumar, K., King, R. W., and Carey, P. R., *Biochemistry* **15**, 2195 (1976).
169. Bayley, P., and Anson, M., *Biochem. Biophys. Res. Commun.* **62**, 717 (1975).
170. Taylor, P. W., King, R. W., and Burgen, A. S. V., *Biochemistry* **9**, 2638 (1970).
171. Mushak, P., and Coleman, J. E., *J. Biol. Chem.* **247**, 373 (1972).
172. Petersen, R. L., Li, T.-Y., McFarland, J. T., and Watters, K. L., *Biochemistry* **16**, 726 (1977).
173. Pocker, Y., and Stone, J. T., *Biochemistry* **7**, 2936 and 3021 (1968).
174. Meldrum, N. U., and Roughton, F. J. W., *J. Physiol. (London)* **80**, 113 (1933).
174a. Whitney, P. L., *Anal. Biochem.* **57**, 467 (1975).
175. Lindskog, S., *Biochemistry* **5**, 2641 (1966).
176. Feeney, J., Burgen, A. S. V., and Grell, E., *Eur. J. Biochem.* **34**, 107 (1973).
177. Coleman, J. E., *J. Biol. Chem.* **242**, 5212 (1967).
178. Ward, R. L., *Biochemistry* **9**, 2447 (1970).
179. Ward, R. L., and Cull, M. D., *Arch. Biochem. Biophys.* **150**, 436 (1972).
180. Hofmeister, F., *Arch. Exp. Pathol. Pharmacol.* **24**, 247 (1888).
181. Fridovich, I., *J. Biol. Chem.* **238**, 592 (1963).

182. Coleman, J. E., *NASA Spec. Publ.* **188**, 141 (1969).
183. Lindskog, S., *Struct. Bonding (Berlin)* **8**, 153 (1970).
184. Riepe, M. E., and Wang, J. H., *J. Biol. Chem.* **243**, 2779 (1968).
185. Taylor, J. S., and Coleman, J. E., *J. Biol. Chem.* **246**, 7058 (1971).
186. Ward, R. L., *Biochemistry* **8**, 1879 (1969).
187. Ward, R. L., and Fritz, K. J., *Biochem. Biophys. Res. Commun.* **39**, 707 (1970).
188. Morpurgo, L., Rotilio, G., Agró, A., and Mandovi, B., *Arch. Biochem. Biophys.* **170**, 360 (1975).
189. Khalifah, R. G., *Biochemistry* **16**, 2236 (1977).
190. Khalifah, R. G., Strader, D. J., Bryant, S. H., and Gibson, S. M., *Biochemistry* **16**, 2241 (1977).
191. Taylor, P. W., Feeney, J., and Burgen, A. S. V., *Biochemistry* **10**, 3866 (1971).
192. Taylor, P., *J. Pharm. Sci.* **64**, 501 (1975).
193. Bertini, I., Luchinat, C., and Scozzafava, A., *J. Am. Chem. Soc.* **99**, 581 (1977).
194. Bertini, I., Luchinat, C., and Scozzafava, A., *Biochim. Biophys. Acta* **452**, 239 (1976).
195. Gerber, K., Ng, F. T. T., Pizer, R., and Wilkins, R. G., *Biochemistry* **13**, 2663 (1974).
196. Norne, J. E., Lilja, H., Lindman, B., Einarsson, R., and Zeppezauer, M., *Eur. J. Biochem.* **59**, 463 (1975).
197. Lanir, A., and Navon, G., *Biochim. Biophys. Acta* **341**, 65 and 75 (1974).
198. Yeagle, P. L., Lochmüller, C. H., and Henkens, R. W., *Proc. Natl. Acad. Sci. U.S.A.* **72**, 454 (1975).
199. Giannini, I., and Sodini, G., *NATO Adv. Study Inst. Ser., Ser. C* **18**, 423 (1975).
200. Stein, P. J., Merrill, S. P., and Henkens, R. W., *J. Am. Chem. Soc.* **99**, 3194 (1977).
201. Sund, H., and Theorell, H., *in* "The Enzymes" (P. D. Boyer, ed.), 2nd ed., Vol. 7, p. 27. Academic Press, New York, 1963.
201a. Sund, H., *in* "Biological Oxidations" (T. P. Singer, ed.), p. 603. Wiley (Interscience), New York, 1968.
