The Effect of Metals on Hydrogen–Tritium Exchange of Proteins*

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ABSTRACT: The effect of metals on the hydrogen–tritium exchange of several proteins has been examined. The rate of exchange of conalbumin (ovotransferrin) is decreased by the chelation of iron or copper. Examination of the exchange as a function of iron concentration shows that only the two metal ions specifically bound to the protein are effective in retarding hydrogen exchange. The hydrogens affected are of a slowly exchanging type with an *average* half-life of 1.5–2 hr in the absence of metal. The data suggest that the effect of metal bindings on exchange is complex and that while hydrogens in some regions of the protein are protected from exchange there are other regions of the protein that may become exposed to exchange.

Binding of iron or copper caused no significant change in the optical rotatory dispersion of conalbumin between 300 and 200 mμ. The exchange of lysozyme, serum albumin, and hemoglobin was not affected by the presence of iron, nor was ribonuclease exchange affected by copper under conditions where copper is bound to the protein. Chelation of zinc by apocarbonic anhydrase caused a slight *increase* in the number of rapidly exchanging hydrogens. Binding of the inhibitor, acetazolamide, to carbonic anhydrase did not affect exchange.

Hydrogen exchange is one of the most general and sensitive methods of examining conformational changes of proteins. The effect upon hydrogen exchange of many of the parameters known to influence protein structure, such as pH and temperature, have been extensively studied (Hvidt and Nielsen, 1966). Conformational changes may also occur upon the binding of small molecules (cofactors, substrates) to a protein, as suggested by the induced-fit hypothesis of Koshland and Neet (1968), and thereby affect hydrogen exchange. This effect has been observed in several cases. For example, Hvidt and Kagi (1963) showed that the binding of nicotinamide–adenine dinucleotide lowered the exchange rate of alcohol dehydrogenase, and DeLuca and Marsh (1967) demonstrated a similar effect by the binding of substrates by luciferase.

It has been suggested that metal binding may cause extensive conformational changes in proteins. Evidence has been presented that divalent cations change glutamine synthetase from a "relaxed" to a "taut" form (Shapiro and Stadman, 1967). However, the effect of metals on the hydrogen exchange of proteins has not been investigated. Work in this laboratory (Emery, 1967) showed that the chelation of iron by small peptides affects the exchange of the peptide amide bonds, which are not involved in the chelation, and we decided to examine this phenomenon in a protein system. Conalbumin (ovotransferrin) was chosen because the specific binding of iron and copper by this protein has been extensively investigated by other chemical and physical techniques (Feeney and Komatsu, 1966). Our results indicate that metal binding by this protein affects hydrogen exchange in a complex manner, retarding exchange in some regions of the protein while apparently accelerating exchange in other regions.

Materials and Methods

Conalbumin (type I), prepared by the method of Woodworth and Schade (1959), bovine hemoglobin, bovine serum albumin, and lysozyme were purchased from Sigma Chemical Co. Bovine pancreas ribonuclease was obtained from Calbiochem. Bio-Gel P-2 was purchased from Bio-Rad Laboratories and tritiated water from New England Nuclear Corp. Bovine carbonic anhydrase was prepared by a method similar to that used by Armstrong *et al.* (1966) to prepare the human enzyme, using DEAE-Sephadex-A50 (Pharmacia) for the final purification step.

Exchange Method. The gel filtration technique of Englander (1963) was used to follow tritium exchange. In a typical experiment, 20 mg of protein was dissolved in 0.5 ml of the appropriate buffer containing 0.4 mCi of tritiated water. Exchange-in was allowed to proceed for 48 hr at 25°. The solution was then passed through a 2.5 × 5 cm column of Bio-Gel P-2 (50–100 mesh, short column) and the eluate was collected in a volume of 5 ml, which was then divided into two equal portions. To one portion the metal (ferric citrate or cupric sulfate solutions) was added and the pH was immediately readjusted, if necessary, to the original value. At appropriate time intervals, each solution was

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then passed through 1.8 × 20 cm columns of the same resin (long column). All columns were previously equilibrated with the buffer used for the exchange-in. The time for passage through the short column was about 3 min and through the long column about 7–8 min. Control experiments showed that the short column was effective in removing more than 99.9% of the THO used for the exchange-in, and removal of THO by the long columns was quantitative. For the exchange-in experiments, THO (25 mCi/ml) was added to the protein solution to give a final specific activity of about 0.4 mCi/ml, and at the appropriate time aliquots of the solution were freed of THO by passage directly through long columns.

