Biochem. J. (1978) **171**, 313–320
Printed in Great Britain

Incorporation and Release of Inorganic Phosphate in Horse Spleen Ferritin

By AMYRA TREFFRY and PAULINE M. HARRISON
Department of Biochemistry, University of Sheffield, Sheffield S10 2TN, U.K.

(*Received* 13 *September* 1977)

When ferritin is reconstituted from Fe and apoferritin *in vitro* in the presence of P_i, the product obtained differs both from native ferritin and from ferritin reconstituted in the absence of P_i. When the latter is incubated with P_i the product resembles native ferritin with respect both to the pattern of P_i incorporated per molecule or per Fe atom and to the ease of release of this P_i relative to Fe release. It is concluded that much of the P_i of native ferritin is adsorbed on surfaces of ferritin iron-core crystallites. The results also suggest that P_i is not present at the intracellular site of Fe incorporation into ferritin, but is added after Fe.

The Fe-storage protein ferritin, commonly found in abundance in liver and spleen, has a high capacity for Fe, which it stores in micellar form in the centre of a protein shell (Harrison, 1977). Up to 4500 Fe atoms may be stored, but this capacity is not normally utilized to the full. Ferritin preparations usually average no more than, and often less than, 3000 Fe atoms/molecule. The micellar nature of the iron complex has been established by electron microscopy (Farrant, 1954) and Mössbauer spectroscopy (Blaise *et al.*, 1965), and the small inorganic particles within the protein shell are actually microcrystals, as shown by electron and X-ray diffraction (Haggis, 1965; Harrison *et al.*, 1967) and high-resolution dark-field electron microscopy (Massover & Cowley, 1973). The size of the protein cavity (about 8nm across; Hoare *et al.*, 1975) sets an upper limit on the size of the iron-core microcrystals, but even in relatively full molecules this upper limit is not always reached, since molecules can contain more than one microcrystalline particle. Chemically ferritin iron cores have been shown to be a hydrated iron oxide–phosphate complex of approximate composition $(FeOOH)_8(FeO{-}OPO_3H_2)$ in the horse spleen protein (Michaelis *et al.*, 1943). The P_i/Fe ratio is rather higher than average in Fe-poor and rather lower than average in Fe-rich molecules (Fischbach *et al.*, 1969). This observation, together with the finding that some 77% of the P_i is lost from the iron micelles when they are separated from their protein shells by alkali treatment (Granick & Hahn, 1944), suggests that a considerable proportion of the P_i is bound loosely at surface sites and is not an intimate part of the atomic structure of the microcrystalline particles. This suggestion is supported by experiments by Van Kreel *et al.* (1972), who showed that the specific radioactivities of P_i in ferritin molecules of different Fe contents, isolated from livers perfused with media containing $[^{32}P]P_i$, were constant and equal to the specific radioactivity of the P_i remaining in supernatants of liver homogenates. These authors concluded that ferritin phosphate and liver P_i are in dynamic equilibrium. No $[^{32}P]P_i$ was bound to apoferritin in their experiments.

In previous studies (Macara *et al.*, 1972) it has been shown that a product closely resembling ferritin can be obtained by reconstitution from apoferritin and Fe^{2+} ions in the presence of an oxidant, but in the absence of added P_i. In the experiments outlined below we compare the properties of ferritin reconstituted with P_i and Fe together in the reaction mixture with a reconstituted ferritin in which P_i was added after Fe in a separate step. We also compare properties of these ferritins with those of native ferritin and of native ferritin that had been incubated with $[^{32}P]P_i$. One of the properties that we have used for comparison is the release of P_i as a function of micellar Fe release. If P_i is surface-bound we should expect it to be released largely ahead of the Fe, that is a 'last-in-first-out' principle would be observed, such as we have found previously when ^{59}Fe was added to preformed micelles (Hoy *et al.*, 1974*b*). Preliminary accounts of this work have been presented (Harrison *et al.*, 1977; Treffry *et al.*, 1977).

Materials and Methods

Chemicals used were AnalaR grade (BDH Chemicals, Poole, Dorset, U.K.). Horse spleen ferritin (twice-crystallized, Cd-free) was purchased from Miles Laboratories, Slough, Bucks., U.K. Ferritin fractions of different Fe content were obtained by sucrose-density-gradient centrifugation as described previously (Hoy & Harrison, 1975). Apoferritin was prepared from ferritin by dialysis against mixtures of equal volumes of 0.1M-thioglycollic acid and 0.1M-

sodium acetate (pH4.25), followed by exhaustive dialysis against glass-distilled water and precipitation with $(NH_4)_2SO_4$ (35%, w/v).

Ferritin Fe was measured either directly by its A_{420} ($A_{1cm}^{1\%Fe} = 100$) or, after reduction, as its 2,2'-bipyridine complex (Drysdale & Munro, 1965).