202. Negelein, E., and Wulff, H.-J., *Biochem. Z.* **293**, 351 (1937).
203. Bonnischen, R. K., and Wassen, A. M., *Arch. Biochem. Biophys.* **18**, 361 (1948).
204. Ulmer, D. D., and Vallee, B. L., *Adv. Chem. Ser.* **100**, 201 (1971).
205. Theorell, H., and Bonnischen, R., *Acta Chem. Scand.* **5**, 1105 (1951).
206. Theorell, H., Nygaard, P., and Bonnischen, R., *Acta Chem. Scand.* **9**, 1148 (1955).
207. Vallee, B. L., and Hoch, F. L., *J. Biol. Chem.* **225**, 185 (1957).
208. Drum, D. E., Li, T.-K., and Vallee, B. L., *Biochemistry* **8**, 3783 (1969).
209. Jörnvall, H., and Harris, J. I., *Eur. J. Biochem.* **13**, 565 (1970).
210. Jörnvall, H., *Eur. J. Biochem.* **16**, 25 (1970).
211. Åkeson, H., *Biochem. Biophys. Res. Commun.* **17**, 211 (1964).
211a. Zeppezauer, E., Søderberg, B.-O., Brändèn, C.-I., Åkeson, Å., and Theorell, H., *Acta Chem. Scand.* **21**, 1099 (1967).
211b. Brändèn, C.-I., *Arch. Biochem. Biophys.* **112**, 215 (1965).
212. Brändén, C.-I., Eklund, H., Nordström, B., Boiwe, T., Söderlund, G., Zeppezauer, E., Ohlsson, I., and Åkeson, A., *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2439 (1973).
213. Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, I., Boiwe, T., and Brändén, C.-I., *FEBS Lett.* **44**, 200 (1974); Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Tapia, O., Brändén, C.-I., Ohlsson, I., Boiwe, T., Soderberg, B. O., and Åkeson, Å., *J. Mol. Biol.* **102**, 27 (1976).
214. Harris, I., *Nature (London)* **203**, 30 (1964).
215. Kagi, H. R., and Vallee, B. L., *J. Biol. Chem.* **235**, 3188 (1960).
216. Hayes, J. E., and Velick, S. F., *J. Biol. Chem.* **207**, 225 (1954).
217. Dickinson, M., *Eur. J. Biochem.* **41**, 31 (1974).
217a. Sytkowski, A. J., and Vallee, B. L., *Proc. Natl. Acad. Sci. U.S.A.* **73**, 344 (1976).

218. Vallee, B. L., Coombs, T. L., and Williams, R. J. P., *J. Am. Chem. Soc.* **80**, 401 (1958).
219. Brändén, C.-I., Jörnvall, H., Eklund, H., and Furugren, B., *in* "The Enzymes" (P. D. Boyer, ed.), 3rd ed., Vol. 11, Part A, p. 103. Academic Press, New York, 1975.
220. Scrutton, M. C., *Inorg. Biochem.*, **1**, 394 (1973).
221. Mildvan, A. S., *in* "The Enzymes" (P. D. Boyer, ed.), 3rd ed., Vol. 2, p. 526. Academic Press, New York, 1970.
222. Weiner, H., *Biochemistry* 8, 526 (1969).
222a. Hughes, M., and Prince, R. H., *Chem. Ind. (London)* p. 648 (1975); *J. Inorg. Nucl. Chem., Bio-Inorg. Sec.* **40**, 703, 713, and 719 (1978).
223. Burton, K., and Wilson, T. H., *Biochem. J.* **54**, 86 (1953).
224. Kosower, E. M., *Biochim. Biophys. Acta* **56**, 474 (1962).
224a. Blumenstein, M., and Raftery, M. A., *Biochemistry* **11**, 1643 (1972).
224b. Birdsall, B., and Feeney, J., *J. Chem. Soc., Perkin Trans. 2* p. 1643 (1972).
224c. Sarma, R. H., Moore, M., and Kaplan, N. O., *Biochemistry* **9**, 549 (1970).
224d. Sarma, R. H., and Kaplan, N. O., *Biochemistry* **9**, 557 (1970).
225. Barrio, J. R., Tolman, G. L., Leonard, N. J., Spencer, R. D., and Weber, G., *Proc. Natl. Acad. Sci. U.S.A.* **70**, 941 (1973).
226. Boyer, P. D., and Theorell, H., *Acta Chem. Scand.* **10**, 447 (1956).
227. Sloan, D. S., Maitland-Young, J., and Mildvan, A. S., *Biochemistry* **14**, 1998 (1975).
228. For summary, see M. Hughes and R. H. Prince, *Bioorg. Chem.* **6**, 137 (1977).
228a. Woenkhaus, C., and Zumpe, P., *Z. Naturforsch. B*, **23**, 484 (1968).
