Biosynthesis of Ferritin by Rat Liver Slices*

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(Received for publication, November 13, 1964)

Iron greatly accelerates the biosynthesis of the protein moiety of the iron storage protein ferritin (1, 2). This phenomenon is therefore of general interest as an example of the adaptive control of specific protein synthesis. As a possible tool for studying the process in vitro, rat liver slices were tested for their ability to incorporate labeled amino acids into ferritin. In this report we describe some of the properties of rat ferritin, a method for its isolation from liver slices, evidence for its biosynthesis in incubated slices, and the effects of pretreatment of the rats with iron and with actinomycin.

**EXPERIMENTAL PROCEDURE**

**Materials**

**Ferritin**—This compound was isolated from the livers of iron-treated rats as carrier to aid in the isolation of labeled ferritin from rat liver slices. Donor animals received 75 mg of iron per kg in the form of iron dextran (Imferon, Lakeside Laboratories, Milwaukee) intraperitoneally 3 days before they were killed. The method of isolation of carrier ferritin was similar to that described below for liver slices under “Methods” although on a 1000-fold larger scale (200 mg of ferritin from 400 g of liver). Furthermore, in the large scale isolation the extract before application to the ion exchange column was fractionated with 2 M ammonium sulfate. The precipitate was desalted by dialysis into 3 M buffer that substituted for the gel filtration step in the small scale isolation. The immunochromatographic step was not involved in the large scale preparation. The carrier ferritin was stored as an ammonium sulfate precipitate at 4° and dialyzed against 0.9% NaCl before use.

Crystallization of rat ferritin in high yield by cadmium ions had been found in this laboratory1 to be feasible only if chloride ions was also present. A chloride requirement does not appear to have been mentioned in earlier reports of crystallization of rat ferritin (3, 4). Under suitable conditions rat ferritin yielded crystals of the same appearance as those that are well known for horse spleen ferritin (5). Cadmium sulfate alone, however, at low concentrations yielded an amorphous precipitate and at high concentrations only a few round, flat crystals of a relatively high solubility (Fig. 1). Preliminary trials indicated that a mixture of 60 mM CdSO4 and 80 mM NaCl (in 20 mM 2,4,6-collidine-HCl, pH 7.4) produced abundant, sharp crystals with little amorphous precipitate, the sharpness and yield being rather sensitive to variation in the concentration of Cd and Cl ions. Yet crystallization proved not to be a convenient or reliable means for the quantitative isolation of rat ferritin, especially on a small scale. It was useful, however, as an additional purification step in the preparation of antigen for the induction of antibody production in rabbits.

Precipitation with antibody was adopted for the isolation of labeled ferritin from liver slices, but an adsorption and elution step prior to the precipitin reaction was deemed important because of the possibility of nonspecific adsorption of labeled proteins from the crude tissue extracts. Conditions for adsorption on carboxymethylcellulose proved to be very exacting and a large fraction of the ferritin was always lost. The difficulty appears to be that the isoelectric point of rat ferritin is close to that of the ion exchange agent. Estimates of the isoelectric point of rat ferritin were obtained by minimum solubility (Fig. 2) and electrophoretic mobility (Fig. 3) and indicate a value of about pH 4.1.

**Diethylaminoethyl Cellulose**—The adsorption of rat ferritin onto DEAE-cellulose (Selectacel No. 70, Lot 1447, Schleicher and Schuell) at pH 5.6 was firm at ionic strength below 5 mM in sodium acetate buffer. A large fraction of the total was eluted at ionic strength 10 mM (pH 5.6) and further discrete, smaller fractions were eluted at progressively higher ionic strengths with buffers of the same pH. This behavior bears some similarity to that observed independently by Mnur, Green, and Carleton (4), although the phenomenon of fractional elution was not mentioned. The eluent (50 mM) chosen for routine use in the present experiments was sufficient to elute the main fractions together. This buffer left a relatively small residue of ferritin at the top of the column, most of which was eluted in fractions with more concentrated buffers, but at least part of which might represent denaturation artifacts.

**Antiserum**—For the present tracer experiments, a cross-reacting, anti-horse ferritin rabbit antiserum was employed instead of homologous antiserum. This choice was made because of a greater confidence in the purity of horse spleen ferritin than the rat liver ferritin then available for use in the induction of antibody. There was therefore less danger of the concomitant induction of antibodies against nonferritin liver proteins. Horse spleen ferritin isolated by the usual method (5) has met many criteria of purity and the sample used for immunization was repeatedly re-crystallized from 0.25 M CdSO4.

Rabbits were treated by subcutaneous injection with 10 mg of ferritin in Freund's adjuvant (8) and the resulting antiserum was preserved with phenol (0.4%). Sufficient cross-reacting antibody was present in 1 ml to precipitate about 1.2 mg of rat ferritin.

**Tracers**—The radiolabeled samples employed were dL-leucine-1-14C, 2.7 mc per mmole from the Isotopes Specialties Company.