$Na_3{}^{32}PO_4$ obtained from The Radiochemical Centre, Amersham, Bucks., U.K., was added to Na_2HPO_4/NaH_2PO_4 solutions to give the required radioactivity. Samples were counted for radioactivity in a Triton/toluene scintillation 'cocktail' (Turner, 1968) with a Packard model 3385 liquid-scintillation spectrometer. Where applicable, samples were bleached with thioglycollic acid to prevent colour quenching. C.p.m. were corrected to d.p.m. by using a chemical quench curve. All ^{32}P counts were also corrected for decay during the counting of successive vials.

Apoferritin concentrations were measured either by A_{280} ($A_{1cm}^{1\%} = 9.0$) or by the method of Lowry *et al.* (1951), with apoferritin as standard. Spectrophotometric measurements were made in a Unicam SP.1800 double-beam spectrometer.

Phosphate analysis

A modification of published methods was necessary because of the presence of thioglycollic acid in the solutions to be analysed. The method adopted was based on those of Anner & Moosmayer (1975) and of Kallner (1975). Solution A was made of the following stock solutions: (a) 1% ammonium molybdate; (b) 24% (v/v) H_2SO_4; (c) 1g of poly(vinyl alcohol) in 100ml of boiling water to which 18.5mg of Malachite Green was added after filtration. Solutions a, b and c were mixed in the proportions 5:3:5 (by vol.) and the mixture was stored in the dark. Solution B was made just before use by mixing 4vol. of solution A with 1vol. of 5M-urea. Then 0.65ml of solution B and 0.2–1.35ml of a sample were mixed, made up to 2ml with water and the A_{630} was measured.

A standard curve was constructed with 1–14μmol of phosphate.

Ferritin reconstitution

Reconstitution in the absence of P_i was essentially like that used by Macara *et al.* (1972). Oxidant ($KIO_3/Na_2S_2O_3$) and Fe [as $(NH_4)_2Fe(SO_4)_2$] were added to apoferritin in 20mM-imidazole/HCl buffer, pH7.0, in the molar proportions $(NH_4)_2Fe(SO_4)_2/KIO_3/Na_2S_2O_3$, 1:1:4, the amount of Fe being calculated to give the desired degree of reconstitution.

When P_i was added to the reconstitution mixture the ratio total P_i/total Fe added did not exceed 0.2 and the Fe concentration was kept low (0.2 or 0.3mM) to avoid precipitation.

Reconstitution was carried out in ten steps (i.e. ten additions of Fe) to give a final concentration of 2mM (or 3mM)-ferritin Fe. Relatively large volumes were used so that concentrations could be kept low, typically about 177ml final volume. After dialysis against water the reconstituted ferritin was concentrated in a 50ml or 8ml ultrafiltration cell (Amicon, High Wycombe, Bucks, U.K.) with a Diaflo PM-10 membrane. The P_i added in these experiments was labelled with ^{32}P so that its distribution among different ferritin fractions and also its release could subsequently be measured by radioactivity counting.

In other experiments ferritin was reconstituted with Fe first and the product was then incubated overnight in 20mM-imidazole/HCl buffer, pH7.0, containing $H^{32}PO_4{}^{2-}$ ions. Native ferritin was treated similarly. Concentrations were adjusted to give $[HPO_4{}^{2-}]/[Fe] = 0.15$. Samples were extensively dialysed to remove unbound P_i in an ultrafiltration cell against 7–10vol. of water.

Release of labelled P_i

Ferritin solutions containing bound $H^{32}PO_4{}^{2-}$ were incubated overnight with unlabelled anion ($PO_4{}^{3-}$, $P_2O_7{}^{4-}$, $HCO_3{}^-$, $SO_4{}^{2-}$) in 20mM-imidazole/HCl, pH7.0. The sample was then placed in an 8MC ultrafiltration cell with a PM-10 membrane and a small amount of free solution passed through the membrane under pressure. The concentration of $[^{32}P]P_i$ in this solution was measured by radioactivity counting and this enabled the concentration of $[^{32}P]P_i$ bound to protein inside the cell to be calculated.

Release of P_i and Fe

A sample (5ml) of ferritin solution (5–6mM in Fe) was placed in an 8MC ultrafiltration cell and a solution made 20mM in thioglycollic acid and 20mM in sodium acetate, pH4.25, in the reservoir. The reservoir and the cell containing the ferritin solution were subjected to pressure [approx. 34.5kPa (5lbf/in^2)] and the reservoir solution was then allowed to pass into the cell, the volume inside the cell being kept constant. Thus the concentration of thioglycollate in the cell gradually increased, giving a gradual release of Fe from the ferritin iron cores. The flow rate was adjusted to about 4ml/h, and 1ml fractions were collected. Each fraction was assayed for released Fe^{2+} ions by the bipyridine method and for any P_i released along with Fe either by counting radioactivity or by the colorimetric procedure.