229. Luisi, P. L., Baici, A., Bonner, F. J., and Aboderini, A. A., *Biochemistry* **14**, 362 (1975).
230. Shore, J. D., and Santiago, D., *J. Biol. Chem.* **250**, 2008 (1975).
231. Weiner, H., Iweibo, I., and Coleman, P. L., *Wenner-Gren Cent. Int. Symp. Ser.* **18**, 619 (1972).
232. Iweibo, I., and Weiner, H., *J. Biol. Chem.* **250**, 1959 (1975).
233. Csopak, H., Lindman, B., and Lilja, H., *FEBS Lett.* **9**, 189 (1970).
234. Ward, R. L., *Biochemistry* 8, 1879 (1969).
235. Lindman, B., Zeppezauer, M., and Å. Åkeson, *Wenner-Gren Cent. Int. Symp. Ser.* **18**, 603 (1972).
236. Zeppezauer, M., Lindman, B., Forsen, S., and Lindqvist, I., *Biochem. Biophys. Res. Commun.* **37**, 137 (1969).
237. Prince, R. H., and Woolley, P. R., unpublished work.
238. Coleman, P. L., and Weiner, H., *Biochemistry* **12**, 1707 (1973).
239. Coleman, P. L., and Weiner, H., *Biochemistry* **12**, 1705 (1973).
240. Takahashi, M., and Harvey, R. A., *Biochemistry* **12**, 4743 (1973).
241. Young, J. M., and Wang, J. H., *J. Biol. Chem.* **246**, 2815 (1971).
242. Hughes, M., Ph.D. Thesis, University of Cambridge, England (1975).
243. Drott, H. R., Santiago, D., and Shore, J. D., *FEBS Lett.* **39**, 21 (1974).
244. Mildvan, A. S., Waber, L., Villafranca, J. J., and Weiner, H., *Wenner-Gren Cent. Int. Symp. Ser.* **18**, 745 (1972).
245. Reynolds, C. H., Morris, D. L., and McKinley-McKee, J. S., *Eur. J. Biochem.* **14**, 14 (1970).
246. McFarland, J. T., Walters, K. L., and Petersen, R. L., *Biochemistry* **14**, 624 (1975).
247. Abeles, R. H., Hutton, R. F., and Westheimer, F. H., *J. Am. Chem. Soc.* **79**, 712 (1957).
248. Samama, J.-P., Zeppezauer, J.-F., Biellmann, J.-F., and Brändén, C.-I., *Eur. J. Biochem.* **81**, 403 (1977).

249. Abdallah, M. A., Biellmann, J.-F., Samama, J.-P., with Wrixon, A. D., *Eur. J. Biochem.* **64**, 351 (1976).
250. Jacobs, J. W., McFarland, J. T., Wainer, I., Jeanmaier, D., Ham, C., Hamm, K., Wnuk, M., and Lam, M., *Biochemistry* **13**, 60 (1974).
