blue. After 15 minutes the solvent was removed (air stream) and a solution of the residue in the minimum volume of dichloromethane placed on a column of activated, acid-washed alumina wet with petroleum ether. The latter solvent eluted a small fraction as blue-green solution (not identified but possibly 1,3-dibromoazulene) and dichloromethane–petroleum ether (1:4) removed a small amount of material which exhibited ultraviolet and visible spectra identical with those of di-1-azulyl disulfide (II).

The principal blue band was eluted with 3:7 dichloromethane–petroleum ether and evaporation (dry air stream) of the solvent from this fraction left 21 mg. (50%) of a blue oil thought to be S-acetyl-3-bromo-1-azulenethiol (XIII). It was unstable in a nitrogen or air atmosphere and was not obtained analytically pure. Attempts to prepare trinitrobenzeneate and picrate derivatives failed. A cyclohexane solution showed λ_{max} in mμ (D_{max}) at 234 (1.30), 294 (1.75), 300 (1.76), 355 (0.24), 372 (0.31), 595 (1.37), 643 (1.19) and 713 (0.44). The infrared spectrum in the region from 5–10 mμ was quite similar to that of S-acetyl-1-azulenethiol (IX).

Reaction of 1-Thiocyano-3-bromoazulene (XII) with Zinc, Acetic Acid and Acetic Anhydride.[18]—To a stirred solution of 45 mg. (0.17 mmole) of 1-thiocyano-3-bromoazulene and 0.2 g. of sodium acetate in 5 ml. of glacial acetic acid and 5 ml. of acetic anhydride was added 0.5 g. of zinc dust. The combined extracts were washed with dilute potassium hydroxide, dried over sodium sulfate, and evaporated (air stream) to dryness. A solution of the residual oil in the minimum volume of dichloromethane was placed on a column of activated, acid-washed alumina wet with petroleum ether. The latter solvent developed faint pink and green zones and a large blue band. The pink zone disappeared and the green zone became more diffuse and finally colored most of the column. Elution with 2:1 dichloromethane–petroleum ether removed the green material which was obtained as an unstable solid (14 mg.). This substance contained sulfur and halogen and its infrared spectrum had no peak in the carbonyl region. It was possibly bis-(3-bromoazulyl)disulfide. The large blue band separated into a blue band and a smaller purple band. The former was eluted with 4:1 dichloromethane–ether and evaporation of the solvent (air stream) left 16 mg. (33%) of blue oil. A cyclohexane solution of this material had the same ultraviolet, visible and infrared spectra as that of the product from the bromination of S-acetyl-1-azulenethiol (see above).

When Florisil was used as the adsorbent the yields of products and the elution solvents were: 2 mg. of green solid (benzene–petroleum ether), 29 mg. (60%) of blue oil (4:1 benzene–dichloromethane) and 3 mg. of a purple oil of unknown identity (4:1 benzene–dichloromethane). The spectra of the blue oil were the same given above except for the presence of a maximum at 614 mμ.

SEATTLE, WASH.

(18) The directions given are taken in part from results obtained by Mr. Lanny L. Replogle.

[CONTRIBUTION FROM THE GIBBS CHEMICAL LABORATORY, HARVARD UNIVERSITY]

The Kinetics of the Reaction of Human Erythrocyte Carbonic Anhydrase. II. The Effect of Sulfanilamide, Sodium Sulfide and Various Chelating Agents

BY ROBERT P. DAVIS[1]

RECEIVED MAY 2, 1959

The kinetic behavior of human erythrocyte carbonic anhydrase was studied by a rapid recording electrometric method for the measurement of changes in hydrogen ion activity accompanying the hydration of carbon dioxide at *p*H 7.00 and 1.5° in lightly buffered solutions. The chemical inhibition of carbonic anhydrase by sulfanilamide, sodium sulfide and various chelating agents was studied at an enzyme concentration corresponding to a zinc concentration of 2.7×10^{-2} micromole per liter. Sulfanilamide and sodium sulfide were strong non-competitive inhibitors. The association constants for binding of the inhibitor by the enzyme and by the enzyme–substrate compound were identical; for sulfanilamide this constant was $1.3 \times 10^{5}\ M^{-1}$, and for sulfide (expressed as total sulfide concentration) it was $3.3 \times 10^{6}\ M^{-1}$. Methylamine, ethylenediamine, ethylenediamine tetraacetate and 1,10-phenanthroline were without inhibitory effect on short incubation with the enzyme. The inference from the sulfide inhibition data is that carbon dioxide does not bind at the zinc atom of the enzyme molecule. Sulfanilamide may bind at the metal site, but inhibition data cannot critically determine this question. From the lack of inhibition by chelating agents, it is inferred that the zinc atom is firmly bound in carbonic anhydrase by the electrons ordinarily involved in chelation of inorganic zinc. The possible role of zinc in contributing an hydroxyl group to the catalytic process is discussed.