Rate of Fe release

This was measured in ferritin fractions obtained by centrifugation in a sucrose density gradient

(Hoy *et al.*, 1974*a*). The amount of Fe/ml was kept constant in all fractions and was released with 20mM-thioglycollic acid in 20mM-sodium acetate buffer, pH4.25, in the presence of 12.8mM-bipyridine. The rate of release was followed at 520nm as the appearance of the bipyridine–Fe complex.

Results

Incorporation of P_i into ferritin

When ferritin was reconstituted in the presence of P_i the latter was incorporated in the ratio P_i/Fe added, approx. 0.15. On incubation of reconstituted ferritin, P_i was taken up to the extent of P_i/Fe = 0.025. When native ferritin (which already contained P_i/Fe = 0.126) was incubated, only a small amount of $[^{32}P]P_i$ was incorporated (ratio = 0.008).

Release of bound P_i by unlabelled anions

Table 1 shows the results of incubating bound labelled P_i with unlabelled P_i or other anions. Some 67.8% of the $[^{32}P]P_i$ bound by native ferritin was released from the protein by unlabelled P_i and 61.1% by pyrophosphate. P_i bound to reconsituted ferritin (on incubation) was less readily released, but again both orthophosphate and pyrophosphate were nearly equally effective. Very little P_i was removed from that ferritin that had been reconstituted in the presence of P_i, and again there was little difference in the effectiveness of orthophosphate and pyrophosphate.

P_i incorporation as a function of Fe

The P_i contents of fractions of native and reconstituted ferritins of different Fe contents separated by centrifugation through sucrose gradients are shown in Figs. 1(*a*)–1(*d*). Results are expressed as P_i incorporated per molecule in Figs. 1(*a*) and 1(*b*) and per Fe atom in Figs. 1(*c*) and 1(*d*). The distributions of P_i/molecule and P_i/Fe atom are indistinguishable for native ferritin and ferritin reconstituted with Fe and then incubated with $[^{32}P]P_i$. Fe-poor molecules (including any apoferritin present) bind little or no P_i. The amount of P_i/molecule increases continuously with Fe content although less steeply for the Fe-rich fractions, whereas the ratio P_i/Fe atom is greatest for Fe-poor fractions and decreases with Fe content. The distribution of $[^{32}P]P_i$ radioactivity among fractions was quite different for ferritin reconstituted in the presence of P_i. P_i incorporation per molecule showed a peak in the region 2250–2500 Fe atoms/molecule (Fig. 2*b*), and a shoulder was observed in the same region of Fig. 1(*d*).

Table 1. *Release of $[^{32}P]P_i$ from ferritin by external anions*

Experiments were done in 20mM-imidazole buffer, pH7.0. Solutions were 3mM in ferritin Fe and external anions. Free $[^{32}P]P_i$ was separated by ultrafiltration.

Ferritin ...	Phosphate released (%)		
Anion	Reconstituted in the presence of $[^{32}P]P_i$	Native incubated with $[^{32}P]P_i$	Incubated with $[^{32}P]P_i$ after reconstitution
Control	1.8	29.5	7.3
SO_4^{2-}	1.9	36.2	11.2
HCO_3^-	2.2	35.5	10.37
$P_2O_7^{4-}$	15.3	61.1	28.4
HPO_4^{2-}	14.4	67.8	34.2

Effect of P_i on Fe distribution in reconstituted ferritin

It has been observed previously (Macara *et al.*, 1972) that the reconstitution procedure that we have used in the absence of P_i tends to give a distribution of Fe atoms/molecule approaching 'all-or-none', i.e. much of the protein has reached a high degree of Fe saturation, whereas most of the remainder contains little or no Fe (see Fig. 2*b*). The presence of P_i in the reconstitution mixture had a marked effect on the Fe distribution, as shown in Fig. 2(*a*). A very sharp peak was obtained at a saturation of about 1500 Fe atoms/molecule. In the reconstitution for which results are shown, the molar ratio of P_i to Fe added was 0.15, but the P_i was all added before reconstitution with Fe, whereas the Fe was added in ten equal amounts. This may account for the presence of a small peak with relatively high Fe content, which could have been produced after free P_i had been depleted. It was also found that the amount of Fe that could be incorporated into ferritin in the presence of this concentration of P_i was relatively low. After an average of 2000 Fe atoms/molecule had been added to the system, precipitation occurred, apparently of Fe that could not be incorporated into ferritin.