Protein concentrations were determined by absorbance at 280 mμ using the following millimolar extinction coefficients: conalbumin, 88; iron and copper conalbumin, 122; hemoglobin, 120; bovine serum albumin, 46; lysozyme, 37; ribonuclease, 9.5; carbonic anhydrase and apocarbonic anhydrase, 57. Radioactivity was determined as described by Emery (1967). The number of tritiums per molecule was calculated from the specific activity of the protein and the specific activity of the THO used for the exchange. The latter value was determined by dilution of an aliquot of the solutions prior to passage through the columns. At least two fractions of protein were collected at each time interval to verify constancy of specific activity (hydrogens per molecule). The points of the exchange curves are averages of these values. The over-all precision of the method is about 10%.

Results

Hydrogen–tritium exchange curves of conalbumin in the presence and absence of iron are shown in Figure 1. Warner and Weber (1953) showed that iron is complexed by conalbumin between pH 6 and 11 although the complex is less stable at the lower pH. In our experiments complex formation, as judged by the appearance of color, was evident at both pH 6.5 and 8 but there is no significant effect of iron on the hydrogen exchange at the lower pH. At pH 8, the exchange is faster than at the lower pH because of the known catalysis of exchange by hydroxide ion, and the exchange in the absence of metal is almost complete by the end of 24 hr. However, at this higher pH the exchange is markedly affected by iron. Addition of iron to the tritiated protein results in the protection of about 55 hydrogens, and these hydrogens remain unexchanged for periods of up to 72 hr. The general phenomenon is reproducible, but the exact number of hydrogens affected varies from 40 to 60 (see below). A different preparation of conalbumin, kindly provided by Dr. Richard Michaud, yielded similar results. At pH 9, the exchange curves (not shown) are shifted downward, as expected due to the increased hydroxide ion concentration, but they remain parallel to those at pH 8 and some 50 hydrogens are again protected by iron. The effect of cupric ion, believed to bind at the same sites as iron, was also examined at pH 9. The exchange of copper conalbumin was identical with iron

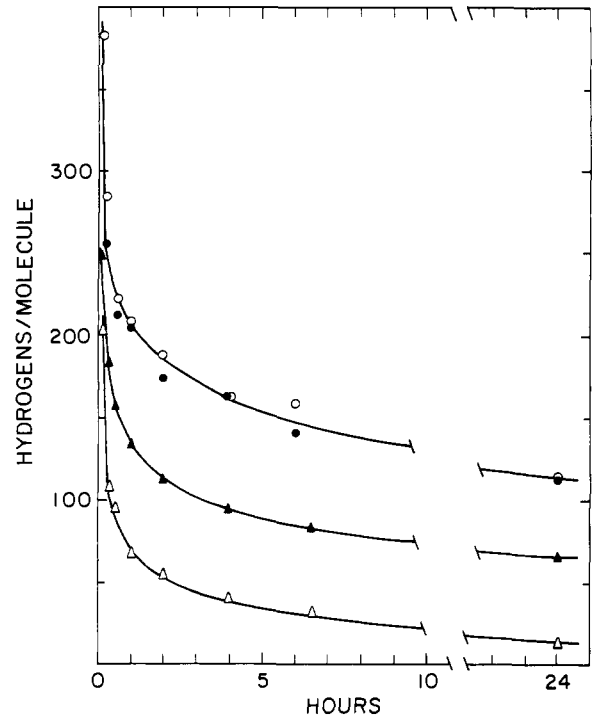

FIGURE 1: Exchange-out (loss of tritium) of conalbumin and iron conalbumin. Conalbumin (20 mg) was incubated for 48 hr at 25° in THO 0.1 M in Tris and $NaHCO_3$ (pH 8.0) or for 24 hr in 0.05 M phosphate (pH 6.5). THO was removed and exchange followed as described in Methods. Where indicated, iron conalbumin was formed by addition of a 2–3 fold molar excess of iron (citrate) after removal of THO. (—○—) Conalbumin, pH 6.5; (—●—) iron conalbumin, pH 6.5; (—△—) conalbumin, pH 8.0; (—▲—) iron conalbumin, pH 8.0.

conalbumin at this pH, about 50 hydrogens being protected.