Introduction

A previous report[2] discussed the results of a study of the basic kinetic mechanism of the human erythrocyte carbonic anhydrase catalyzed hydration of carbon dioxide. From the ionic inhibition data it was possible to determine explicitly the values of the rate constants for each step of the Michaelis–Menten mechanism. It was of interest to extend the study to possible modes of chemical inhibition beyond the previously studied non-specific ionic effects in order to elucidate the chemical aspects of the catalysis in contrast to the kinetic details.

Mann and Keilin[3] first reported the inhibition of carbonic anhydrase by sulfanilamide. Their observations were extended to a large series of compounds of the sulfonamide derivative by Miller, Dessert and Roblin,[4] culminating in the synthesis of acetazoleamide, a potent inhibitor of carbonic anhydrase.[5] Davenport[6] interpreted his data on the inhibition of carbonic anhydrase by sulfanilamide to demonstrate a competitive mechanism of inhibition. The data, however, demonstrated only the mass action principles in the inhibition but could not distinguish critically among the various conceivable mechanisms of enzyme inhibition. Since no information was available on the mechanism of inhibition, further study of this problem seemed in order.

The role of the zinc atom in the carbonic anhydrase molecule is not yet known. The data of

(1) Junior Fellow of the Society of Fellows, Harvard University. Department of Medicine, University of North Carolina, School of Medicine, Chapel Hill, North Carolina.

(2) R. P. Davis, THIS JOURNAL, **80**, 5209 (1958).

(3) T. Mann and D. Keilin, *Nature*, **146**, 164 (1940).

(4) W. H. Miller, A. M. Dessert and R. O. Roblin, Jr., THIS JOURNAL, **72**, 4893 (1950).

(5) T. H. Maren, E. Mayer and B. C. Wadsworth, *Bull. Johns Hopkins Hosp.*, **95**, 199 (1954).

(6) H. W. Davenport, *J. Biol. Chem.*, **158**, 567 (1945).

Tupper, Watts and Wormall[7] and of Scott and Mendive[8] suggest that zinc is firmly bound in the protein molecule. For other zinc enzymes, notably the pyridine nucleotide dependent metallodehydrogenases, the metal appears to be the active center of the enzyme[9-13] combining stoichiometrically with diphosphopyridine nucleotide[10,11,13] and showing competitive inhibition with various zinc-binding reagents.[12,13] No similar data are available for carbonic anhydrase. Keilin and Mann[14] proposed that zinc was the functional active center for carbonic anhydrase and that sulfanilamide inhibition, which they had demonstrated, might arise by reaction of the sulfanilamide at the zinc atom.[3] In an effort to clarify these issues, an investigation of the effect of sulfanilamide, sodium sulfide and various chelating agents on the kinetics of the reaction of human erythrocyte carbonic anhydrase was undertaken and the results are reported below.

Experimental Details

The apparatus used in these studies has been described in detail in the previous report.[2] The rate of the reaction was measured by the automatic recording of the rate of formation of hydrogen ion as measured by a glass–calomel electrode system in a DuBridge–Brown d.c. amplifying circuit employing an FP-54 electrometer tube and coupled to a Leeds and Northrop Company Speedomax Recording Millivoltmeter. The initial voltage signal was balanced by a Type K-1 potentiometer, since the apparatus is most accurate near the null point. Only initial rates were determined.

The studies were conducted at 1.50 ± 0.05° at *p*H 7.00 ± 0.02 (at the experimental temperature) in 0.002 *M* phosphate buffer. Stirring was accomplished by polyethylene enclosed magnetic stirring bars driven externally. The cleaning of glassware and preparation of water have been previously described.[2]

All inorganic reagents were Mallinckrodt Analytical, Baker C.P. and Merck Reagent Grades employed without further purification. Sulfanilamide and 1,10-phenanthroline were obtained from Fisher Scientific Company and were likewise not further purified. The methylamine, ethylenediamine and ethylenediamine tetraacetate were kindly provided by Dr. F. Harris and had been purified from material obtained from the best available commercial sources.

