Slow Tritium–Hydrogen Exchange in Some Cyclic Peptide Chelates*

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ABSTRACT: The Englander technique (Englander, S. W., *Biochemistry 2*, 798 (1963)) was adapted to the study of tritium–hydrogen exchange of low molecular weight peptides. Two cyclic hexapeptides (ferrichrome and ferrichrome A) were both found to have from two to four slowly exchanging hydrogens per molecule. Not only the number of slowly exchanging hydrogens, but also their rate of exchange were found to be pH dependent. Half-lives up to 7 hr were observed at 30°. Slow exchange was dependent upon the presence of the 1 mole of iron chelated by these compounds. Removal of the iron from the peptides resulted in rapid exchange of all amide hydrogens ($t_{1/2} < 5$ min); iron added in excess of 1 mole/mole of peptide yielded exchange data identical with those found for the stoichiometric chelate. Based on the known crystallographic conformation of ferrichrome A, we assign two of the slowly exchanging hydrogens to the buried amide groups (Orn_1 and Orn_2) and two hydrogens, whose presence or absence depends on the pH, to across the ring hydrogen bonds (amide group nitrogens of Orn_3 and Ser_2). Ferrioxamine B, a trihydroxamate iron chelate containing two amide bonds, showed two slowly exchanging hydrogens with a half-life of 5 hr at pH 4.3 and 4°. The pH dependence of exchange rate was very sharp with a minimum at pH 4.3. Removal of the iron resulted in rapid exchange of the amide hydrogens. We suggest that the slowness of exchange of these amide hydrogens is due to hydrogen bonding between the amide NH and the hydroxylamino oxygen in a manner analogous to that found in the X-ray structure of ferrichrome A. Other peptides were examined by the exchange technique: glutathione, oxidized glutathione, cyclo(Ala-Ala-Gly-Ala-Gly-Gly), and vasopressin. None was found to have slowly exchanging hydrogens. We discuss the relevance of our results to the interpretation of hydrogen-exchange data of proteins based on the Linderstrøm–Lang model.

The rate of exchange of peptide-bond hydrogens in proteins is generally much slower than that found in small peptides and random-chain polypeptides. This slowness of exchange is generally attributed to hydrogen bonding or burying of the peptide bond by the secondary and tertiary structure of the protein. The interpretation of hydrogen-exchange data is very complex and has been the subject of much discussion and speculation (Harrington *et al.*, 1966; Schellman and Schellman, 1964; Hvidt and Neilsen, 1966). Much of the ambiguity in the interpretation of exchange data is because of the unavailability of simple model compounds that contain slowly exchanging peptide hydrogens.

Ferrichrome A is a heteromeric cyclic peptide containing iron chelated by three hydroxamic acid residues (Emery and Neilands, 1961). Recently, the X-ray crystallographic structure of this compound has been published (Zalkin *et al.*, 1966). Examination of a space-filling model based on the X-ray structure revealed that several of the peptide hydrogens were buried by the side chains which chelate the iron over the peptide ring, and this suggested that these hydrogens might be of the slowly exchanging class. Experiments presented in this paper show that from two to four of the six peptide-bond hydrogens of ferrichrome A exhibit a remarkably slow pH-dependent exchange similar to that found in proteins. The relevance of this finding to the interpretation of hydrogen-exchange data of proteins is discussed.

Experimental Section

Materials. Ferrichrome and ferrichrome A were crystallized from the culture fluid of a *Ustilago sphaerogena* fermentation as previously described (Garibaldi and Neilands, 1955). Iron was removed from the ferrichrome compounds by methods previously described (Emery and Neilands, 1960). Desferriferrioxamine (Desferal) was a gift from Dr. Walter Barrett, Ciba Pharmaceutical Co., Summit, N. J. Ferrioxamine B was prepared by addition of 3.1 mmoles of ferric ammonium sulfate to 3 mmoles of Desferal and purification of the resulting chelate by paper electrophoresis at pH 5 (Emery, 1965). Following elution from the paper with water the substance was lyophilized. Arginine-vasopressin and cyclo(Ala-Ala-Gly-Ala-Gly-Gly) were generous gifts from Dr. Saul Lande and Dr. V. Prelog, respectively. Bio-Gel P-2 was purchased from Bio-Rad Laboratories and tritiated water from New England Nuclear Corp.

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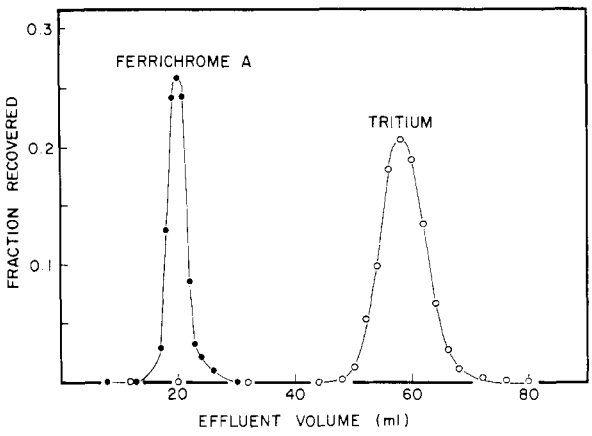


FIGURE 1: Separation of ferrichrome A from tritiated water using Bio-Gel P-2 (50–100 mesh). The column was 1.5 × 20 cm with a flow rate of 2 ml/min. Ferrichrome A (2 mg) dissolved in 0.5 ml of tritiated water (1 μc/ml) was placed on the column and eluted with water at a temperature of 30°.