Effect of Iron on Exchange of Other Proteins. In order to establish that the effect of iron on conalbumin hydrogen exchange is due to specific binding of the metal, the hydrogen exchange of several other proteins was examined. The data of Figure 2 show that iron does not influence the exchange of hemoglobin, bovine serum albumin, or lysozyme at the pH shown. The possibility exists that the metal might influence exchange at some other pH. Nevertheless, we tentatively conclude that the mere presence of iron does not affect the exchange process.

Hydrogen Exchange "Titration" of Conalbumin. To establish further the specific nature of iron in protecting from exchange certain hydrogens of conalbumin, the protective effect was examined as a function of iron concentration. In this experiment, iron of varying concentration was added to a solution of tritiated conalbumin immediately after removal of excess THO by passage through the short column. Exchange-out of tritium was allowed to proceed for 4 hr and the number of hydrogens protected was determined by comparison with an aliquot of the same protein solution to which no iron was added. The number of protected hydrogens as a function of iron concentration

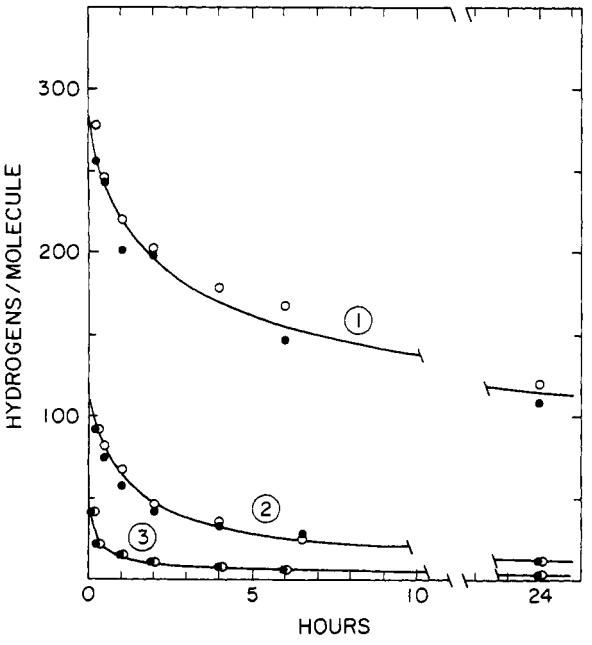

FIGURE 2: Exchange-out of hemoglobin, bovine serum albumin, and lysozyme. Exchange-in was carried out for 48 hr at 25° in 0.05 M phosphate (pH 6.5) (hemoglobin) or 0.1 M Tris (pH 8.0) (serum albumin and lysozyme). Sodium bicarbonate (0.1 M) was also present in the albumin and lysozyme experiments: (1) hemoglobin, (2) bovine serum albumin, and (3) lysozyme. Solid symbols indicate the presence of iron (Figure 1).

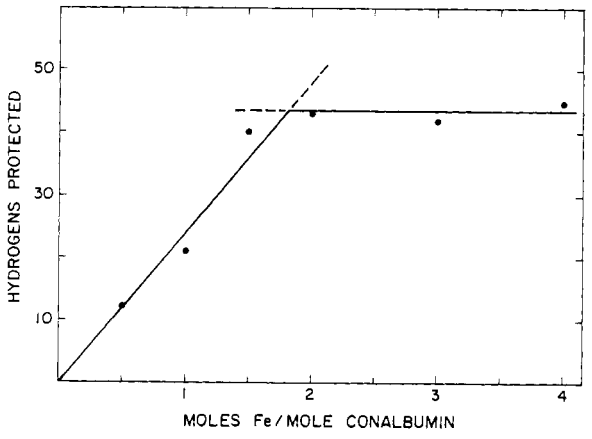

FIGURE 3: The effect of iron concentration on the protection of hydrogen exchange of conalbumin. Exchange was performed at pH 8.0, 25°, as described in Figure 1. Hydrogens/molecule was determined at the end of a 4-hr exchange-out period and compared to the value found when no iron was added. The difference between these values is expressed as hydrogens protected.