*This work was supported by Grant GB-2080 of the National Science Foundation, awarded to Professor H. Tarver and one of the authors (R.A.F.), and by a grant to one of us (R.A.F.) from the Research Committee of the University of California School of Medicine.

1 R.A. Fineberg, unpublished observations.
Inc., Lot E 5445, and 7.2 mc per mmole from the Volk Radiochemical Company, Lot 3488, respectively, and nn-leucine-4,5-T, 100 mc per mmole, Lot 418 from the Volk Radiochemical Company.

Actinomycin—The sample of actinomycin D employed was No. 3053 from the National Cancer Institute and showed an absorption maximum at 435 nm in Tyrode's solution. The dose administered was computed from the observed absorbance, assuming $\varepsilon_M = 25,400$ as suggested by Kahan, et al. (9).

Methods

The animals providing the liver slices were immature, Long-Evans strain rats. In each of the first four experiments, with animals 2 to 3 months old, 175 to 250 g, one noninjected rat served as control while iron was administered to one or two siblings of the same sex. The animals for Experiment 5, weighing 50 to 60 g, were taken at random from two just weaned litters 30 days old. Iron was given intraperitoneally as 0.4% ferric ammonium citrate in 0.9% NaCl except in Experiment 1, where iron dextran (Imferon) was used. The dose and timing are indicated in Table I. Actinomycin D (Experiment 5) was injected in a total dose of 18 mg per kg into four subcutaneous sites as a 0.015% solution in 0.9% NaCl containing 10% propylene glycol. Propylene glycol in 0.9% NaCl was given to the controls. The actinomycin was given 1 hour before the iron, which in turn was given 34 hours before the animals were killed. The subcutaneous route was chosen for the antibiotic so as to avoid direct contact of the liver with the undiluted solution.

Incubations—After decapitation of the animals, the livers were removed and chilled. Slices averaging 0.35 mm were

Incubations—After decapitation of the animals, the livers were removed and chilled. Slices averaging 0.35 mm were prepared from the peripheral portions of the lobes with a Mickle chopper (10). The sliced tissue was weighed (0.4 to 0.5 g) and transferred into flat-bottomed vials (25 mm outer diameter) containing 4 volumes of chilled Tyrode's solution. Labeled leucine was added and the vials were incubated for 24 hours at

![Fig. 1. Solubility of rat ferritin in cadmium sulfate. Samples of once crystallized ferritin (dialyzed against 50 mM Na$_2$SO$_4$) were mixed with an equal volume of unbuffered CdSO$_4$ of different concentrations and equilibrated for 30 minutes at 25°. An aliquot of the supernatant fluid after centrifugation was diluted in 0.4 M CdSO$_4$ and analyzed for ferritin iron by measuring absorbance at 400 nm. The results (filled circles) are plotted as percentage of original. At 100% the concentration of ferritin was about 1.8%. Another aliquot of the supernatant was analyzed for protein by the method of Lowry et al. (6) and yielded essentially the same curve. Analysis of the supernatants from suspensions kept for 5 days at 4° gave the values shown by open circles. The drop in concentration of ferritin in the last two tubes was accounted for by the presence of (imperfect) crystals at 5 days, but not at 30 minutes.

![Fig. 2. Solubility of rat ferritin (in 1 M ammonium sulfate) as a function of pH. Samples of the same ferritin preparation used for Fig. 1 were equilibrated for 10 minutes at room temperature with 1 M ammonium sulfate containing 50 mM sodium acetate buffer at different pH values. After centrifugation, the absorbance at 400 nm was taken as a measure of the ferritin remaining in solution.

![Fig. 3. pH dependence of apparent mobility of rat ferritin in paper electrophoresis, corrected for electroendosmosis. Samples of the same ferritin preparation employed for the data in Figs. 1 and 2 were subjected to electric fields of the order of 10 v per cm for 2 hours in a water-cooled paper electrophoresis apparatus (7). Sodium formate buffers were used below pH 4, sodium acetate above pH 4, and each gave the same results at pH 4. After the papers had been dried, the migration of the center of density of the ferritin spot was measured relative to that of caffeine used as an uncharged standard to correct for endosmosis. The correction for endosmosis is subject to error from any retardation of ferritin due to interaction with the paper. The interaction is expected to be greater at lower ionic strength and on the acid side, and this presumably accounts for the main inflection, especially in the lower curve.](http://www.jbc.org/ by guest on August 15, 2017)
37° under 3% CO₂-97% O₂ in a Dubnoff incubator shaking at 2 c.p.s.

**Extraction of Ferritin**—After incubation the slices were homogenized in their suspending medium for 2 minutes at room temperature with a Teflon pestle homogenizer, with 1.0 ml of 0.9% NaCl for rinse. Carrier ferritin (1 mg in 0.2 ml) was added just before homogenization. The homogenate was heated at 70° for 1 minute with stirring, was cooled and acidified to pH 5.5 (±0.1) with 0.25 M acetic acid, and was centrifuged at 12000 × g to yield a slightly opalescent extract.