Absorption coefficient at 420nm

Native ferritin has an average absorption coefficient, $A_{1cm}^{1\%Fe}$, at 420nm of approx. 100 (Hoy *et al.*, 1974*a*). We now find that this value also gives a good approximation for reconstituted ferritin in the middle range of Fe contents. A comparison of numbers of Fe atoms/molecule measured by using this coefficient with that measured by the probably more accurate bipyridine method is shown in Fig. 3(*a*) for reconstituted ferritin fractions of different Fe contents. At the lower range of Fe contents considerable divergence in estimated Fe atoms/mole-

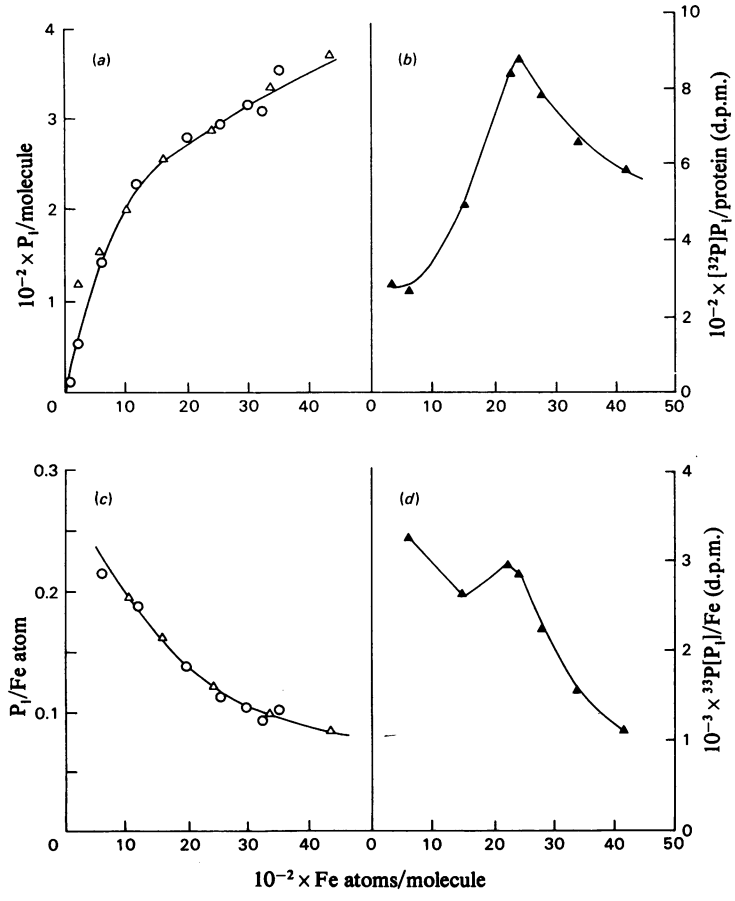

Fig. 1. *Distribution of P_i/molecule (a and b) and P_i/Fe atom (c and d) in ferritin molecules of different Fe contents*
Reconstitution and incubation with P_i were carried out as described in the Materials and Methods section. Fractionation was carried out by sucrose-density-gradient centrifugation. Fractions (1 ml) from the gradient tubes were analysed for protein and Fe by colorimetric procedures and for P_i either colorimetrically or by scintillation counting of ^{32}P. (*a*) and (*c*): ○, native ferritin; △, reconstituted ferritin incubated with $[^{32}P]P_i$, pH 7.0. Values of d.p.m. for reconstituted ferritin were scaled to fit the curve for native ferritin. (*b*) and (*d*): ferritin reconstituted in the presence of $[^{32}P]P_i$. Arbitrary scale.

cule was obtained by the two methods, probably as a result of experimental error.

With ferritin that has been reconstituted in the presence of P_i there is a much larger discrepancy between the two estimates. Fig. 3(*b*) shows that the assumption of an absorption coefficient of 100 now gives estimated Fe contents much lower than with the bipyridine method, i.e. the value 100 is too high for this product. This may be because P_i interferes with the formation of the polynuclear Fe(III)–O–Fe(III) bridged structure, which gives ferritin its red–brown colour.

Rates of Fe release with thioglycollate

Fig. 4 shows the rates of release of Fe from ferritin fractions of different Fe contents obtained by sucrose-density-gradient centrifugation of ferritins reconstituted with and without P_i. The concentration of Fe was constant at 2.5 μg/ml for each fraction. The number of protein molecules/ml therefore decreased as the number of Fe atoms/molecule increased. Fe was released by using thioglycollate in the presence of bipyridine as described in the Materials and Methods section. No release of Fe was observed in the absence of thioglycollate. Thioglycollate gave most rapid release from the Fe-poor fractions of ferritin reconstituted without P_i, a result similar to those obtained previously with native ferritin fractions, when Fe was removed with 1,10-phenanthroline (Hoy *et al.*, 1974*a*) or cysteine (Crichton *et al.*, 1975). Except for Fe-poor fractions, release from ferritin reconstituted in the presence of P_i was much greater at similar Fe