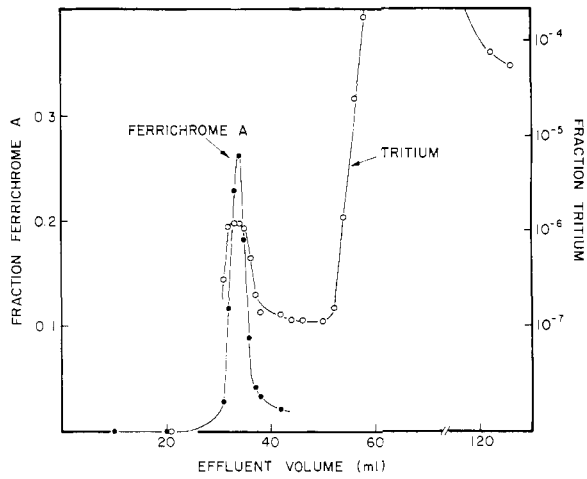


FIGURE 2: Incorporation of slowly exchanging tritium into ferrichrome A. Ferrichrome A (2 mg) was dissolved in 0.5 ml of tritiated water (1 mc/ml), heated to 80° for 15 min, cooled, and passed through a 1.8 × 27 cm Bio-Gel P-2 column with a flow rate of 2 ml/min. The temperature was 4°.

Methods. Isotopic equilibration with tritium (exchange-in) was carried out by dissolving 10–15 mg of the given compound in 0.5 ml of tritiated water (1 mc/ml) and heating to 80° for 15 min. The cooled sample was diluted with 2 ml of the appropriate buffer and the back-exchange (exchange-out) of tritium from the compound was followed by the two-column technique of Englander (Englander, 1963) using Bio-Gel P-2 (50–100 mesh) instead of Sephadex. All columns were previously equilibrated with buffer: 0.005 M tartrate (pH 3.0), 0.005 M acetate (pH 4.7), or 0.005 M phosphate (pH 7.0). The short column measured 2.5 × 5 cm and the long columns 1.8 × 27 cm. The concentrations of ferrichrome (ϵ_{425} 2895) and ferrioxamine (ϵ_{440} 2560) in the eluted fractions were determined spectrophotometrically. The molar extinction coefficient at 440 mμ for ferrichrome A was carefully redetermined and found to be 3360 M^{-1} cm^{-1}. There is some discrepancy between this value and the value of 3740 previously reported (Emery and Neilands, 1960); the redetermined (lower) value was used in this work. Concentrations of nonchelated peptides were also determined spectrophotometrically at suitable wavelengths in the ultraviolet (225–240 mμ).

The radioactivity of 0.5-ml aliquots of the eluate was determined in a Beckman CPM-100 scintillation counter using 10 ml of modified Bray's solution (dioxane containing 20% naphthalene, 1% PPO, and 0.025% POPOP).[1] Under these conditions the counting efficiency for tritium was 34% with a background of 30 cpm. Samples were counted to a standard deviation of ±5%. Individual color quench correction curves were prepared for all iron chelates; the maximum correction was 12%, but in most cases samples of about 0.05 mg were counted for which the correction was 5% or less.

Results

Removal of Tritium by the Englander Method. The Englander method has found wide acceptance for the study of tritium exchange of proteins. Our initial experiments indicated that this technique could also be applied to small peptides by the use of Bio-Gel P-2. In the two-column technique we found that 99.5–99.9% of the tritiated water was removed from the peptides by the first short column in a time of 2–3 min. The tritium content of the solvent was thus sufficiently lowered so that back-exchange of tritium into the solute could be considered negligible during subsequent incubation of the small column eluate. Passage of aliquots of the short-column eluate through the long columns resulted in quantitative removal of the remaining tritium in the solvent. Figure 1 shows the separation of ferrichrome A from tritiated water of specific activity 1 μc/ml, which approximates the radioactivity of the solvent after passage through the short column. At this level of radioactivity any incorporation of tritium into the peptide would not result in a measurable number of counts in the peptide fraction. Similar elution patterns were obtained for the other peptides studied; no detectable counts were found in the peptide-containing fractions.

A preliminary one-column experiment was next performed to determine if ferrichrome A might in fact contain slowly exchanging hydrogens. A sample of ferrichrome A previously equilibrated with tritiated water (1 mc/ml) was subjected to a one-column run at 4°. The peak of radioactivity emerging with the