The substrate solution was prepared by bubbling purified carbon dioxide through water at a constant known pressure and the concentration calculated from Henry's Law. All rates were corrected for the experimentally determined non-enzymatic rates at the appropriate substrate concentration.

Carbonic anhydrase was prepared from human erythrocytes according to a slightly modified procedure described by Keilin and Mann and previously discussed.[2] The enzyme concentration corresponded to a zinc concentration of 2.7×10^{-8} *M* in the reaction mixture.[15]

All reagents in the assay mixture except substrate were premixed in the reaction vessel and allowed to equilibrate for several minutes at the reaction temperature prior to the initiation of the reaction by the addition of the substrate.

(7) R. Tupper, R. W. E. Watts and A. Wormall, *Biochem. J.*, **50**, 429 (1952).

(8) D. A. Scott and J. R. Mendive, *J. Biol. Chem.*, **140**, 445 (1941).

(9) B. L. Vallee, *Advances in Protein Chem.*, **10**, 317 (1955).

(10) F. L. Hoch and B. L. Vallee, *J. Biol. Chem.*, **221**, 491 (1956).

(11) H. Theorell, R. Bonnichsen and A. P. Nygaard, *Acta Chem. Scand.*, **9**, 1148 (1955).

(12) B. L. Vallee, F. L. Hoch, S. J. Adelstein and W. E. C. Wacker, THIS JOURNAL, **78**, 5879 (1956).

(13) S. J. Adelstein and B. L. Vallee, *J. Biol. Chem.*, **233**, 589 (1958).

(14) D. Keilin and T. Mann, *Biochem. J.*, **34**, 1163 (1940).

(15) The zinc analyses were kindly performed by Dr. Bert Vallee, Biophysics Research Laboratory of the Department of Medicine, Harvard Medical School and the Peter Bent Brigham Hospital, Boston, Mass., by an ashing procedure followed by dithiozonium complexing and spectrophotometric analysis.

Results and Discussion

Sulfanilamide.—The data on sulfanilamide inhibition of carbonic anhydrase are shown in Table I. From the plot of these data according to the

TABLE I

THE EFFECT OF SULFANILAMIDE ON THE RATE OF ENZYMATIC HYDRATION OF CARBON DIOXIDE (μM SEC.$^{-1}$) AT *p*H 7.00 IN 0.002 *M* PHOSPHATE BUFFER

Enzyme concentration 2.7×10^{-8} *M*

CO_2 (*M*)	Sulfanilamide (*M*) None	4.0×10^{-6}	8.0×10^{-6}	1.2×10^{-5}
7.57×10^{-4}	6.21	3.95	3.38	2.52
15.1×10^{-4}	11.6	7.20	5.32	4.39
37.8×10^{-4}	20.4	14.0	10.2	8.14
60.6×10^{-4}	..	16.0	..	..
75.7×10^{-4}	28.7	17.5	14.3	11.7
11.4×10^{-3}	..	24.6	16.9	12.2
15.1×10^{-3}	36.8	..	..	..

method of Lineweaver and Burk[16] (Fig. 1), it is evident that the Michaelis constant, K_m, is invariant and that the mechanism of sulfanilamide

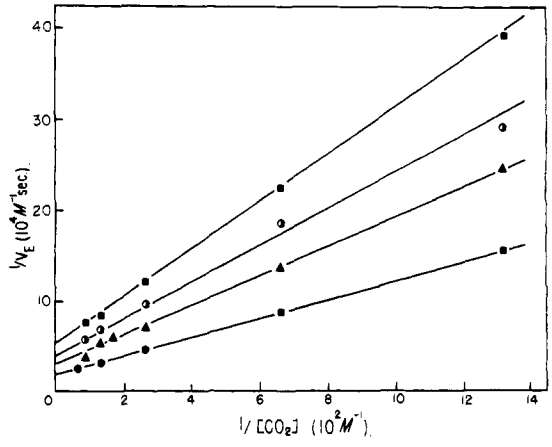

Fig. 1.—The effect of sulfanilamide on the enzymatic hydration of carbon dioxide at *p*H 7.00 in 0.002 *M* phosphate buffer at 1.5°, a plot of inverse rate against inverse CO_2 concentration at various concentrations of sulfanilamide: ●, none; ▲, 4.0×10^{-6} *M*; ◑, 8.0×10^{-6} *M*; ■ 1.2×10^{-5} *M*.