is shown in Figure 3. The stoichiometry of binding of metal to protein is 2:1 (Warner and Weber, 1953). These data show that the effect of iron is proportional to its concentration until the two bindings sites of the protein are saturated, after which excess iron produces no additional effect. This experiment was repeated at pH 6.5 but the data obtained were very erratic (see Discussion). These results prove, however, that the alteration of hydrogen exchange of conalbumin by iron is due to the unique binding of the metal at the specific metal-binding sites of the protein.

Nature of the Protected Hydrogens. In the experiments described above, the metal was added to the tritiated protein solution immediately after removal of THO by passage through the short column, a procedure requiring 3 or 4 min. At pH 8 or 9 the exchange of unprotected side chain and amide hydrogens would be complete within this time period, so that the hydrogens protected by metal must be of a rather slowly exchanging type even in the absence of metal. The nature of the protected hydrogens was further investigated by delaying the time of addition of iron after passage of the protein through the short column. The data obtained are shown in Table I and suggest

TABLE I: Effect of Iron on the Hydrogen Exchange of Conalbumin.

Time of Iron Addn to Tritiated Conalbumin after THO Removal	Hydrogens/ Conalbumin Found after 6-hr Exchange-out	Hydrogens Protected
0	88	45
4 min	80	37
30 min	72	29
1 hr	70	27
2 hr	59	16
5 hr	44	1
No iron added	43	

that the *average* half-life of the hydrogens in question is of the order of 1.5 hr in the absence of metal. It is obvious that the exact number of hydrogens protected must depend upon the rapidity with which metal is added to the protein solution after removal of excess THO and this explains why the absolute number of hydrogens found to be protected varies somewhat in different experiments. Moreover, the possibility cannot be overlooked that a number of rapidly exchanging hydrogens, with half-lives of the order of minutes, are also protected by iron but are not seen in our experiments because they have already exchanged during passage through the short column. To test this possibility, THO and iron were added in rapid succession to a solution of the protein, which was then passed through a long column to remove THO; 37 hydrogens were found to be "trapped," and these hydrogens must have half-lives of only seconds. This method was not generally used because of the inherent possibility of removal of metal during passage through the short column.

Exchange-in. The preceding experiments show unequivocally that metal binding by conalbumin retards

the exchange of a number of hydrogens of this protein. It is therefore obvious that upon addition of THO to identical solutions of metalloprotein and apoprotein (exchange-in) these particular hydrogens in the metalloprotein cannot undergo exchange to become isotopically labeled. Should these be the *only* hydrogens affected by metal binding, one would predict that the apoprotein would become more highly labeled than the metalloprotein. However, the exchange-in curves of conalbumin and iron conalbumin show little difference (Figure 4). As in the exchange-out experiments, passage of the protein through the columns, a procedure requiring 7–8 min, removes the rapidly exchanging hydrogens. As shown above, however, the hydrogens protected from exchange by metal are of a slowly exchanging type and would be observed to become labeled in the apoprotein after periods of exchange of several hours in the exchange-in experiments. One can explain the equal labeling of the two proteins during exchange-in by assuming that while some hydrogens are retarded in exchange by metal binding there are other hydrogens that are made available to exchange. The latter group of hydrogens would not be observable in the exchange-out experiments, not having become labeled during the initial equilibration with THO.

