The Mobilization of Iron from Ferritin by Chelating Agents*

Leon Pape,† Jagir S. Multani, Clyde Stitt, and Paul Saltman‡

ABSTRACT: The mobilization of iron from ferritin at rates and in quantities of physiological significance can be accomplished using specific low molecular weight chelating agents. Removal of the iron does not involve reduction of Fe^{3+} to Fe^{2+} and can be carried out under mild conditions of temperature and neutral pH.

Iron is more rapidly released from noncrystalline ferritin in which the ferric hydroxide micelle is only partially surrounded with protein subunits. Noncrystallizable ferritin is in equilibrium with ferritin and may play an important intermediate role in the *in vivo* mobilization of iron by chelates. These findings confirm and extend the model proposed in the preceding paper (Pape, L., Multani, J. S., Stitt, C. F., and Saltman, P. (1968), *Biochemistry 7*, 606) for the synthesis of ferritin and the mobilization of its iron involving chelation reactions in which redox systems are not required.

In the previous paper (Pape *et al.*, 1968) we have discussed some aspects of the chemistry and biochemistry of iron in ferritin. Since Laufberger's (1937) original work there have been continuous efforts to elucidate the mechanisms by which iron is mobilized from ferritin for metabolic use. A number of models have been proposed. Michaelis *et al.* (1943) demonstrated the *in vitro* removal of ferritin iron by reduction with dithionite ($Na_2S_2O_6$) in the presence of bipyridine or phenanthroline at pH 4.6. Reduction and removal of ferritin iron has also been carried out using physiological agents including cysteine, glutathione, and ascorbic acid. In view of these results, efforts have been directed at finding a suitable "physiological" redox system. Mazur and Green (1959) claimed that xanthine oxidase was responsible for ferritin iron reduction and mobilization in the liver. Mazur and Carleton (1963) further suggested that ATP[1] and ascorbate may be involved in an energy-linked reduction and mobilization of the iron. However, several agents which severely inhibit xanthine oxidase such as allopurinol have little or no effect on iron metabolism (Davis and Deller, 1966). Charley *et al.* (1960) demonstrated that small chelate molecules were required to facilitate the transport of iron from serum to tissue in mammalian cells and the uptake was independent of metabolic inhibitors enhanced uptake and much of the iron accumulated was incorporated into ferritin.

The participation of chelating agents in the regulation and control of iron metabolism by a variety of plants, bacterial, and animal systems is clearly recognized and is well documented (Wallace, 1963; Brown and Tiffen, 1965; Neilands, 1957; Saltman, 1965). It has been demonstrated that both steric considerations as well as binding affinities influence the rate of iron exchange between chelator and transferrin (Bates *et al.*, 1967a,b). Although the environment at the iron binding site of transferrin is quite unlike that in ferritin, we were led to examine the possibility that small chelate molecules were also capable of removing iron from ferritin under physiological conditions. We will demonstrate that chelators can remove iron from ferritin in the absence of redox reactions, and that the chemical nature of the attacking chelating agent regulates the rate of iron mobilization.

Materials and Experimental Methods

Preparation of Ferritin. Ferritin was prepared from horse spleen using the general techniques described by Granick (1942). Following maceration in distilled water and heat extraction at 80°, the homogenate was filtered while hot and the precipitate was discarded. The clear filtrate was adjusted to pH 4.6 and centrifuged at 1280 rpm for 30 min. The precipitate was discarded and ferritin was crystallized from the supernatant by the addition of $CdSO_4$ to a final concentration of 5%. The crystals were separated by centrifugation, dissolved in 2% $(NH_4)_2SO_4$, and recrystallized with $CdSO_4$. The supernatant of the second crystallization was retained as the noncrystalline fraction. Both the crystalline and noncrystalline ferritin were dialyzed several days against distilled water to remove salt. After dialysis, the preparations were adjusted to pH 7.4 and centrifuged to remove any sediment.

Dialysis Techniques. Aliquots (5 ml) of crystalline or noncrystalline ferritin containing from 0.2 to 0.7 mg/ml of iron was placed in No. 27 Visking sacs for dialysis. The sacs were then washed with iron-free water to remove all contaminants adhering to the outside and

* From the Division of Biology, University of California at San Diego, La Jolla, California 92037. *Received June 14, 1967.* This work was supported by grants from the John A. Hartford Foundation, the National Institutes of Health (AM05484), and the California Division of the American Cancer Society.

† Present address: Department of Physics, California State College at Los Angeles.

‡ Research career development awardee, U. S. Public Health Service.

[1] Abbreviation used: ATP, adenosine triphosphate.