inhibition is non-competitive. Calculation of the inhibition index[17,18]

$$\varphi = \frac{V_E}{V_I} - 1$$

where V_E = uninhibited enzymatic rate, and V_I = rate in presence of inhibitor, and treatment of the inhibition index as a function of substrate concentration in the manner described by Kistiakowsky and Shaw[18] (Fig. 2) enable the derivation of the association constants for the inhibitor and both enzyme and enzyme–substrate compound

$$E + I \rightleftarrows EI, K_{EI} \quad (1)$$

$$ES + I \rightleftarrows ESI, K_{ESI} \quad (2)$$

From this derivation and on the assumption that

(16) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

(17) F. H. Johnson, H. Eyring and W. Kearns, *Arch. Biochem.*, **3**, 1 (1943).

(18) G. B. Kistiakowsky and W. H. R. Shaw, THIS JOURNAL, **75**, 866 (1953).

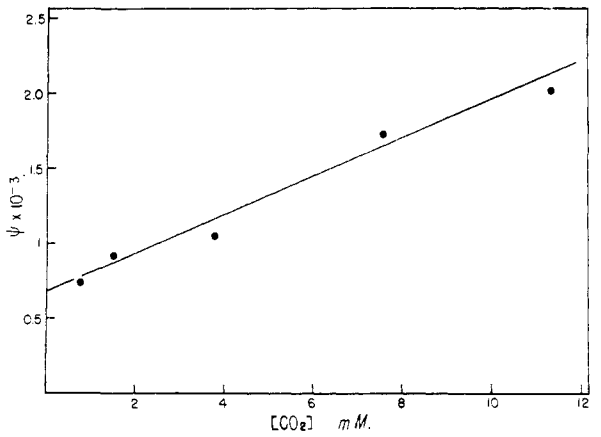

Fig. 2.—The inhibition index for sulfanilamide and carbonic anhydrase at *p*H 7.00 in 0.002 *M* phosphate buffer at 1.5°, a plot of ψ against CO_2 concentration, where $\psi = (\varphi/[I])(S + K_m)$ and $\varphi = (V_E - V_I)/V_I$.

one mole of sulfanilamide is bound per mole of enzyme, it is found that

$$K_{EI} = K_{ESI} = 1.3 \times 10^5\ M^{-1}$$

The non-competitive inhibition of carbonic anhydrase by sulfanilamide is in contrast to the competitive mechanism proposed by Davenport[6] for this inhibition. Davenport's suggestion was based upon the relation between the degree of inhibition and the logarithm of the inhibitor concentration. This relationship is the same for all mechanisms of reversible enzyme inhibition. The finding by Davenport[6] that for the inhibition of carbonic anhydrase by thiophene-2-sulfonamide the degree of inhibition depended only on the inhibitor concentration and was independent of a fourfold change in substrate concentration is likewise compatible only with *non-competitive* inhibition.

Since sulfide inhibition (to be discussed below) is also non-competitive, the possibility still exists that sulfanilamide binds at the zinc atom as proposed by Mann and Keilin, although this cannot be critically determined at present inasmuch as inhibition data cannot exclude the possibility that sulfanilamide binds at a third site other than the zinc atom and other than the locus of carbon dioxide binding.

TABLE II

THE EFFECT OF SODIUM SULFIDE ON THE RATE OF ENZYMATIC HYDRATION OF CARBON DIOXIDE (μM SEC.$^{-1}$) AT *p*H 7.00 IN 0.002 *M* PHOSPHATE BUFFER

Enzyme concentration 2.7 × 10^{-8} *M*

CO_2(*M*)	Na_2S(*M*) None	5.0 × 10^{-8}	1.5 × 10^{-7}	3.0 × 10^{-7}
7.57 × 10^{-4}	7.96	6.93	5.42	3.65
15.1 × 10^{-4}	13.4	12.1	9.50	5.95
37.8 × 10^{-4}	22.6	19.6	16.7	11.2
75.7 × 10^{-4}	32.8	28.8	20.9	14.5
11.4 × 10^{-3}	42.3	33.0	25.4	19.9

The association constants for carbonic anhydrase and sulfanilamide show a rather high order of inhibition. No studies were made of the inhibition with acetazoleamide, reportedly an even more potent inhibitor of carbonic anhydrase than its parent compound, sulfanilamide.[5] Its mechanism of inhibition would presumably be the same as that of sulfanilamide. The association constant for human erythrocyte carbonic anhydrase and sulfanilamide is slightly lower than that determined by Davenport. This difference may well be due to species variation, the errors of the different methods or the difference in ionic strength of the assay mixture.