[1] Abbreviations used: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

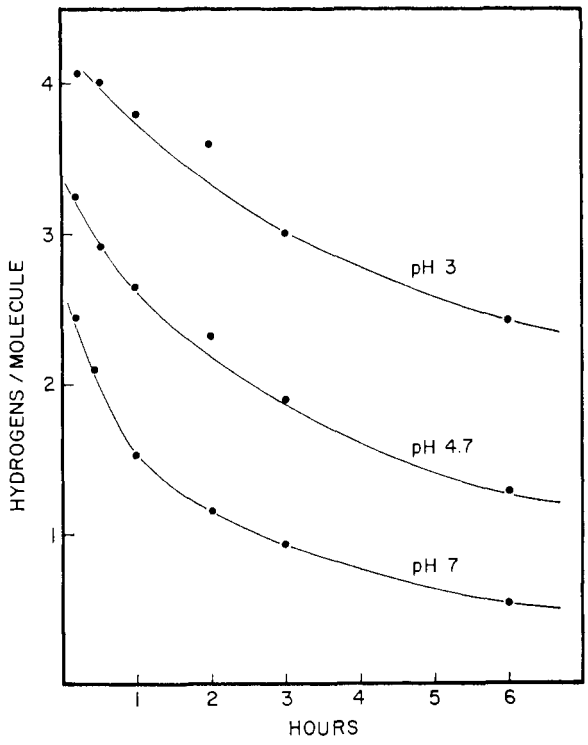


FIGURE 3: Exchange-out curves (loss of tritium) of ferrichrome A. Ferrichrome A (10 mg) was heated in 0.5 ml of tritiated water (1 mc/ml) at 80° for 15 min and cooled, and 2 ml of the appropriate buffer was added. Ferrichrome A was then separated from THO by the two-column technique (see Experimental Section) and its specific activity, expressed as hydrogens per molecule, was determined. The temperature was 30°.

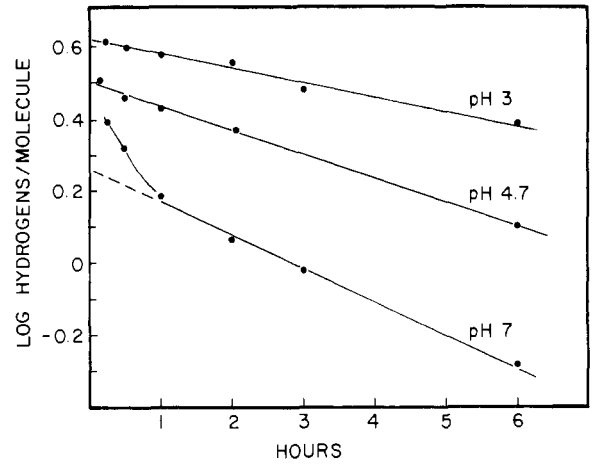


FIGURE 4: Semilog plots of the curves of Figure 3 showing first-order nature of the rates of tritium exchange-out from ferrichrome A.

peptide (Figure 2) indicated the presence of three to four atoms of tritium per molecule of ferrichrome A. Passage through the long column required about 10 min, suggesting that these hydrogens were indeed of the slowly exchanging class. It should be emphasized that in all subsequent experiments we used the two-column technique in which the large tritium peak was removed by the initial short column, so that the resolution of peptide from tritiated solvent was comparable to that shown in Figure 1. In addition, several successive fractions from each column were routinely analyzed and always were found to have constant specific activity (tritiums per molecule).

Tritium Exchange of Ferrichrome A. Exchange experiments with ferrichrome A at 4° showed that the half-life of the slowly exchanging hydrogens was of the order of 24 hr, so a higher temperature was chosen. The back-exchange of fully tritiated ferrichrome A at 30° and several pH values is shown in Figure 3.[2] It is apparent that from 2 to 4 of the 11 exchangeable hydrogens present in ferrichrome A exhibit a very slow rate of exchange. These kinetic curves are strikingly similar to those found by many investigators for proteins both with respect to the rate of exchange and pH dependence (Hvidt and Nielsen, 1966). First-order plots of the log hydrogens per molecule as a function of time are shown in Figure 4, and from these data half-lives of 7.1, 4.5, and 3.0 hr were found at pH 3.0, 4.7, and 7.0, respectively. The number of hydrogens/molecule extrapolated to zero time was 4.2, 3.2, and 1.8 at the respective pH values. The deviation of the early points at pH 7 is often found in similar experiments with proteins and represents the exchange of one or more hydrogens of a more rapidly exchanging class.

Tritium Exchange of Ferrichrome. Ferrichrome is a structural analog of ferrichrome A and it was of interest to compare the behavior of these two compounds. Figure 5 shows the back-exchange of fully tritiated ferrichrome at 30°. Although the exchange rates are faster than those observed in ferrichrome A, they are still several orders of magnitude slower than found in simple peptides. First-order plots showed the half-lives to be 48, 94, and 14 min at pH 3.0, 4.7, and 7.0, respectively. The number of slowly exchanging hydrogens extrapolated to zero time was 3.8, 2.7, and 1.8 at the respective values of pH. No differences were found in the optical rotatory dispersion of ferrichrome between 230 and 600 mμ taken in the same buffers as used in the exchange experiments.

Tritium Exchange of Ferrioxamine B. Ferrioxamine B (Bickel *et al.*, 1960) contains three hydroxamic acid groups chelated to iron and two nonchelated peptide bonds (Figure 6). At 25° and pH 4.7 the half-life of two hydrogens was found to be about 16 min. The kinetics could be followed more conveniently at 4° (Figure 7). The half-life of the two hydrogens at this lower temperature and pH 4.7 was found from a first-order plot to be 3.3 hr. Numerous experiments with

[2] In this paper, the term tritiated will always be understood to mean equilibration of nitrogen-, oxygen-, or sulfur-bound hydrogens with THO.