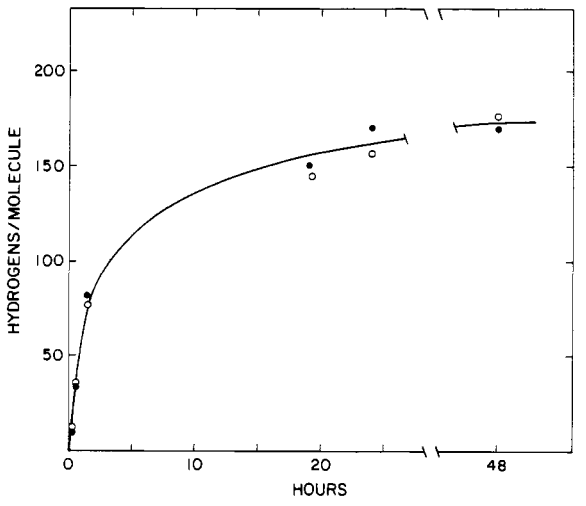

FIGURE 4: Exchange-in of conalbumin and iron conalbumin at pH 8.0 and 25°. (—○—) Conalbumin; (—●—) iron conalbumin.

Evidence in support of the above hypothesis is shown in Figure 5. In this experiment an initial exchange-out experiment is performed on the tritiated conalbumin and iron conalbumin. After 24 hr, the presence of the metal has "trapped" about 50 hydrogens in the protein. Tritiated water is now added to both protein solutions and exchange-in is followed as described previously. It is seen that the iron conalbumin remains more highly labeled than the apoprotein and its degree of labeling asymptotically approaches a significantly higher level than ever achieved during the 48-hr tritiation of the protein in the absence of metal. This behavior is difficult to explain by a "tightening up" of the protein structure, a process that would decrease the total number of hydrogens available for equilibration with tritiated water. The observation is compatible, however, with the idea that binding of metal to the tritiated protein locks in a group of tritium atoms but at the same time exposes other regions of the protein to exchange.

Optical Rotatory Dispersion. To determine if conformational changes of conalbumin upon iron binding could be verified by an independent technique, the optical rotatory dispersions of the protein and its iron or copper complex were compared. As Ulmer and Vallee (1963) showed, iron chelation by conalbumin gives rise to an anomalous Cotton effect in the visible region which is due to asymmetry of the complexing sites. However, we observed that at 300 mμ the dispersion curves of conalbumin, iron conalbumin, and copper conalbumin become identical and remain so

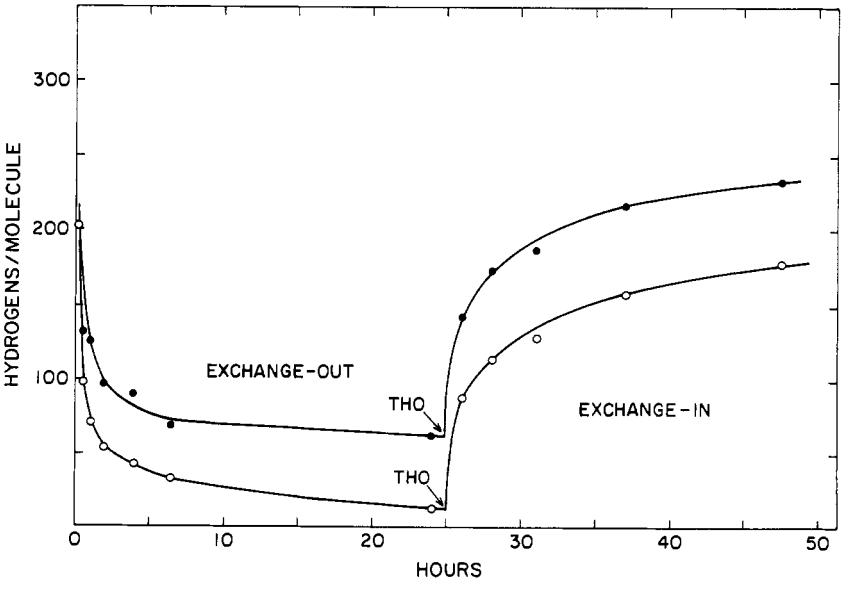

FIGURE 5: The exchange-in of iron conalbumin already containing trapped hydrogens. Exchange was measured at pH 8.0 and 25° after equilibrating the conalbumin for 48 hr with THO as described in Figure 1. (—○—), Conalbumin; (—●—), iron conalbumin.