Sodium Sulfide.—The inhibition of carbonic anhydrase by sodium sulfide at *p*H 7.00 (Table II, Fig. 3) is likewise non-competitive and, from a

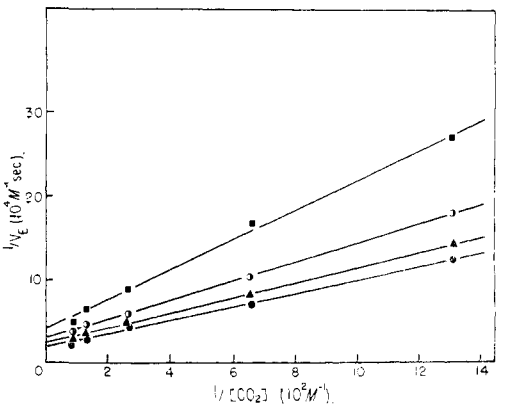

Fig. 3.—The effect of sodium sulfide on the enzymatic hydration of carbon dioxide at *p*H 7.00 in 0.002 *M* phosphate buffer at 1.5°, a plot of inverse rate against inverse CO_2 concentration at various concentrations of Na_2S: ●, none; ▲, 5.0 × 10^{-8} *M*; ◑, 1.5 × 10^{-7} *M*; ■, 3.0 × 10^{-7} *M*.

similar derivation of the inhibition index, it is found that

$$K_{EI} = K_{ESI} = 3.3 \times 10^6\ M^{-1}$$

Chelating Agents.—No inhibition was observed with methylamine, ethylenediamine and 1,10-phenanthroline at concentrations of 1.25 × 10^{-5}, 2.22 × 10^{-5} and 1.67 × 10^{-4} to 2.5 × 10^{-3} *M*, respectively, approximately 10^3 – 10^5 times the enzyme concentration of the assay mixture. All the inhibition by 5 × 10^{-3} *M* ethylenediamine tetraacetate could be accounted for on the basis of the ionic strength inhibition effect previously described[2] (Table III).

The non-competitive behavior of sodium sulfide inhibition is strong evidence that the zinc of carbonic anhydrase is not the site of carbon dioxide binding. At *p*H 7.00, the sulfide is in the chemical form of HS^- and H_2S, roughly in equimolar amounts. Which species was responsible for the inhibition of carbonic anhydrase was not determined inasmuch as the *p*H dependence of this inhibition was not studied. With equality of K_{EI} and K_{ESI}, the ease of inhibitor binding by the enzyme is independent of the presence or absence of carbon dioxide on the substrate-binding site.

The non-competitive behavior as well as the lack of inhibition by various zinc-chelating agents under the conditions studied makes difficult any firm conclusion on the role of the zinc atom in the carbonic anhydrase molecule. Smith[19] has proposed that for carbonic anhydrase the enzyme–substrate compound is in the form of a zinc-carbonate or

(19) E. L. Smith, *Proc. Natl. Acad. Sci., Wash.*, **35**, 80 (1949).

TABLE III

THE EFFECT OF CHELATING AGENTS ON THE RATE OF ENZYMATIC HYDRATION OF CARBON DIOXIDE (μM SEC.$^{-1}$) AT pH 7.00 IN 0.002 M PHOSPHATE BUFFER

Enzyme concentration 2.7×10^{-8} M

	Chelating Agent (M)							
		Methyl-amine	Ethylene-diamine	EDTA·Na pH 7.00	1,10-Phenanthroline			
CO_2(M)	None	1.25×10^{-5}	2.22×10^{-5}	5×10^{-3}	1.67×10^{-4}	1.67×10^{-3}	3.3×10^{-3}	2.5×10^{-3}
37.8×10^{-4}	22.6	22.9	23.2	16.2	24.7	21.1	20.6	
15.1×10^{-4}	13.3							13.3

zinc-subcarbonate complex. This suggestion, made from an analogy with metal-peptidases, must be incorrect in that the inhibition studies of carbonic anhydrase with sulfide indicate that no direct ligand is formed between carbon dioxide and the zinc of carbonic anhydrase. The inhibition with sulfide is interesting also in view of the demonstration by Neuberg and Mandl[20] that zinc will not form an insoluble sulfide in the presence of amino acids. This chelation of zinc by amino acids must, then, involve at least one of the electrons participating in sulfide bond formation. The binding of zinc in carbonic anhydrase may be different from that of the amino acid–zinc complex in the light of the failure of metal chelating agents to inhibit carbonic anhydrase significantly, despite profound and immediate inhibition with sodium sulfide. The strong and instantaneous inhibition by sodium sulfide suggests that other electrons of the zinc atom are involved in sulfide formation than in the chelation of the zinc in the enzyme molecule.