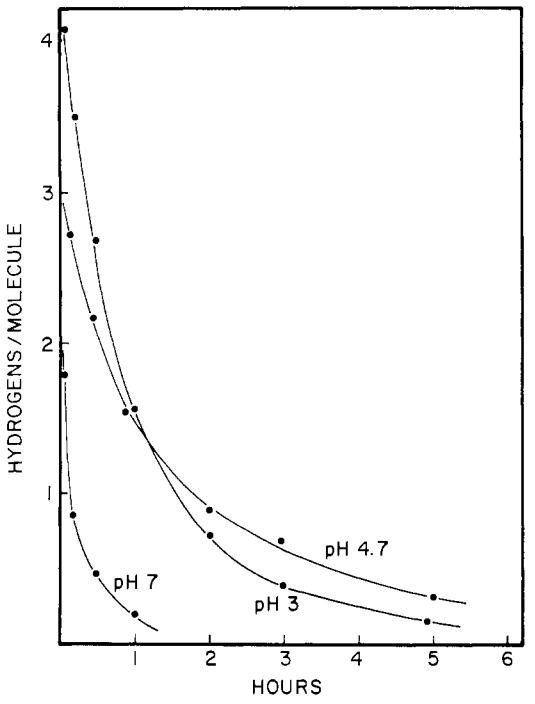


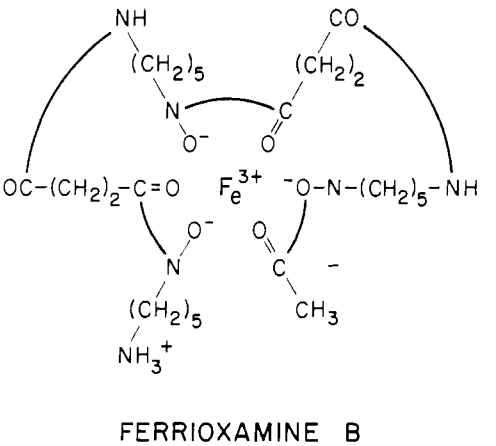
FIGURE 5: Exchange-out curves (loss of tritium) of ferrichrome. The conditions and procedure were exactly those of Figure 3.

this compound always resulted in the finding of two slowly exchanging hydrogens when the kinetic data were extrapolated back to zero time. Since the half-life of hydrogens on a primary amino group is 1 sec or less (Schellman and Schellman, 1964), the two hydrogens found must be the two peptide NH hydrogens. The pH dependence of exchange of ferrioxamine was much more pronounced than for the ferrichromes (Table I). A minimum rate of exchange was found at pH 4.3 ($t_{1/2}$ = 5 hr). Table II summarizes the exchange results found for ferrichrome, ferrichrome A, and ferrioxamine.

Control Experiments. In view of the rather unexpected

TABLE I: Effect of pH on the Half-Life of the Two Amide Hydrogens of Ferrioxamine B at 4°.

pH	Half-Life (min)
3.0	28
3.6	72
3.9	150
4.1	250
4.3	295
4.7	200
5.6	86
6.4	9
7.0	<2



FERRIOXAMINE B

FIGURE 6: Structure of ferrioxamine B. The three hydroxamate groups are octahedrally chelated about the ferric ion.

finding of slowly exchanging hydrogens in relatively low molecular weight peptides, a number of control experiments were performed. As discussed later, the observed slowness of exchange is undoubtedly a consequence of the conformational rigidity imposed upon these molecules by chelation of the iron. The iron-free compounds were therefore examined. After equilibration with tritiated water, desferriferrichrome, desferriferrichrome A, and Desferal (desferriferrioxamine B) all showed a very rapid back-exchange with rates

TABLE II: Half-Lives of Amide Hydrogens of Ferrichrome, Ferrichrome A, and Ferrioxamine B.

	pH		
	3.0	4.7	7.0
Ferrichrome, 30°	48[a] (3.8)[b]	94 (2.7)	14 (1.8)
Ferrichrome A, 30°	426 (4.2)	270 (3.2)	180 (1.8)
Ferrioxamine B, 4°	28 (2.2)	200 (2.2)	<2 (2.2)
Unprotected amide, 4°	44[c]	2	0.01
Unprotected amide, 30°	2	0.1	0.0004

[a] All half-lives are in minutes. [b] Values in parentheses are the number of slowly exchanging hydrogens per molecule extrapolated to zero time. [c] These values are calculated from

$$t_{1/2} = \frac{0.69}{50(10^{-\mathrm{pH}} + 10^{\mathrm{pH}-6})10^{0.05(t-20)}\ \mathrm{min}^{-1}}$$

(Hvidt and Nielsen, 1966).

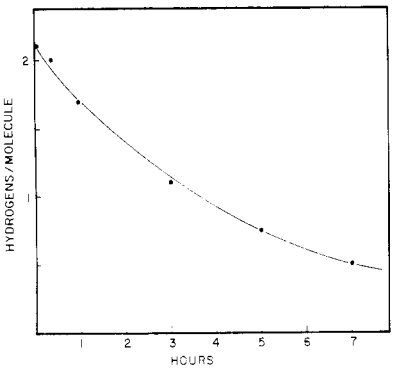


FIGURE 7: Exchange-out (loss of tritium) of ferrioxamine B at pH 4.7 and 4°. The procedure was exactly that of Figure 3.