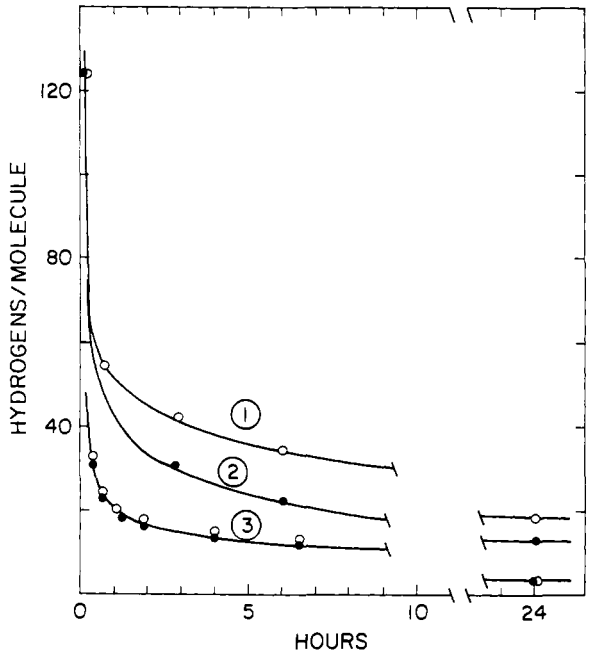

FIGURE 6: Hydrogen exchange of apocarbonic anhydrase, carbonic anhydrase, and ribonuclease. Ribonuclease exchange was carried out in 0.2 M acetate (pH 5.5). Carbonic anhydrase exchange was done in 0.025 M Tris (pH 7.4). After removal of THO, 0.002 M cupric chloride or 0.0025 M zinc acetate was added to ribonuclease and apocarbonic anhydrase (closed symbols), respectively, and exchange compared to the metal-free proteins (open symbols): (1) apocarbonic anhydrase, (2) carbonic anhydrase, and (3) ribonuclease.

to 200 mμ. In particular, there is no significant difference in the deep trough at 233 mμ usually associated with helical content.

Other Protein–Metal Systems. Figure 6 shows hydrogen exchange curves of ribonuclease and carbonic anhydrase. Under conditions where ribonuclease is reported to bind 5 atoms of copper (Girotti and Breslow, 1968), copper does not influence the exchange of this protein. Zinc was removed from carbonic anhydrase by the method of Coleman (1965) until 84% of the enzymic activity was lost. After equilibration of the apoenzyme for 48 hr with THO, the exchange-out in the presence of zinc, which fully restored enzymic activity, was compared to the apoenzyme. The effect of zinc binding by carbonic anhydrase is much less than that caused by binding of iron by conalbumin and is in the opposite direction (see Discussion). Addition of the strongly bound inhibitor, acetazolamide, did not significantly affect the hydrogen exchange of the zinc enzyme.

Discussion

The binding of a small molecule or ion to a protein can influence hydrogen exchange in several possible ways. The bound substance may physically shield a portion of the protein and make certain hydrogens inaccessible to solvent. A local conformation change of the protein at or near the binding site might affect hydrogens in that region (induced fit). Or, a general change in conformation could occur and influence hydrogen exchange at sites of the protein that are distant from the binding site. DiSabato and Ottesen (1965) found that nucleotide binding impairs the exchange of about 200 hydrogens of lactic dehydrogenase, and these authors felt that it would be unlikely that such a small bound molecule could "bury" that many hydrogens. It is even more difficult to visualize how the unhydrated ferric and cupric ions chelated by conalbumin could shield an extensive area of the protein. Our finding that binding of acetazolamide to carbonic anhydrase does not influence hydrogen exchange supports the belief that small molecules do not effectively shield a significant number of hydrogens from the exchange process.