The non-reactivity of carbonic anhydrase to various metal chelating agents also suggests that the zinc is quite firmly bound in the enzyme molecule, as has been inferred from the inability of radioactive zinc to exchange with the zinc of carbonic anhydrase of erythrocytes[7] and from the resistance of the zinc to electrodialysis.[8] The electrons ordinarily involved in the chelate formation may already be tied in the enzyme–metal compound with an association constant greater than that for the metal–chelate formation. The association constant for carbonic anhydrase and its zinc must be at least 10^{21}, since the over-all association constant for the binding of inorganic zinc by ethylenediamine tetraacetate is $10^{16.21}$ and no inhibition was demonstrated at a molar ratio of chelating agent to enzyme zinc of 10^5.

In their extensive and detailed studies of zinc enzymes, Vallee and his associates[12] have shown that it is often necessary to preincubate some pyridine nucleotide dependent metallodehydrogenases with 1,10-phenanthroline and other chelating agents before inhibition is demonstrable. The preincubation period (for 50% inhibition) varied with the enzyme and, from these data, it was possible to calculate rate constants for the chelation of these zinc enzymes. The times varied from one minute to many hours, relatively consistently for the different enzymes no matter which chelating agent was used although the absolute time depended on the agent as well as the enzyme. Adelstein and Vallee[13] have shown also that the chelation and concomitant inhibition of beef liver glutamic dehydrogenase by 1,10-phenanthroline is instantaneous and instantaneously reversed by inorganic zinc ion.

Vallee, Coombs and Williams[22] have demonstrated that, for several zinc enzymes, the absorption spectrum of the inhibited enzyme–zinc–chelate with 1,10-phenanthroline and 8-hydroxyquinoline-5-sulfonic acid is the same as that of the chelate of inorganic zinc with these agents. Klotz and Loh Ming,[23] however, have shown significant spectral shifts on zinc-mediated binding of some chelating agents by protein. Since on dialysis of liver alcohol dehydrogenase zinc–1,10-phenanthroline chelate no 1,10-phenanthroline zinc chelate was demonstrable in the dialysate, Vallee, *et al.*, inferred that the zinc was not removed from the enzyme during enzyme zinc-chelate formation but that a double ligand of zinc was formed with both enzyme protein and chelating agent. The liver alcohol dehydrogenase–1,10-phenanthroline complex was the single chelate, however, which demonstrated a shift of absorption maximum on ligand formation like the mixed chelates of Klotz and Loh Ming. Whether or not other enzymes lose their zinc on chelation has not been conclusively shown. Frieden[24] has shown that beef liver glutamic dehydrogenase is split into four fragments by 1,10-phenanthroline and by DPN·H suggesting that the zinc may act to cement these molecular fragments into the enzyme unit and demonstrating a significant molecular change on chelation of the zinc. The long incubation needed in the chelation of some zinc enzymes with 1,10-phenanthroline, 8-hydroxyquinoline, etc., may be a reflection of a high association constant for the apoenzyme and zinc or the possibility that in chelation with these agents it is necessary to open a tight helical configuration of the enzyme protein molecule. The lack of chelation of carbonic anhydrase zinc may be a reflection of a more internal location of this metal in the carbonic anhydrase molecule than in other zinc enzymes where chelation results in competitive inhibition and where a superficial location is necessary in the substrate binding function of those enzymes. In the studies reported here, the incubation period of the chelating agent with the enzyme was quite brief, about five to ten minutes as needed for proper temperature equilibration of the assay mixture prior to the addition of the substrate. A preliminary series of experiments[25] involving 24 hours of incubation of 1,10-phenan-

(20) C. Neuberg and I. Mandl, *Arch. Biochem.*, **19**, 149 (1948).

(21) G. Schwarzenbach and E. Freitag, *Helv. Chim. Acta*, **34**, 1503 (1951).