TABLE III: Maximum Half-Lives for the Amide Hydrogens of Model Peptides.

	Half-Life (min)	Temp (°C)	pH
Glutathione	2	4	5.5
Glutathione (oxidized)	3	4	4.7
Desferriferrichrome A	3	4	4.7
Cyclo(Ala-Ala-Gly-Ala-Gly-Gly)	3	30	4.7
Desferriferrioxamine B	4	30	4.7
Vasopressin	2	30	4.7

expected for simple peptides. Equally rapid exchange was found for such diverse peptides as glutathione, oxidized glutathione, arginine-vasopressin, and cyclo-(Ala-Ala-Gly-Ala-Gly-Gly). A hydroxamic acid iron chelate, $[\text{fusarinine}]_3Fe^{III}$ (Emery, 1965), which contains no peptide bonds but does have 12 hydrogens bonded to oxygen and nitrogen, was also examined. This substance emerged from the column with no significant radioactivity (<0.1 hydrogen/molecule) after 10 min at 25° and pH 4.7. The specific activities of all of the above compounds were too low for accurate calculations of rate constants. The results, summarized in Table III, are therefore only approximate and represent maximum values for the half-lives of the exchange of the peptide hydrogens.

It seemed possible that the differences of exchange rates observed for ferrichrome, ferrichrome A, and ferrioxamine B might be related in some way to differences in the strength with which they chelate iron. To test this hypothesis, the three β-methylglutaconic acid groups of ferrichrome A were catalytically hydrogenated to β-methylgutaric acid groups. This alteration affects the affinity of the hydroxamate groups for iron (Emery, 1960; Anderegg *et al.*, 1963). A tritium-exchange experiment with this derivative of ferrichrome A at pH 4.7 and 30° yielded results identical with those found for ferrichrome A itself.

Iron chelated by hydroxamic acids is rapidly exchangeable with inorganic iron (Lovenberg and Rabinowitz, 1963). Although the mechanism of this exchange is unknown, it is obvious that such a process must cause transient conformational changes in the ligand, which in turn might influence hydrogen exchange of the peptide bonds. However, iron (citrate) at a concentration of 4.6×10^{-6} M in excess of the iron chelated by ferrichrome had no observable effect upon the tritium exchange of ferrichrome at pH 3.0 even though exchange of the metal is known to take place rapidly under these conditions.

Isotope Effects. Englander (1963) found a small but definite isotope effect in his tritium-exchange studies of ribonuclease. He observed this effect by initially bringing the protein to equilibration with tritium using deuterium oxide rather than water as the bulk solvent. Figure 8 shows exchange experiments in which ferrichrome was tritiated in either water or 99.8% D_2O as solvent. For the latter experiment, sufficient tritiated water (sp act. 25 mc/ml) was added to 99.8% D_2O to give a final specific activity of 1 mc/ml, the same as that used in all other experiments. The data show no detectable isotope effect. These experiments also demonstrate the reproducibility of the technique. Many duplicate runs, not shown, using different columns and different samples of ferrichrome indicated that the over-all reproducibility was about ±5%, corresponding to 0.1–0.2 hydrogen/molecule.

Effect of Urea. As one might expect, urea drastically affects the hydrogen exchange of proteins. Figure 8 shows the effect of 5 M urea upon the hydrogen exchange of ferrichrome at pH 4.7 and 30°. A total of four slowly exchanging hydrogens was observed, rather than the usual three found under these conditions in the absence of urea. The kinetics are no longer first order, but the data can be fit by assuming two hydrogens exchanging with a first-order rate constant of 1.2 hr^{-1}, and two independently exchanging hydrogens with a first-order rate constant of 0.69 hr^{-1} (Linderstrøm-Lang, 1958). The rate constant for the three hydrogens found in the absence of urea is 0.44 hr^{-1}.

Exchange of Ferrichrome A Crystals. Very few studies have been undertaken to compare hydrogen exchange of proteins in the crystalline state with exchange data for the same proteins in solution. In view of the known conformation of ferrichrome A in the crystal, we were curious to make such a comparison with this compound. Figure 9 shows the loss of tritium from crystals of ferrichrome A which were obtained by crystallization of this compound, after heating to 80°, from tritiated water. Very small crystals, with an average edge of 0.01 mm, were purposely prepared by rapid crystallization. Ferrichrome A forms square plates and the third dimension was too small to measure conveniently. The finding of 10 of the possible 11 exchangeable hydrogens after 10 min indicates that heating to 80° is probably sufficient for

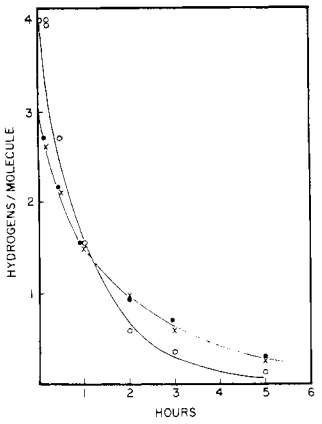


FIGURE 8: Exchange-out of protonated (—●—) and deuterated (—×—) ferrichrome against water at pH 4.7 and 30°. The effect of 5 M urea (—○—) on the exchange of protonated ferrichrome at the same pH and temperature is also shown. The line drawn through the urea points is a theoretical curve calculated assuming two groups, each containing two hydrogens, and the hydrogens of each group exchanging with independent first-order rate constants of 1.2 and 0.69 hr^{-1}, respectively.