251. Dickenson, C. J., and Dickinson, F. M., *Biochem. J.* **147**, 303 (1975).
252. Dickinson, F. M., and Dickenson, C. J., *Biochem. J.* **147**, 541 (1975).
253. Shore, J. D., Gutfreund, H., Brooks, R. L., Santiago, D., and Santiago, P., *Biochemistry* **13**, 4185 (1974).
254. McFarland, J. T., and Chu, Y.-H., *Biochemistry* **14**, 1140 (1975).
255. Mildvan, A. S., and Weiner, H., *Biochemistry* **8**, 552 (1969).
256. Mildvan, A. S., and Weiner, H., *J. Biol. Chem.* **244**, 2465 (1969).
257. Jörnvall, H., *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2295 (1973).
258. Sloan, D. L., and Mildvan, A. S., *Biochemistry* **13**, 1711 (1974).
259. McFarland, J. T., Chu, Y. H., and Jacobs, J. W., *Biochemistry* **13**, 65 (1974).
260. Bernhard, S. A., Dunn, M. F., Luisis, P. L., and Schack, P., *Biochemistry* **9**, 185 (1970).
261. Taniguchi, S., Yonetani, T., and Sjöberg, B., *Acta Chem. Scand.* **23**, 255 (1969).
262. Gilleland, M. L., and Shore, J. D., *Biochem. Biophys. Res. Commun.* **40**, 230 (1970).
263. Dunn, M. F., and Hutchinson, J. S., *Biochemistry* **12**, 4882 (1973).
264. Klinman, J. P., *J. Biol. Chem.* **247**, 7977 (1972).
265. Klinman, J. P., *J. Biol. Chem.* **250**, 2569 (1975).
266. Wratten, C. C., and Cleland, W. W., *Biochemistry* **4**, 2442 (1965).
267. Wratten, C. C., and Cleland, W. W., *Biochemistry* **2**, 935 (1963).
268. Ulmer, D. D., and Vallee, B. L., *Ad. Chem. Ser.* **100**, 195 (1971).
269. Williams, R. J. P., *Inorg. Chim. Acta Rev.* **5**, 137 (1971).
270. Mildvan, A. S., *in* "The Enzymes" (P. D. Boyer, ed.) 3rd ed., Vol. 2, p. 530 et seq. Academic Press, New York, 1970.
271. Waldschmidt-Leitz, E., *Physiol. Rev.* **11**, 358 (1931).
272. See, for example, Quiocho, F. A., and Lipscomb, W. N., *Adv. Protein Chem.* **25**, 1 (1971).
273. Coleman, J. E., *Prog. Bioorg. Chem.* **1**, 159 (1971); Ludwig, M. L., and Lipscomb, W. N., *Inorg. Biochem.* **1**, 438 (1973).
273a. Bishop, W. H., Quiocho, F. A., and Richards, F. M., *Biochemistry* **5**, 4077 (1966).
274. Van Wart, H. E., and Vallee, B. L., *Biochem. Biophys. Res. Commun.* **75**, 732 (1977).
275. Jones, M. M., Hunt, J. B., Storm, C. B., Evans, P. S., Carson, F. W., and Pauli, W. J., *Biochem. Biophys. Res. Commun.* **75**, 253 (1977).
276. Suh, J., and Kaiser, E. T., *Chem. Commun.* p. 106 (1976).
277. Lipscomb, W. N., Hartsuck, J. A., Quiocho, F. A., and Reeke, G. N., *Proc. Natl. Acad. Sci. U.S.A.* **64**, 28 (1969).
278. Hartsuck, J. A., and Lipscomb, W. N., *Enzymes* **3**, 1 (1971).
279. Quiocho, F. A., and Richards, F. M., *Biochemistry* **5**, 4062 (1966).
280. Bishop, W. H., Quiocho, F. A., and Richards, F. M., *Biochemistry* **5**, 4077 (1966).
281. Neurath, H., and Schwert, G. W., *Chem. Rev.* **46**, 69 (1950).
282. Koshland, D. E., *Proc. Natl. Acad. Sci. U.S.A.* **44**, 98 (1958).
283. Koshland, D. E., and Neet, K. E., *Annu. Rev. Biochem.* **37**, 359 (1968).
284. Johansen, J. T., and Vallee, B. L., *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2006 (1973).
285. Johansen, J. T., and Vallee, B. L., *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2532 (1971).
286. Riordan, J. F., and Vallee, B. L., *Biochemistry* **3**, 1768 (1964).
287. Johansen, J. T., and Vallee, B. L., *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2532 (1971).
288. Quiocho, F. A., McMurray, C. H., and Lipscomb, W. N., *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2850 (1972).
289. Johansen, J. T., Livingston, D. M., and Vallee, B. L., *Biochemistry* **11**, 2584 (1972).

290. Scheule, R. K., Van Wart, H. E., Vallee, B. L., and Scheraga, H. A., *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3273 (1977).