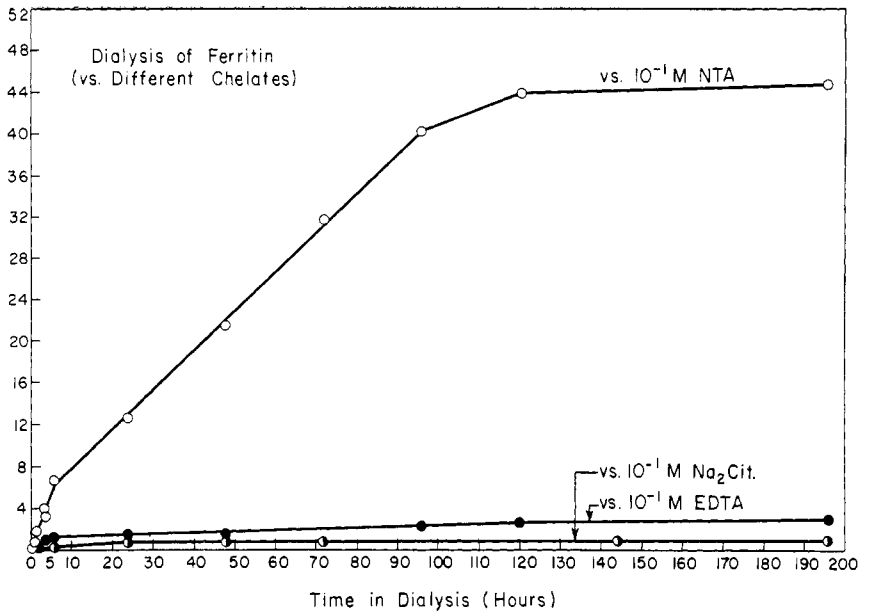

FIGURE 1: Time course of dialysis of iron from crystalline ferritin (0.2–0.7 mg of iron/ml) using nitrilotriacetate, EDTA, and citrate (0.1 M). Solutions were 5×10^{-3} M in Tris-HCl (pH 7.4); total ionic strength, μ 0.2 with NaCl; temperature, 25°.

then suspended in 250-ml erlenmeyer flasks containing 100 ml of chelate solution. Nitrilotriacetic acid (Matheson), disodium EDTA (Mallinckrodt), and trisodium citrate (Mallinckrodt) were used in the preparation of chelate solutions which were 5×10^{-3} M in Tris-HCl buffer (pH 7.4), total ionic strength μ 0.2 with NaCl. The same buffer without chelating agent was used as a control. Dialysis was carried out at 25° with constant stirring. At periodic intervals, 1.0-ml samples of dialysate were removed and total iron was determined using a modification of the method of Bothwell and Mallett (1955). Appropriate corrections were made for changes in dialysate volumes.

Results

Mobilization as a Function of Chelating Agents. The time course of removal of iron from ferritin by nitrilotriacetic acid, EDTA, and citrate, each at 0.1 M, is shown in Figure 1. It is apparent that efficacy of the chelator varies markedly both in rate and total amount of iron removed. The 0.1 M nitrilotriacetate removed about 44% of iron initially present in the ferritin. Equilibrium was reached in 120 hr. EDTA removed only about 3% of the initial iron, while 1% was removed by the sodium citrate. No iron was detectable in the dialysate when crystalline or noncrystalline ferritin solutions were dialyzed against buffer in the absence of chelator for as long as 14 days.

The Effect of Chelator Concentration on Mobilization. The removal of iron is dependent on chelate concentration (Figure 2). Both the rate of removal and the maximum amount of iron removed are reduced when concentrations of nitrilotriacetate are lowered. At 10^{-2} M nitrilotriacetate, 19% of the iron was mobilized, and 12.5% at 10^{-3} M. Comparable reductions were observed at lower concentrations of EDTA and citrate.

Mobilization of Iron from Noncrystalline Ferritin. The dialysis of the noncrystalline protein against the same three chelators gave results similar both in chelate specificity and concentration dependence to those observed for ferritin. The order of effectiveness was nitrilotriacetate > EDTA > citrate. Both amount of iron removed and rate were concentration dependent. As was seen with ferritin (Figure 1), there was a rapid mobilization of iron during the initial 6 hr. During the first 48 hr, there was a marked increase in the removal of iron from noncrystalline ferritin when compared with ferritin (Figure 3). A summary of dialysis data representative times for the various chelating agents is presented in Table I.

Discussion

It is clear that significant amounts of iron can be removed from ferritin by low molecular weight chelating agents without the mediation of a redox system. It has been proposed by Mazur *et al.* (1955) that the mechanism of iron release involves a small quantity of ferrous iron external to the micelles and bound to the ferritin by relatively labile sulfhydryl groups. This reduced iron fraction is in redox equilibrium with the micellular ferric iron, and the removal of the external ferrous iron results in a shift of the equilibrium and consequent mobilization of the metal. We found that when either crystalline or noncrystalline ferritin was dialyzed against buffer in the absence of chelate, no iron was removed.