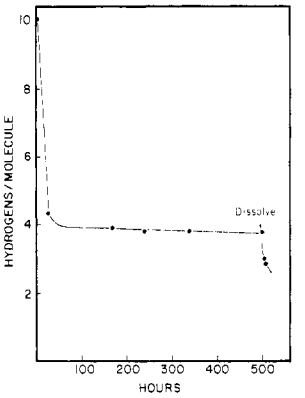


FIGURE 9: Exchange-out of ferrichrome A crystals. About 10 mg of ferrichrome A was crystallized from tritiated water (1 mc/ml) after heating to 80° for 15 min. After washing twice with 10^{-3} N HCl, the crystals were covered with about 5 ml of 10^{-3} N HCl and left at room temperature. At the indicated times, about 1–2 mg of the crystals was removed and dissolved in water, and the specific activity was determined.

tritium equilibration (exchange-in). After 24 hr at room temperature, the number of hydrogens per molecule has dropped to 4 and this number remains constant for a period of 3 weeks. In this experiment the crystals were suspended in water adjusted to pH 3 with hydrochloric acid to prevent dissolution, and it should be remembered that in solution ferrichrome A was also found to have four slowly exchanging hydrogens at this pH. At the end of 3 weeks, the crystals were quickly dissolved by the addition of solid sodium carbonate. The labeled hydrogens remaining were observed to back-exchange at a rate comparable to that found previously for ferrichrome A in solution.

Discussion

A number of small peptides and random coil proteins have previously been examined by the hydrogen-exchange technique: glycylglycine, alanylglycylglycine, *N*-methylacetamide, insulin A chain, oxidized ribonuclease, angiotensin, and gelatin (see Harrington *et al.*, 1966, for references). In all of these examples, the amide hydrogens were found to exchange as a single first-order decay curve with half-lives of the order of several minutes. The much slower exchange of protein amide hydrogens, with half-lives of several hours, is generally attributed to protection from exchange by hydrogen bonding and/or inaccessibility to the solvent due to conformational stability of the protein. One might assume that this phenomenon could also occur in smaller molecules should the amide group find itself in a similar restricted environment. The ferrichrome compounds appear to fulfill this requirement. These compounds are cyclic hexapeptides containing three N^{δ}-hydroxyornithine residues whose side chains, in the form of hydroxamic acid derivatives, chelate one iron atom in the ferric state. The chelation of the iron in effect ties the three ornithine side chains in a knot over one end of the hexapeptide ring. Examination of space-filling molecular models of the ferrichromes shows that the chelation imposes an extremely rigid conformation upon the molecules which results in the burying of the amide hydrogens of ornithine residues 1 and 2 under a tent formed by the ornithine side chains. In addition, the peptide ring resembles an antiparallel pleated sheet with the possibility of two across the ring hydrogen bonds. Only one such bond, involving the α-*N* of ornithine residue 3 and the carbonyl of serine 2, was actually indicated in the crystallographic study (Zalkin *et al.*, 1966), although two such bonds have been found in crystalline cyclohexaglycine (Karle and Karle, 1963).

Our experimental results can be discussed with reference to the above structural considerations. With respect to hydrogen exchange, the ferrichrome compounds are very simple models since there are only six peptide bonds to consider. We can assume that the additional five exchangeable hydrogens of ferrichrome A (two serine hydroxyl and three methylglutaconic carboxyls), not present in ferrichrome, exchange too fast to be seen by the Englander technique. Extrapolation of our semilog plots of exchange data to zero time may not reliably indicate the total number of slowly exchanging hydrogens because of the small slopes involved; however, there appear to be at least two such hydrogens which were always detected in the pH range of 3–7. We believe that the most probable assignment of these hydrogens is to the amide groups of Orn_1 and Orn_2 which are buried under the chelated iron. In any case, their rate of exchange is remarkably

slow, with a half-life of several hours at 30°, compared to a half-life of 2.6 hr found by Englander (1963) for the class III hydrogens of ribonuclease at the same pH but a temperature of 4°. Since an increase of temperature from 4 to 30° is known to increase the exchange rate by 10–20 times (Hvidt and Nielson, 1966), the ferrichrome hydrogens exchange more slowly by at least an order of magnitude, and indeed an experiment with ferrichrome A at 4° indicated a half-life of 20–30 hr. Most important is the fact that the slow exchange is completely dependent upon the presence of the chelated iron. The iron-free ferrichromes and other cyclic peptides showed the rapid exchange expected for small peptides. The drastic conformational change imposed by chelation of iron is shown in Figure 10.