(22) B. L. Vallee, T. L. Coombs and R. J. P. Williams, THIS JOURNAL, **80**, 397 (1958).

(23) I. M. Klotz and W. C. Loh Ming, *ibid.*, **76**, 805 (1954).

(24) C. Frieden, *Biochim. et Biophys. Acta*, **27**, 431 (1958).

(25) R. Davis and B. Fingerman, unpublished data.

throline in high concentration with carbonic anhydrase at room temperature demonstrated slightly more inhibition of the enzyme activity than could be accounted for by simple loss of activity of the enzyme on standing for this period. No studies have yet been done on the reversibility of this inhibition or to clarify whether the chelation involved a partial denaturation of the enzyme. Maren[26] has demonstrated a lack of inhibition of carbonic anhydrase by 1,10-phenanthroline and other chelating agents after five hours of incubation.

In the studies by Adelstein and Vallee[13] sodium sulfide was a less efficient inhibitor of beef liver glutamic dehydrogenase than were 1,10-phenanthroline and 8-hydroxyquinoline-5-sulfonic acid, and about the same in molar efficiency as diethyl dithiocarbonate. From the data of Vallee, Hoch, Adelstein and Wacker,[12] it is apparent that the rate constants for chelation and inhibition of a number of zinc enzymes bear the relation that the rate constant for 1,10-phenanthroline > 8-hydroxyquinoline-5-sulfonic acid > diethyl dithiocarbonate. If these data can be extended to carbonic anhydrase, inhibition with 1,10-phenanthroline should have been quickly apparent at the concentrations used.

It may be that for zinc enzymes there is a wide spectrum of mechanism of binding or association constants for binding of zinc to the apoenzyme with beef liver glutamic dehydrogenase showing the loosest association and readiest chelation, various other pyridine nucleotide metallodehydrogenases and carboxypeptidase making up a middle group with varying ease of chelation of the zinc due to firmer association and carbonic anhydrase representing an extremely strong association of protein with the zinc atom with the result that chelation is not possible with ordinary metal ligating agents.

In the absence of a role of zinc in carbon dioxide binding during carbonic anhydrase catalysis, the possibility exists that zinc is a site of binding for a water molecule or an hydroxyl ion. In the previously reported study of the effect of ions on the reaction of carbonic anhydrase,[2] the data suggested the possibility that the product of the carbonic anhydrase reaction may be bicarbonate ion directly, even at *p*H 7 where the non-enzymatic reaction yields chiefly carbonic acid. For the non-enzymatic reaction, in contrast, hydroxylation of carbon dioxide does not become appreciable until *p*H 8 and higher.[27] The *p*H dependence of carbonic anhydrase activity as determined by Kiese[28] and by Roughton and Booth[29] is compatible with the *p*H dependence of the formation of an enzyme–zinc hydroxide. Chaberek, Courtney and Martell[30] point out the ease of formation of the hydroxo-complex of metal chelates for weakly basic metals like zinc at relatively low *p*H values. Aquo ligands of the zinc will be displaced by hydroxyl groups, as discussed by Klotz and Loh Ming, with the quite possible result that important hydroxyl equilibria of the zinc in carbonic anhydrase at *p*H 7 prevent the chelation of other agents. The function of the protein in carbonic anhydrase, in addition to providing specificity for the reaction, is perhaps the formation of a stable chelate of zinc enabling hydroxylation of zinc and subsequently of carbon dioxide to take place at lower *p*H than for the uncatalyzed reaction. This hypothesis, perhaps strengthened by the disappearance of the neutral salt inhibition above *p*H 8.5,[29] has not yet been tested critically.

Acknowledgments.—This work was supported in part by grants to George B. Kistiakowsky and Harvard University from the Rockefeller Foundation and to the author from the Society of Fellows of Harvard University, to all of whom the author wishes to express his gratitude, as well as to acknowledge the encouragement and generous help and advice by Professor George B. Kistiakowsky.

(26) T. H. Maren, personal communication.

(27) C. Faurholt, *J. chim. phys.*, **21**, 400 (1924).

(28) M. Kiese, *Biochem. Z.*, **307**, 400 (1941).

(29) F. J. W. Roughton and V. H. Booth, *Biochem. J.*, **40**, 319 (1946).

(30) S. Chaberek, Jr., R. C. Courtney and A. E. Martell, THIS JOURNAL, **74**, 5057 (1952).

CAMBRIDGE, MASS.