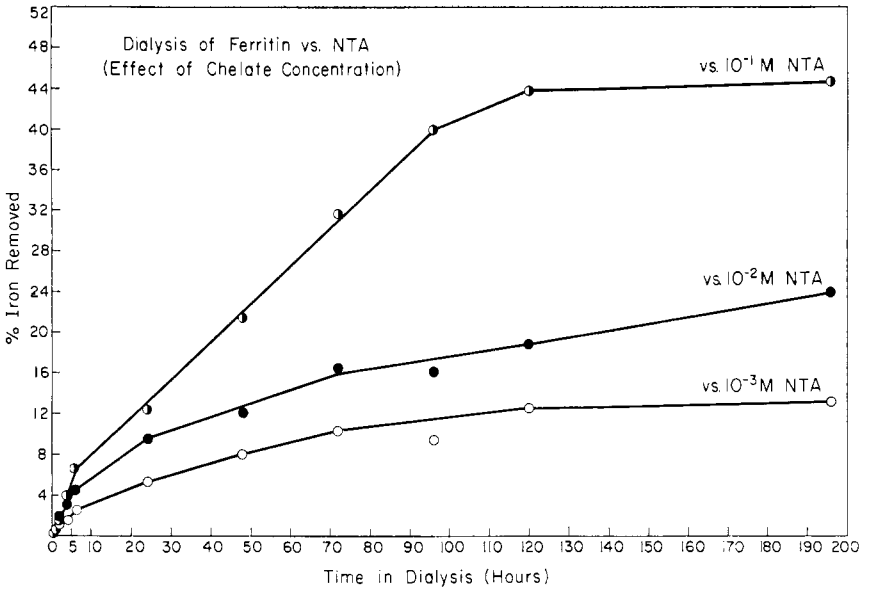

FIGURE 2: Time course of dialysis of iron from crystalline ferritin using nitrilotriacetate at various concentrations. Conditions as described in Figure 1.

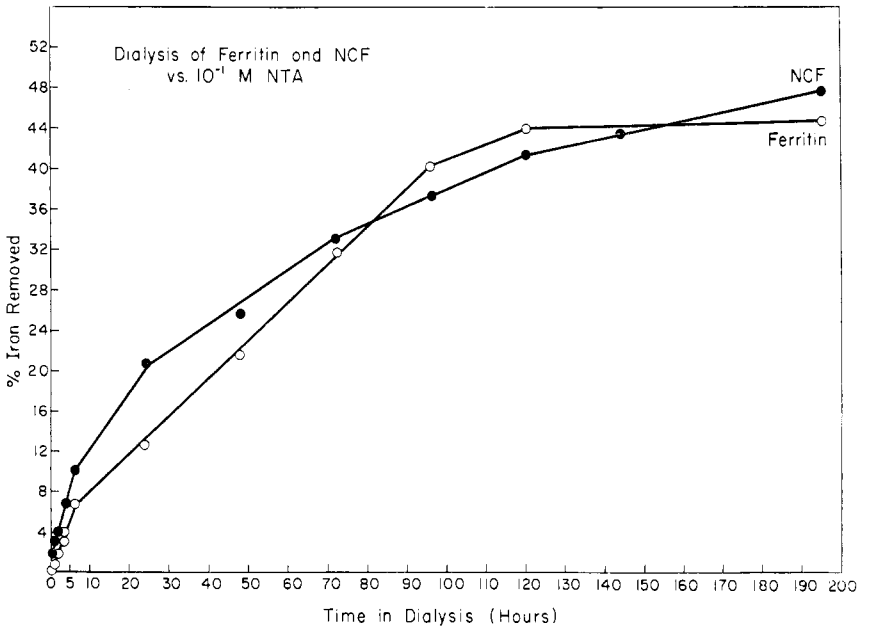

FIGURE 3: Comparison of rates of iron mobilization from crystalline and noncrystalline ferritin using 0.1 M nitrilotriacetate. Conditions as described in Figure 1.

It should be noted that the stability constant of NTA for iron is approximately 10^{-9} that of EDTA for the metal (Chaberek and Martell, 1959). Thus, the avidity of binding of chelator for iron does not uniquely determine the rate or amount of metal mobilized from the micelle. The efficiency of nitrilotriacetate compared with EDTA and citrate in removing ferritin iron indicates that there exists a specific relationship between chelator structure and its ability to solubilize polynuclear ferritin iron. The chemical nature of the iron–chelate also influences biological utilization of the metal. Helbock and Saltman (1967) studying the absorption and utilization of iron–chelates by intact rats demonstrated that both iron–nitrilotriacetate and iron–EDTA were rapidly absorbed across the intestine. Iron–EDTA was quickly excreted *via* the kidneys and not utilized. Iron presented in the nitrilotriacetate complex was, however, concentrated in the liver and

TABLE I: Removal of Iron by Dialysis from Crystalline and Noncrystalline Ferritin as a Function of Concentration and Chemical Nature of Chelate.