The number of observable slowly exchanging hydrogens was found to increase with decreasing pH, and as many as four such hydrogens could be seen at pH 3 for both ferrichrome and ferrichrome A. In addition to the buried hydrogens discussed above, it seems likely that these additional hydrogens are the amide hydrogens of Orn_3 and Ser_2 which may take part in across the ring hydrogen-bond formation. Although these assignments cannot be made with certainty, the X-ray structure does indicate that the two remaining amide hydrogens (Ser_1 and Gly) are completely exposed at the nonchelated end of the molecule and have no adjacent groups with which to interact, and these hydrogens probably exchange before our first measurements are taken. Presumably the four nonexchanging hydrogens found in suspensions of ferrichrome A crystals at pH 3 (Figure 9) are the same as those found in a solution of ferrichrome A at the same pH. The structural rigidity of the ferrichromes due to the iron chelation makes it seem very likely that the conformation in solution is very similar if not identical with that in the crystalline state. It is possible that the unusually slow exchange of ferrichrome A at pH 3 as compared to ferrichrome and ferrioxamine B is due to association of this molecule at the lower pH, although unpublished ultracentrifugation experiments with the ferrichromes have shown no evidence of association of these molecules at pH 7. Such association would be expected to slow the exchange rates of the amide hydrogens and perhaps even bring side-chain hydroxyl and carboxyl group hydrogens of ferrichrome A into a measurable range.

Complete substitution of exchangeable hydrogens in a protein by deuterium can bring about physical changes due to changing strengths of hydrogen bonds, and this is reflected in differences in tritium-exchange rates (Englander, 1963). The main factor in conformational stabilization in the ferrichromes is chelation of iron, and one would not expect to see such differences with these compounds. The back exchange of ferrichrome was identical whether the equilibration with tritium was carried out in deuterium oxide or water as the bulk solvent. In both cases the exchange-out was done against water as solvent. Thus, the presence of deuterium (or tritium) in these molecules does not bring about any conformational or other change which is reflected in the exchange experiments, so that we can be confident that what we are observing is representative of the molecules dissolved in water alone.

Urea does not show the drastic effect on hydrogen exchange of the ferrichromes that is found with proteins. This is not surprising in view of the lack of isotope effect discussed above. "Denaturation" of the ferrichromes is more analogous to removal of iron which allows the ornithine side chains to "unfold" and thus expose the peptide ring, and we have seen that this leads to rapid hydrogen exchange. However, the effect of urea on the exchange of ferrichrome is not negligible (Figure 8). Four hydrogens, rather than the usual three, are now found at pH 4.7. The kinetics of exchange can be explained by assuming that there are two classes, each containing two hydrogens, exchanging independently. If our previous speculations are correct, the two more slowly exchanging hydrogens are those buried under the chelated iron and the other two are the across the ring hydrogen bonds. We believe that the effect of urea is on the rate of exchange of the amide hydrogen *per se* and not due to conformation changes of the molecule, as will be discussed below.

Ferrioxamine B is structurally very different from the ferrichrome compounds (Figure 6) and the amide hydrogens cannot be buried in the manner in which two hydrogens are buried in the ferrichromes. Ferrioxamine does in fact show a much more rapid hydrogen exchange than the ferrichromes. Nevertheless, at the pH of minimum exchange a half-life of 5 hr was found for the two amide hydrogens, a value considerably greater than found for simple peptides. As for the ferrichromes, the slowness of exchange is completely dependent upon chelated iron. The arrangement of the three hydroxamate bonds in ferrioxamine B is similar to the ferrichromes in that they can arrange themselves octahedrally about the iron, thus forming an especially stable hexadentate chelate. Examination of molecular models shows that there is enough flexibility in the 1-amino-5-hydroxylamino moieties to allow the two amide groups to be tucked back in toward the iron so that the hydrogens of these groups may hydrogen bond to the hydroxylamino oxygens, in a manner analogous to that observed in the crystal structure of ferrichrome A, and thus impede hydrogen exchange. The more rapid exchange of these two hydrogens compared to the ferrichromes would then be merely a consequence of the fact that only this type of hydrogen bonding, and not additional protection by burying, is possible. Although this hypothesis is attractive, more data are needed before we can definitely conclude that hydrogen bonds are present in any of these compounds in solution.

The existence of across the ring hydrogen bonds in cyclic peptides in solution has been a matter of much speculation. Our results suggest, but by no means prove, that such bonds may be present in the ferrichromes. If this is the case, however, the primary factor stabilizing such bonds is chelation of iron; removal of the metal leads to disappearance of all slowly exchanging hydrogens, indicating that the across the ring hydrogen bonds do not in themselves play a