291. Prince, R. H., and Spencer, R. C., *Inorg. Chim. Acta* **3**, 54 (1969).
292. Alcock, N. W., Prince, R. H., and Spencer, R. C., *J. Chem. Soc. A* p. 2383 (1968).
293. Spilburg, C. A., Bethune, J. L., and Vallee, B. L., *Biochemistry* **16**, 1142 (1977).
294. Alter, G. M., Leussing, D. L., Neurath, H., and Vallee, B. L., *Biochemistry* **16**, 1142 (1977).
295. Vallee, B. L., Riordan, J. F., and Coleman, J. E., *Proc. Natl. Acad. Sci. U.S.A.* **49**, 109 (1963).
296. Kaiser, E. T., and Kaiser, B. T., *Acc. Chem. Res.* **5**, 219 (1972).
297. Diamond, I., Swenerton, H., and Hurley, L. S., *J. Nutr.* **101**, 77 (1971).
298. Coleman, J. E., and Vallee, B. L., *J. Biol. Chem.* **235**, 390 (1960).
299. Coleman, J. E., and Vallee, B. L., *J. Biol. Chem.* **236**, 2244 (1961).
300. Coleman, J. E., and Vallee, B. L., *J. Biol. Chem.* **236**, 3430 (1962).
301. Coleman, J. E., and Vallee, B. L., *Biochemistry* **1**, 1083 (1962).
302. Vallee, B. L., and Riordan, J. F., *Brookhaven Symp. Biol.* **21**, 91 (1968).
303. Simpson, R. T., Riordan, J. F., and Vallee, B. L., *Biochemistry* **2**, 616 (1963).
304. Riordan, J. F., and Vallee, B. L., *Biochemistry* **2**, 1460 (1963).
305. Roholt, O. A., and Pressman, D., *Proc. Natl. Acad. Sci. U.S.A.* **58**, 280 (1967).
306. Johansen, J. T., and Vallee, B. L., *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2532 (1971).
307. Wacker, H., Lehky, P., Fischer, E. H., and Stein, E. A., *Helv. Chim. Acta* **54**, 473 (1971).
308. Riordan, J. F., Sokolovsky, M., and Vallee, B. L., *Biochemistry* **6**, 3609 (1967).
309. Vallee, B. L., Riordan, J. F., Bethune, J. L., Coombs, T. L., Auld, D. S., and Sokolovsky, M., *Biochemistry* **7**, 3547(1968).
310. Davies, R. C., Riordan, J. F., Auld, D. S., and Vallee, B. L., *Biochemistry* **7**, 1090 (1968).
311. Auld, D. S., and Vallee, B. L., *Biochemistry* **10**, 2892 (1971).
312. Makinen, M. W., Yamamura, K., and Kaiser, E. T., *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3882 (1976).
313. Breslow, R., and Wernick, D. L., *Proc. Natl. Acad. Sci. U.S.A.* **74**, 1303 (1977).
314. Hayes, D. M., and Kollman, P. A., *J. Am. Chem. Soc.* **98**, 7811 (1976).
315. Barber, A. K., and Fischer, J. R., *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2970 (1972).
316. Vallee, B. L., *Ann. N.Y. Acad. Sci.* **158**, 377 (1969).
317. Reck, G. R., Walsh, K. A., Hermodson, M. A., and Neurath, H., *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1226 (1971).
318. Fersht, A., "Enzyme Structure and Mechanism" Freeman, San Francisco, California, 1977.
318a. Dickerson, R. E., and Geis, I., "Structure and Function of Proteins." Harper, New York, 1969.
318b. Elmore, D. J., "Peptides and Proteins." Cambridge Univ. Press, New York, 1968.
318c. Laidler, K. J., and Bunting, P. S., "The Chemical Kinetics of Enzyme Action," 2nd ed. Oxford Univ. Press, London and New York, 1973.
319. "Specialist Periodical Reports: Amino-acids, Peptides and Proteins" Chem. Soc. London.
320. Williams, A., "Introduction to the Chemistry of Enzyme Action" McGraw Hill, New York, 1969.
321. Dodgson, K. S., Spencer, B., and Williams, K., *Nature (London)* **177**, 432 (1956).
322. Gutfreund, H., "An Introduction to the Study of Enzymes." Blackwell, Oxford, 1965.

323. Fromm, H. J., "Initial Rate Enzyme Kinetics." Springer-Verlag, Berlin and New York, 1975.
324. Wong, J. T.-F., "Kinetics of Enzyme Mechanisms." Academic Press, New York, 1975.
325. Segel, I. H., "Enzyme Kinetics." Wiley, New York, 1975.
326. Cornish-Bowden, A., "Principles of Enzyme Kinetics." Butterworth, London, 1975.
327. Cleland, W. W., *Enzymes* **2**, 1 (1970).
327a. Cleland, W. W., *in* "The Enzymes" (P. D. Boyer ed.), 2nd ed., Vol. 2, p. 8 et seq. Academic Press, New York, 1970.
328. Dalziel, K., *Enzymes* **10**, 2 (1975).
329. For a readable account, see Zuckerkandl, E., *in* "Bioorganic Chemistry," p. 53, Freeman, San Francisco, California, 1968.
330. Zuckerkandl, E., and Pauling, L., *in* "Evolving Genes and Proteins" (V. Bryson and H. J. Vogel, eds.), p. 97. Academic Press, New York, 1965.
331. Zuckerkandl, E., and Pauling, L., *in* "Horizons in Biochemistry" (M. Kasha and B. Pullman, eds.), p. 189. Academic Press, New York, 1962.
332. See Gutfreund, H., "An Introduction to the Study of Enzymes," pp. 278, 279, and 289. Blackwell, Oxford, 1965.