It is not possible on the basis of exchange data alone to decide whether the effect of metal binding on hydrogen exchange is due to local or general conformation changes. Our exchange data are very similar to those observed upon the binding of nicotinamide–adenine dinucleotide to alcohol dehydrogenase, which retards the exchange of about 50 hydrogens (Hvidt and Kägi, 1963). These authors suggested that this effect is due to the conversion of a group of very rapidly exchanging hydrogens into a very slowly exchanging class. Later, however, Hvidt and Nielsen (1966) offered an alternative explanation, namely, that *all* of the measurably exchanging hydrogens are affected. The latter interpretation was used by Ulmer and Kägi (1968), who found that reduction of ferricytochrome *c* retarded hydrogen exchange due to the fact that "the structure of the reduced is significantly more compact than of the oxidized cytochrome." There is little definitive evidence that conalbumin changes its size or shape upon metal chelation. Small differences in sedimentation coefficients, diffusion constants, and frictional ratios have been reported that indicate a slightly more compact molecule upon chelation (Fuller and Briggs, 1956). We were unable to detect any differences in the ultraviolet optical rotatory dispersion of the free and complexed protein. However, our experiments indicate that chelation causes about 50 hydrogens to be *protected* from exchange while another group of hydrogens are simultaneously *exposed* to exchange. This is not due to the conversion of a rapidly exchanging class of hydrogens to a slowly exchanging class because the data of Table I show that the *average* half-life of these hydrogens is about 1.5 hr in the absence of iron. Of course, very rapidly exchanging hydrogens, which are lost during passage of the tritiated protein through the short column, and very slowly exchanging hydrogens, which do not become labeled during the 48-hr incubation of the metal-free protein, are not observable, a difficulty inherent in all hydrogen exchange experiments. It seems quite possible, therefore, that many other hydrogens are influenced by metal chelation, which suggests that more than local conformation changes occur. Should iron-binding convert conalbumin from a "loose" into a "compact" form, then the iron-free protein would become more labeled in THO

(exchange-in) than the complexed protein, but this is not observed (Figure 5). The conformation changes caused by metal chelation must, therefore, be quite complex and cause a quickening of exchange in some regions and a slowing in other regions.

The lack of effect of iron upon the exchange of conalbumin at pH 6.5 is probably due to the much weaker binding of metal under more acid conditions (Warner and Weber, 1953). Kinetic evidence has shown that the binding of iron by conalbumin is cooperative, *i.e.*, binding of the second metal ion is accelerated by the first bound metal. Consequently, a solution of conalbumin containing less than 2 equiv of metal consists of molecules containing two bound metal ions and molecules containing no bound metal; a species of protein containing one bound metal has not been obtained (Woodworth, 1966). It is not surprising, therefore, to find that with less than 2 equiv of iron present the effect on hydrogen exchange (Figure 3) is proportional to iron concentration, because we are looking at an average exchange of two species of protein. Similar results are obtained when other parameters, such as optical rotation (Vallee and Ulmer, 1962) or absorbance (Warner and Weber, 1953), are followed as a function of iron concentration. However, it has been suggested (R. Michaud, personal communication) that at lower pH a species of conalbumin may be obtained containing just one atom of chelated iron. Attempts to follow exchange at pH 6.5 as a function of iron concentration were unsuccessful, probably due to removal of iron and acceleration of exchange during passage through the column. Such an approach in principle could determine whether or not the conformation changes are entirely due to binding of the first metal, which "sets up" the conformation for binding of the second metal, as suggested by Woodworth (1966).

Preliminary experiments with other metalloproteins indicate that our results with conalbumin cannot be generalized. Binding of copper to ribonuclease did not affect hydrogen exchange under the conditions of our experiment. Binding of zinc to apocarbonic anhydrase appears to slightly accelerate exchange (Figure 6). This result is not incompatible with the iron conalbumin system in which binding of iron apparently retards and accelerates exchange in different regions. However, X-ray data have shown that the apocarbonic anhydrase is isomorphous with the zinc enzyme (Fridborg *et al.*, 1967). The slight effect of zinc binding here may be due to charge effect rather than conformation changes.

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