Incorporation and Release of Inorganic Phosphate in Horse Spleen Ferritin

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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 M"ossbauer spectroscopy (Blaise et al., 1965), and the small inorganic particles within the protein shell are actually microcristals, 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 microcristals, but even in relatively full molecules this upper limit is not always reached, since molecules can contain more than one microcristalline particle. Chemically ferritin iron cores have been shown to be a hydrated iron oxide-phosphate complex of approximate composition (FeOOH)_x(FeO-OP_2H_5) in the horse spleen protein (Michaelis et al., 1943). The P_i/Fe ratio is rather higher than average in Fe-poors 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 microcristalline particles. This suggestion is supported by experiments by Van Kreele 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 [32P]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 [32P]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 [32P]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 ^59Fe was added to preformed micelles (Hoy et al., 1974b). Preliminary accounts of this work have been presented (Harrison et al., 1977; Treffry et al., 1977).

Materials and Methods

Chemicals used were AnalR 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 (pH 4.25), followed by exhaustive dialysis against glass-distilled water and precipitation with (NH₄)₂SO₄ (35%, w/v).

Ferritin Fe was measured either directly by its A₄₂₀(Fe) (A₄₂₀(Fe) = 100) or, after reduction, as its 2,2'-bipyridine complex (Drysdale & Munro, 1965).

Na₃²³PO₄ obtained from The Radiochemical Centre, Amersham, Bucks., U.K., was added to Na₂HPO₄/NaH₂PO₄ 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 ³²P counts were also corrected for decay during the counting of successive vials.

Apoferitin concentrations were measured either by A₂₈₀ (A₂₈₀ = 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) H₂SO₄; (c) 1 g of poly(vinyl alcohol) in 100 ml of boiling water to which 18.5 mg 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 4 vol. of solution A with 1 vol. of 5M-urea. Then 0.65 ml of solution B and 0.2–1.35 ml of a sample were mixed, made up to 2 ml with water and the A₆₃₀ was measured.

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

**Ferritin reconstitution**

Reconstitution in the absence of P₁ was essentially like that used by Macara et al. (1972). Oxidant (KIO₃/Na₂S₂O₃) and Fe [as (NH₄)₂Fe(SO₄)₂] were added to apoferitin in 20 mm-imidazole/HCl buffer, pH 7.0, in the molar proportions (NH₄)₂Fe(SO₄)₂/KIO₃/Na₂S₂O₃: 1:1:4. The amount of Fe being calculated to give the desired degree of reconstitution.

When P₁ was added to the reconstitution mixture the ratio total P₁/total Fe added did not exceed 0.2 and the Fe concentration was kept low (0.2 or 0.3 mm) to avoid precipitation.

Reconstitution was carried out in ten steps (i.e. ten additions of Fe) to give a final concentration of 2 mm (or 3 mm)-ferritin Fe. Relatively large volumes were used so that concentrations could be kept low, typically about 177 ml final volume. After dialysis against water the reconstituted ferritin was concentrated in a 50 ml or 8 ml ultrafiltration cell (Amicon, High Wycombe, Bucks, U.K.) with a Diaflo PM-10 membrane. The P₁ added in these experiments was labelled with ³²P 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 20 mm-imidazole/HCl buffer, pH 7.0, containing H³²PO₄⁻ ions. Native ferritin was treated similarly. Concentrations were adjusted to give [HPO₄²⁻]/[Fe] = 0.15. Samples were extensively dialysed to remove unbound P₁ in an ultrafiltration cell against 7–10 vol. of water.

**Release of labelled P₁**

Ferritin solutions containing bound H³²PO₄⁻ were incubated overnight with unlabelled anion (PO₄³⁻, P₂O₇⁴⁻, HCO₃⁻, SO₄²⁻) in 20 mm-imidazole/HCl, pH 7.0. The sample was then placed in an 8 MC ultrafiltration cell with a PM-10 membrane and a small amount of free solution passed through the membrane under pressure. The concentration of [³²P]P₁ in this solution was measured by radioactivity counting and this enabled the concentration of [³²P]P₁ bound to protein inside the cell to be calculated.

**Release of P₁ and Fe**

A sample (5 ml) of ferritin solution (5–6 mm in Fe) was placed in an 8 MC ultrafiltration cell and a solution made 20 mm in thioglycollic acid and 20 mm in sodium acetate, pH 4.25, in the reservoir. The reservoir and the cell containing the ferritin solution were subjected to pressure [approx. 34.5 kPa (5 lbf/in²)] 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 4 ml/h, and 1 ml fractions were collected. Each fraction was assayed for released Fe²⁺ ions by the bipyridine method and for any P₁ 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 1978
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(Hoy et al., 1974a). The amount of Fe/ml was kept constant in all fractions and was released with 20 mm-thioglycollic acid in 20 mm-sodium acetate buffer, pH 4.25, in the presence of 12.8 mm-bipyridine. The rate of release was followed at 520 nm as the appearance of the bipyridine-Fe complex.