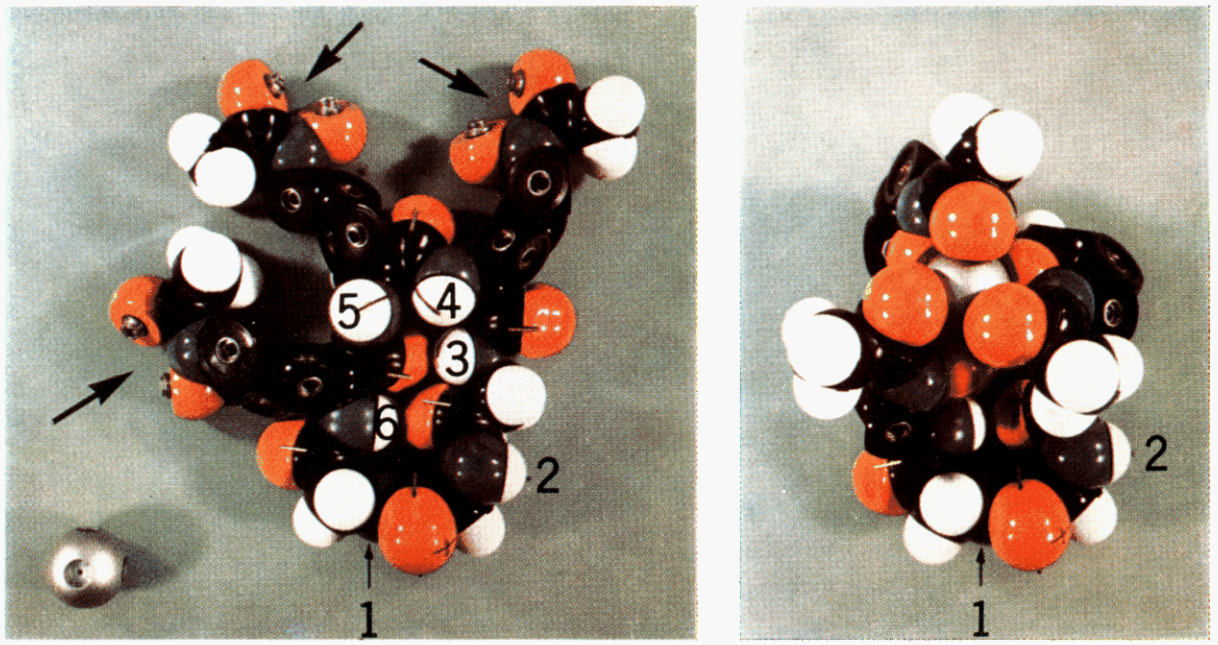


FIGURE 10: Studies on the structure of ferrichrome. (a) Structure of ferrichrome from which the iron atom (lower left) has been removed and the three substituted ornithine side chains put in a fully extended conformation. Heavy arrows indicate the three hydroxamate groups. The NH hydrogens of the cyclic hexapeptide ring are numbered according to Zalkin *et al.* (1966): 1 (not visible), Gly_1; 2, Gly_2; 3, Gly_3; 4, Orn_1; 5, Orn_2; 6, Orn_3. In ferrichrome A, serine replaces glycine at positions 2 (Ser_1) and 3 (Ser_2), and β-methylglutaconate replaces acetate as the hydroxamate acyl moieties. Hydrogens have been removed from side-chain methylene carbons. (b) The effect of iron chelation by the three hydroxamate groups. All atoms of the peptide ring are in the same positions as in part a.

significant role in the secondary structure of these peptides. Attempts to detect such hydrogens by the Englander technique with the iron-free peptides in nonaqueous media have not been successful.

Relevance to Protein Hydrogen Exchange. Most investigators interpret hydrogen exchange of proteins by a model proposed by Linderstrøm-Lang (1958) which assumes that a stable conformation (A_1) is in equilibrium with one or more other conformations (A_2) and that *only* the latter undergo exchange, each amide group exchanging with a rate constant (k_3) which is that found for small peptides (Table II).

$$A_1 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} A_2 \xrightarrow{k_3} \text{exchange}$$

The rate of exchange of any given group in the protein is then a function of k_1 and k_2, the sum of which is often referred to as the protein's motility. The effect of pH on k_3 is known from studies of model peptides; thus, a study of the effect of pH upon the rate of hydrogen exchange of a protein presumably yields information about the effect of pH on the equilibrium between A_1 and A_2 (see, for example, Segal and Harrington, 1967). If the amide hydrogens in conformation A_1 are in fact able to undergo exchange without prior "opening up" to conformation A_2, then hydrogen-exchange data become too complex to interpret, a possibility acknowledged by Linderstrøm-Lang (1958).

The effect of pH on both the number and rate of slowly exchanging hydrogens in the ferrichrome compounds is strikingly similar to that found for proteins except, of course, that we are dealing with two to four hydrogens instead of hundreds or thousands. The effect of pH is not at all what it is on other model peptides studied, where an increase of two pH units increases the rate of hydrogen exchange by 50–100 times. Increasing the pH from 4.7 to 7.0 only increases the rate of exchange of ferrichrome A by 50%, and even the six- to sevenfold increased rate of exchange of ferrichrome is much less than predicted for simple peptides. In terms of the Linderstrøm-Lang model discussed above, one would have to postulate that a nonexchanging conformation is in equilibrium with an exchanging conformation. Such an equilibrium might be that between the chelated and nonchelated form of the ferrichromes, but that this is not the case is shown by the lack of effect of excess iron upon the hydrogen exchange. Added iron should push the equilibrium toward the nonexchanging chelated form and slow the exchange rate, but this does not occur. Ferrichrome has no ionizable groups in the pH range studied, and the optical rotatory dispersion between 230 and 600 mμ is identical at pH 3, 4.7, and 7. These facts, plus the structural rigidity imposed by the

chelated iron, lead us to doubt the presence of an "opened up" exchanging form of the molecule, but rather we believe that it is likely that exchange takes place directly from a single conformation. The greatly reduced rate of exchange of a given amide group hydrogen may then be a reflection of its local environment, for example, steric factors, planarity of the amide bond, neighboring charged groups, etc. Leichtling and Klotz (1966) reached a similar conclusion by examining the influence of some of these parameters on hydrogen exchange of poly-L-glutamic acid in D_2O–dioxane. The effect of pH on the intrinsic exchange rate of a given amide group may thus vary from one group to another, in which case the determination of k_3 by a study of simple model peptides is not justified.

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