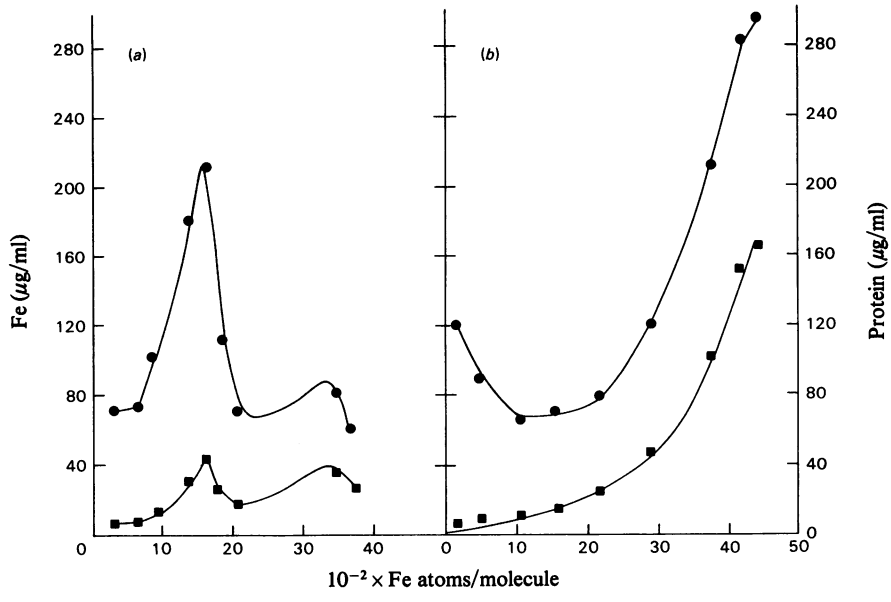

Fig. 2. *Distribution of Fe and protein in reconstituted ferritins*

(*a*) Reconstitution in the presence of P_i. P_i was added first in a single step, then Fe in ten steps. Final molar ratio of P_i/Fe = 0.15. (*b*) Reconstitution in the absence of P_i. Reconstitutions were carried out in 20mM-imidazole buffer, pH7.0, with $KIO_3/Na_2S_2O_3$ as oxidant. $(NH_4)_2Fe(SO_4)_2$ was added to give 4000 Fe atoms/protein molecule. After reconstitution solutions were dialysed, concentrated and fractionated on sucrose density gradients. Each fraction was assayed for protein (●) and Fe (■) colorimetrically.

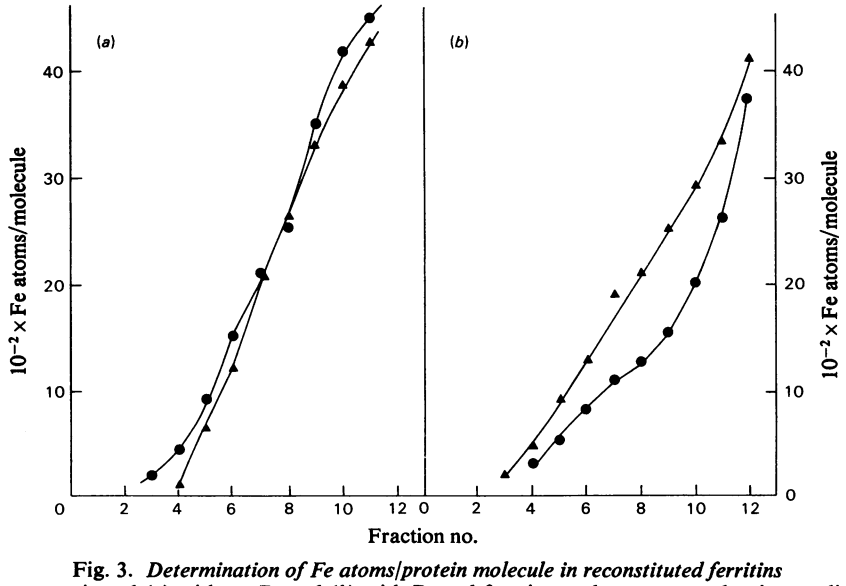

Fig. 3. *Determination of Fe atoms/protein molecule in reconstituted ferritins*

Ferritins were reconstituted (*a*) without P_i and (*b*) with P_i and fractionated on sucrose density gradients. Fractions were assayed for protein by the method of Lowry *et al.* (1951) and for Fe either (●) directly by measuring A_{420} ($A^{1\%Fe}_{1cm}$ = 100) or (▲) as the Fe–bipyridine complex estimated against a standard curve at 520nm.