Results

Incorporation of P\textsubscript{i} into ferritin

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

Release of bound P\textsubscript{i} by unlabelled anions

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

P\textsubscript{i} incorporation as a function of Fe

The P\textsubscript{i} contents of fractions of native and reconstituted ferritins of different Fe contents separated by centrifugation through sucrose gradients are shown

<table>
<thead>
<tr>
<th>Anion</th>
<th>Reconstituted in the presence of [\textsuperscript{32}P]P\textsubscript{i}</th>
<th>Native incubated with [\textsuperscript{32}P]P\textsubscript{i}</th>
<th>Incubated with [\textsuperscript{32}P]P\textsubscript{i} after reconstitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.8</td>
<td>29.5</td>
<td>7.3</td>
</tr>
<tr>
<td>SO\textsubscript{4}\textsuperscript{2-}</td>
<td>1.9</td>
<td>36.2</td>
<td>11.2</td>
</tr>
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<td>HCO\textsubscript{3}\textsuperscript{-}</td>
<td>2.2</td>
<td>35.5</td>
<td>10.37</td>
</tr>
<tr>
<td>P\textsubscript{2}O\textsubscript{4}\textsuperscript{2-}</td>
<td>15.3</td>
<td>61.1</td>
<td>28.4</td>
</tr>
<tr>
<td>HPO\textsubscript{4}\textsuperscript{2-}</td>
<td>14.4</td>
<td>67.8</td>
<td>34.2</td>
</tr>
</tbody>
</table>

Effect of P\textsubscript{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\textsubscript{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. 2b). The presence of P\textsubscript{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\textsubscript{i} to Fe added was 0.15, but the P\textsubscript{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\textsubscript{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\textsubscript{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 420 nm

Native ferritin has an average absorption coefficient, \(A_{420}^{1%}\text{cm}\), at 420 nm of approx. 100 (Hoy et al., 1974a). 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): $O$, native ferritin; $\Delta$, 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 $\mu$g/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., 1974a) 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 P1. P1 was added first in a single step, then Fe in ten steps. Final molar ratio of P1/Fe = 0.15. (b) Reconstitution in the absence of P1. Reconstitutions were carried out in 20mM-imidazole buffer, pH 7.0, with KIO3/Na2S2O3 as oxidant. (NH4)2Fe(SO4)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 P1 and (b) with P1 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 A420 (A1%Fe = 100) or (▲) as the Fe–bipyridine complex estimated against a standard curve at 520nm.
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 Pı content (see Fig. 1).

Release of Pı as a function of Fe release

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

Discussion

The results described above show that ferritin reconstituted in the absence of Pı will bind this anion to give a product closely resembling native ferritin with respect to the distribution of Pı 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 Pı 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 [32P]Pı, 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 Pı present in the reconstituted/incubated ferritin. From its 'last-in-first-out' behaviour we may conclude that the additional Pı was taken up into the few unoccupied surface sites, and this conclusion may be supported by the finding that this Pı is quite readily displaced by
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 4–300 K, 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 6.5 nm (65 Å) diameter. It is clear from this analysis that the presence of phosphate during reconstitution results in a slight narrowing of

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**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 thioglycolate, 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).

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**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\(_4\))\(_2\)Fe(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 0.4 nm 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 6.5 nm without P1 to about 4.8 nm in its presence (Fig. 7). Thus P1 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 4.0 nm it appears to be similar to the reconstituted ferritin.

In a previous study (Hoy et al., 1974a) 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, P1-reconstituted ferritins show a faster release than does the P1-free product (Fig. 4). This suggests that, even when the same amount of Fe is incorporated per molecule, the presence of P1 leads to a smaller particle size. The peak in the rate of Fe release from ferritin reconstituted in the presence of P1 is at 2250–2500 Fe atoms/molecule. This corresponds to the peak in the amount of P1 incorporated/molecule and the shoulder in the amount of P1 incorporated/Fe atom shown in Fig. 1. It suggests that the more P1 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 P1 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 P1 (Fig. 1). It may be that these molecules have rapidly added Fe to the exclusion of P1, although they may have accumulated their Fe after P1 was depleted from the system. The release of P1 as a function of Fe release (Fig. 5) also indicates that when P1 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 P1 (Table 1).

The concentration of P1 used in the reconstitution (less than 1 mm) was within or below the physiological range of cell P1. 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 P1 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 P1, as in our 'reconstitution/incubation' experiments.

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References


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