Conditions		% of Total Iron Removed			
Chelate (M)	Sample	2 hr	6 hr	24 hr	120 hr
NTA (10^{-1})	NCF	4.0	10.2	20.7	41.4
	Ferritin	1.3	6.5	12.4	43.9
EDTA (10^{-1})	NCF	1.5	2.7	4.3	14.0
	Ferritin	0.5	1.2	1.4	2.7
Sodium citrate (10^{-1})	NCF	0.0	1.1	2.0	2.8
	Ferritin	0.0	0.2	0.7	0.9
NTA (10^{-2})	NCF	3.7	8.3	11.5	19.0
	Ferritin	1.8	4.5	9.5	18.9
EDTA (10^{-2})	NCF	0.0	1.7	3.3	6.3
	Ferritin	0.0	0.6	1.0	2.1
Sodium citrate (10^{-2})	NCF	0.0	0.5	1.2	2.5
	Ferritin	0.0	0.3	0.4	0.9

spleen, the primary ferritin depots. It has also been demonstrated that iron exchange between chelate and transferrin (Bates *et al.*, 1967a,b) is critically dependent upon the specific chelates used to present or remove the metal. Recent studies concerning the depolymerization of polynuclear iron–citrate complexes by attacking chelators show a marked effect of both the structure of the agent as well as proton concentration (Spiro *et al.*, 1967). We have no knowledge at present concerning the nature of endogenous chelates which may be involved in the mobilization of ferritin iron although many organic and amino acids or carbohydrates may participate.

We were particularly interested in the enhanced rates of iron removal from the noncrystalline fraction. In his early investigations of ferritin, Granick (1942) designated several distinct fractions of the nonhematin iron of horse spleen. These included hemosiderin granules, crystalline ferritin which was soluble in ammonium sulfate, and noncrystalline ferritin which had solubility properties equivalent to ferritin but could not be crystallized with $CdSO_4$. The noncrystalline fraction, present in the supernatant following $CdSO_4$ precipitation of crystalline ferritin, appears to have approximately the same Fe:N:P ratios as the parent ferritin. It was demonstrated in the previous communication (Pape *et al.*, 1968) that the two forms of ferritin are in equilibrium *in vitro*. Further, electron microscopy revealed that noncrystalline ferritin had a disrupted protein outer shell which exposed a greater surface area of the iron micelle. The enhanced rate of iron mobilization from this fraction by attacking chelators is consistent with these observations.

We believe the results presented here extend the involvement of chelating agents in iron metabolism to those mechanisms operative in the mobilization of ferritin and other storage forms of iron. Of primary importance for future research will be the identification of those cellular metabolites which function in this process.

References

Bates, G. W., Billups, C., and Saltman, P. (1967a), *J. Biol. Chem. 242*, 2810.

Bates, G. W., Billups, C., and Saltman, P. (1967b), *J. Biol. Chem. 242*, 2816.

Bothwell, T. H., and Mallett, B. (1955), *Biochem. J. 59*, 599.

Brown, J. C., and Tiffen, L. D. (1965), *Plant Physiol. 40*, 395.

Chaberek, S., and Martell, A. E. (1959), Organic Sequestering Agents, New York, N. Y., Wiley, pp 564, 572.

Charley, P. J., Rosenstein, M., Shore, E., and Saltman, P. (1960), *Arch. Biochem. Biophys. 88*, 222.

Davis, P. S., and Deller, D. J. (1966), *Lancet 2*, 470.

Granick, S. (1942), *J. Biol. Chem. 146*, 451.

Helbock, H. J., and Saltman, P. (1967), *Biochim. Biophys. Acta 135*, 979.

Laufberger, V. (1937), *Bull. Soc. Chim. Biol. 19*, 1575.

Mazur, A., Baez, S., and Shorr, E. (1955), *J. Biol. Chem. 213*, 147.

Mazur, A., and Carleton, A. (1963), *J. Biol. Chem. 238*, 1817.

Mazur, A., and Green, S. (1959), Sulfur in Proteins, New York, N. Y., Academic, p 189.

Michaelis, L., Coryell, C. D., and Granick, S. (1943), *J. Biol. Chem. 148*, 463.

Neilands, J. B. (1957), *Bacteriol. Rev. 21*, 121.

Pape, L., Multani, J. S., Stitt, C. F., and Saltman, P. (1968), *Biochemistry 7*, 606 (this issue; preceding paper).

Saltman, P. (1956), *J. Chem. Ed. 42*, 682.

Spiro, T. G., Bates, G. W., and Saltman, P. (1967), *J. Am. Chem. Soc. 89*, 5559.

Wallace, A. (1963), *Agr. Food Chem. 11*, 103.