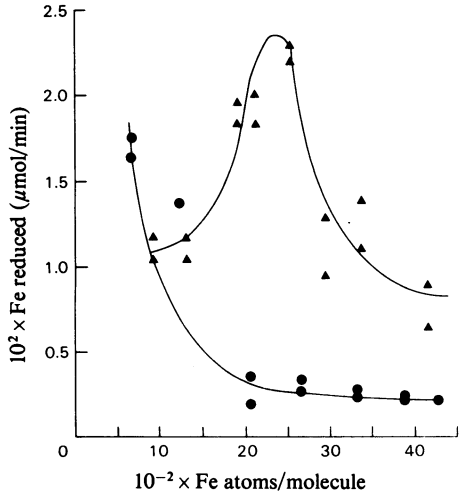

Fig. 4. *Rates of Fe release from reconstituted ferritin fractions at constant Fe concentration* (2.5 μg/ml)
Ferritins were reconstituted (●) without P_i or (▲) with P_i and fractionated on sucrose density gradients. Fe was released with 20mM-thioglycollic acid/20mM-sodium acetate, pH4.3, and release followed by the appearance of the Fe–bipyridine complex.

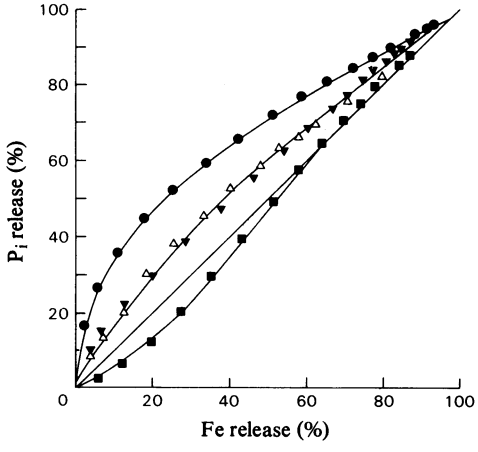

Fig. 5. *Release of Fe and P_i from native and reconstituted ferritins*
Release was carried out in an ultrafiltration cell with 20mM-thioglycollic acid/20mM-sodium acetate, pH 4.3. The volume of the ferritin solutions was kept constant at 5ml. The eluate was collected in 1ml fractions and assayed for iron with bipyridine and for phosphate either as ^{32}P or colorimetrically. ●, Native ferritin incubated with $[^{32}P]P_i$; △, reconstituted ferritin incubated with $[^{32}P]P_i$; ▼, native ferritin; ■, ferritin reconstituted in the presence of $[^{32}P]P_i$.

content, and the distribution pattern showed a marked peak near 2250–2500 Fe atoms/molecule. Thus release of Fe atoms was fastest for molecules containing a relatively high P_i content (see Fig. 1).

Release of P_i as a function of Fe release

Percentage release of P_i is plotted against percentage Fe release in Fig. 5 for (1) native ferritin incubated with $[^{32}P]P_i$, (2) reconstituted ferritin incubated with $[^{32}P]P_i$, (3) native ferritin or (4) ferritin reconstituted in the presence of $[^{32}P]P_i$. Fig. 5 shows that, except in case (4), P_i is released more readily than Fe, whereas in case (4) P_i release initially lags behind that of Fe. For simultaneous release of all P_i and Fe a line of 45° slope would be expected. The release patterns of native ferritin and reconstituted ferritin that had been incubated with $[^{32}P]P_i$ are virtually identical. A comparison of results for cases (1) and (3) indicates that the added P_i was bound less intimately with the Fe than the endogenous P_i of native ferritin. The opposite seems to apply for case (4). Here the relative lag in P_i release may result from the fact that all the P_i was present at the beginning of the experiment, whereas Fe was added in small successive increments. The system may have been partially depleted of P_i before the last additions of Fe were made.

Discussion

The results described above show that ferritin reconstituted in the absence of P_i will bind this anion to give a product closely resembling native ferritin with respect to the distribution of P_i incorporated per molecule or per Fe atom as a function of Fe atoms present in the molecule (Fig. 1), although the absolute amount incorporated was considerably less than that present in native ferritin. The release of this bound P_i also resembled that from native ferritin (Fig. 5). Its release somewhat ahead of Fe when the latter was reduced with thioglycollate suggested that a proportion of it was located on readily accessible surface sites on the iron-core crystallites, although the remainder must be supposed to have been more tightly bound, perhaps by incorporation into stacking faults or discontinuities within the iron-core material (represented diagrammatically in Fig. 6). On incubation with $[^{32}P]P_i$, native ferritin bound a small amount of this anion, which was more readily released than that present in native ferritin or in the reconstituted/incubated ferritin (Fig. 5) and more readily displaced by unlabelled anions than the P_i present in the reconstituted/incubated ferritin. From its 'last-in-first-out' behaviour we may conclude that the additional P_i was taken up into the few unoccupied surface sites, and this conclusion may be supported by the finding that this P_i is quite readily displaced by

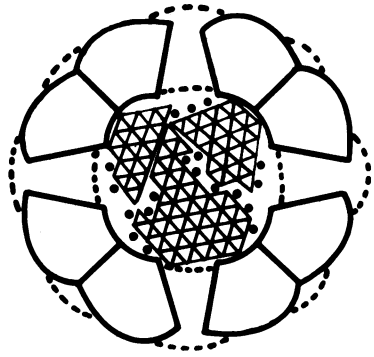

Fig. 6. *Diagrammatic representation of ferritin molecule* The ferritin molecule consists of a multi-subunit protein shell surrounding a microcrystalline iron core of hydrous ferric oxide–phosphate. The molecule is depicted as containing three core crystallites (drawn schematically as a triangular lattice) with P_i ions represented as (solid) circles. Surface P_i ions should be readily displaced, whereas those that are trapped in the centre of the core are not readily released. When Fe is released by reduction to Fe(II) with thioglycollate, surface P_i would be expected to be released first, ahead of most of the Fe in the crystallites and of the trapped P_i. Native ferritin, which contains P_i, may take up some [^{32}P]P_i at vacant or readily exchangeable surface sites (cf. results in Fig. 5).

incubation with pyrophosphate and other anions (Table 1). A smaller percentage of the P_i added to reconstituted ferritin is released, the difference perhaps being due to the incorporation of some of this P_i into the less-accessible stacking faults suggested above.

The product obtained when P_i was present in the reconstitution mixture is very different in several respects from that obtained when Fe and P_i were added separately and consecutively.

The results shown in Fig. 2 indicate that P_i gives rise to a markedly smaller average crystallite size, a large fraction of molecules containing only one-third to one-half of the Fe atoms/molecule present after P_i-free reconstitution. This conclusion is supported by analysis of Mössbauer spectra obtained from our reconstituted samples by J. M. Williams, D. P. Danson & C. Janot (personal communication). Mössbauer spectra were studied over a temperature range 4–300K, and hyperfine field and quadrupole interactions were correlated with particle size distributions assuming that most of the ferritin molecules in the ferritin reconstituted in the absence of P_i contain iron core of 6.5nm (65Å) diameter. It is clear from this analysis that the presence of phosphate during reconstitution results in a slight narrowing of

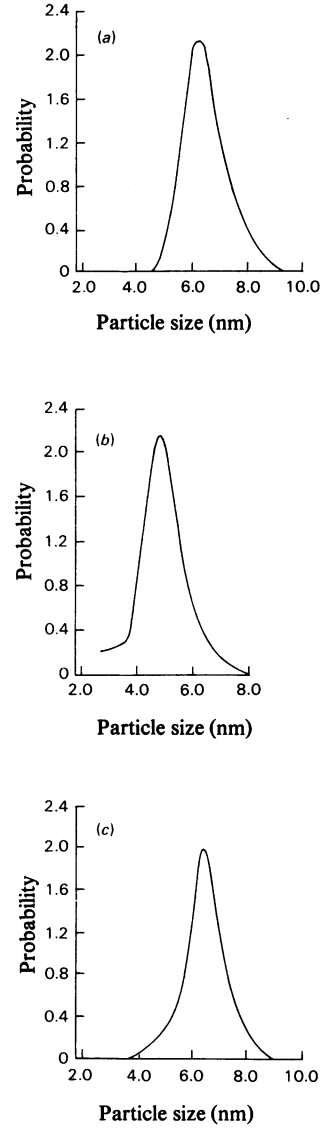

Fig. 7. *Particle-size distributions of iron cores in reconstituted ferritins*
Estimated particle-size distributions are shown in ferritins reconstituted (*a*) without and (*b*) with P_i. A total of 2000 Fe atoms/molecule was added to the reconstitution mixture as $(NH_4)_2Fe(SO_4)_2$ enriched with ^{57}Fe. After reconstitution samples were dialysed, concentrated and freeze-dried. (*c*) Native ferritin fraction containing 1730–2480 Fe atoms per molecule. Particle-size distributions were estimated by analysis of Mössbauer spectra by J. M. Williams, D. P. Danson & C. Janot (personal communication), whom we thank for permission to reproduce this diagram. The ordinate gives the probability of finding particles within 0.4nm of the specified size. Values were assigned to the probability such that the sum of all probabilities was equal to 10.

the particle-size distribution and a shift to smaller average size from about 6.5nm without P_i to about 4.8nm in its presence (Fig. 7). Thus P_i interferes with the regular crystal growth of ferritin iron-core material. Sample C is a native ferritin fraction containing 1730–2480 Fe atoms per molecule. Except for a small contribution from particles smaller than 4.0nm it appears to be similar to the reconstituted ferritin.

In a previous study (Hoy *et al.*, 1974*a*) the relatively fast release of Fe from Fe-poor ferritin as compared with Fe-rich ferritin fractions at constant Fe concentration was explained by the relatively large surface/volume of the small crystallites giving a greater area for Fe release. For the same Fe content, P_i-reconstituted ferritins show a faster release than does the P_i-free product (Fig. 4). This suggests that, even when the same amount of Fe is incorporated per molecule, the presence of P_i leads to a smaller particle size. The peak in the rate of Fe release from ferritin reconstituted in the presence of P_i is at 2250–2500 Fe atoms/molecule. This corresponds to the peak in the amount of P_i incorporated/molecule and the shoulder in the amount of P_i incorporated/Fe atom shown in Fig. 1. It suggests that the more P_i present the smaller are the iron-core particles, and may explain why most molecules only reach a low degree of saturation with Fe in this reconstitution (Fig. 2). It may be that the greater amount of P_i incorporated, giving a more disorganized form of iron core, prevents or inhibits further Fe being taken up. The small number of ferritin molecules containing over 3000 Fe atoms (Fig. 2) contain relatively little P_i (Fig. 1). It may be that these molecules have rapidly added Fe to the exclusion of P_i, although they may have accumulated their Fe after P_i was depleted from the system. The release of P_i as a function of Fe release (Fig. 5) also indicates that when P_i is present during reconstitution it becomes closely integrated with the Fe, and this is also suggested by the difficulty with which it is exchanged with exogenous P_i (Table 1).

The concentration of P_i used in the reconstitution (less than 1mM) was within or below the physiological range of cell P_i. Although the concentration of free cytoplasmic Fe may be below that used in our reconstitution procedure and the physiological donor of Fe to ferritin is not known, we may tentatively conclude from our results that Fe and P_i may be added separately to ferritin within cells. Such 'compartmentalization' could perhaps be achieved if ferritin received its Fe at a membrane site and the ferritin iron oxide complex was subsequently equilibrated with cytoplasmic P_i, as in our 'reconstitution/incubation' experiments.

We thank the Medical Research Council for support, and Mr. A. Doyle for expert technical assistance.

References

Anner, B. & Moosmayer, M. (1975) *Anal. Biochem.* **65**, 305–309

Blaise, A., Chappert, J. & Girardet, J. L. (1965) *C. R. Hebd. Séances Acad. Sci. Ser. D* **261**, 2310–2313

Crichton, R. R., Wauters, M. & Roman, F. (1975) in *Proteins of Iron Storage and Transport in Biochemistry and Medicine* (Crichton, R. R., ed.) pp. 287–294, North-Holland, Amsterdam

Drysdale, J. W. & Munro, H. N. (1965) *Biochem. J.* **95**, 851–857

Farrant, J. L. (1954) *Biochim. Biophys. Acta* **13**, 569–576

Fischbach, F. A., Harrison, P. M. & Hoy, T. G. (1969) *J. Mol. Biol.* **39**, 235–238

Granick, S. & Hahn, P. (1944) *J. Biol. Chem.* **155**, 661–669

Haggis, G. H. (1965) *J. Mol. Biol.* **14**, 598–602

Harrison, P. M. (1977) *Semin. Haematol.* **14**, 55–70

Harrison, P. M., Fischbach, F. A., Hoy, T. G. & Haggis, G. H. (1967) *Nature (London)* **216**, 1188–1190

Harrison, P. M., Banyard, S. H., Russell, S. M. & Treffry, A. (1977) *Iron Metabolism: Ciba Found. Symp.* **51**, 19–40

Hoare, R. J., Harrison, P. M. & Hoy, T. G. (1975) *Nature (London)* **255**, 653–654

Hoy, T. G. & Harrison, P. M. (1975) in *Proteins of Iron Storage and Transport in Biochemistry and Medicine* (Crichton, R. R., ed.), pp. 279–286, North-Holland, Amsterdam

Hoy, T. G., Harrison, P. M., Shabbir, M. & Macara, I. G. (1974*a*) *Biochem. J.* **137**, 67–70

Hoy, T. G., Harrison, P. M. & Shabbir, M. (1974*b*) *Biochem. J.* **139**, 603–607

Kallner, A. (1975) *Clin. Chim. Acta* **59**, 35–39

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Macara, I. G., Hoy, T. G. & Harrison, P. M. (1972) *Biochem. J.* **126**, 151–162

Massover, W. H. & Cowley, J. M. (1973) *Proc. Natl. Acad Sci. U.S.A.* **70**, 3847–3851

Michaelis, L., Coryell, C. D. & Granick, S. (1943) *J. Biol. Chem.* **148**, 463–480

Treffry, A., Banyard, S. H., Hoare, R. J. & Harrison, P. M. (1977) in *Proteins of Iron Metabolism* (Brown, E. B., ed.), pp. 3–11, Grune and Stratton, New York

Turner, J. C. (1968) *Int. J. Appl. Radiat. Isot.* **19**, 557–563

Van Kreel, B. K., Pijnenberg, A. M. C. M., Van Eijk, H. G. & Leijnse, B. (1972) *Biochim. Biophys. Acta* **273**, 243–247