**Chromatography**—A 2-ml sample of extract was filtered through a dextran gel (Sephadex G-25, Pharmacia) column of 30 cm³ total volume. Dilute salt (sodium acetate buffer, pH 5.6, ionic strength = 3 mm) was employed for elution as well as for prior equilibration of the column. The protein fraction, free of the original salts, was collected in 7 ml after the experimentally determined void volume had been discarded. The eluted protein was then applied to the DEAE-cellulose column.

The DEAE-cellulose (100 mg occupying about 1 cm³) had been equilibrated with sodium acetate buffer, pH 5.6, ionic strength = 3 mm. The adsorbed ferritin was washed with 5 ml of the same buffer, and then eluted with 50 ml buffer at the same pH. About 2 ml of eluate was collected, including a faintly visible yellow foreband plus the entire darker orange band.

**Precipitin Reaction**—Half of the eluate was mixed with 0.25 ml of anti-horse ferritin antiserum in a 5-ml conical centrifuge tube and incubated for 20 minutes at 37°. This amount of serum contained antibody well in excess of that required to precipitate all of the ferritin. The other half of the eluate was mixed with normal rabbit serum, and in no case did it produce a visible precipitate. The suspensions were centrifuged at room temperature and the supernatant fluid was decanted and drained. The sediments were washed twice by resuspension each time in 2 ml of 0.9% NaCl at room temperature. The over-all recovery of carrier ferritin added to the original homogenate averaged 35% as measured by the A₃₆₀ of the washed precipitates dissolved in 0.05 M NaOH.

**Isotopic Analysis**—The washed precipitates were dissolved in 1.00 ml of 1 M Hyamine 10X hydroxide in methanol and transferred to glass vials with 15 ml of the dioxane scintillation mixture of Bray (11). The ferritin remained dispersed for more than 24 hours in vials maintained at −5°. Samples and blanks were counted in the Packard Tri-Carb liquid scintillation spectrometer under “balance point” conditions ascertained for ¹⁴C-labeled ferritin or for ¹³C-labeled ferritin, respectively. Counting was repeated at intervals to be sure that the rate had stabilized. Quenching was assessed by recounting after the addition of a known amount of ¹³C- or ¹⁴C-labeled toluene.

**RESULTS**

**Isolation of Ferritin**—Some properties of rat ferritin pertinent to its isolation such as cadmium sulfate solubility and isoelectric point have been presented above. Considerable losses occurred during the DEAE-cellulose chromatography. A part of this loss represented the somewhat more tightly bound ferritin fractions which if desired could be included by eluting at anionic strength of about 200 mm. With a given quantity of DEAE-cellulose, the amount of loss was a negative function of the size of ferritin sample. Recovery experiments carried out separately with the principal, more loosely bound, fractions showed no marked differences among themselves in the degree of their recovery under the conditions of the standard isolation. Subsequent experiments showed that the over-all recovery of added carrier could be increased to 50% by lowering the temperature from 70° to 65° at the heating stage. This may be an underestimate of the recovery of fresh, endogenous ferritin because of the possibility that the carrier ferritin used for the evaluation of the recovery is to some degree denatured. A further subsequent improvement in the isolation was the use of pH 6.0 to facilitate the initial adsorption to DEAE-cellulose, pH 5.6 still being employed for elution. Fresh DEAE-cellulose is used for each specimen since excessive losses were occasionally observed with regenerated gel.

Ferritin can be isolated from rat liver slices by this method without the use of carrier ferritin. Carrier was used to improve the yield and uniformity of recovery because of the low endogenous ferritin levels in the young rats employed.

**Biosynthesis of Ferritin**—The results of the tracer experiments

**Table 1**

I ncorporation of leucine into ferritin by rat liver slices

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Dose of iron</th>
<th>Interval between iron and killing</th>
<th>Concentration of labeled leucine in medium</th>
<th>Uptake of label</th>
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<td>0</td>
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* Actinomycin D, 18 mg per kg subcutaneously, 1 hour before iron.
† In this case, iron dextran was used, but in all other cases, ferric ammonium citrate.
‡ In this case 90% of the leucine was carrier L-leucine.

© The collaboration of Dr. Mary Loewus in obtaining the data depicted in Figs. 1, 2, and 3 is gratefully acknowledged.
with liver slices are given in Table I. In the case of the uninjected control animals (one each in Experiments 1 to 4), the fraction of the administered leucine found in the isolated ferritin was low and relatively constant. In the corresponding specimens from the iron-treated rats, the incorporation averaged 5-fold higher although they were much more variable. Student's t test applied to Experiments 1 to 4 validates the increase due to iron (p = 0.01).

In Experiment 5 the slices from the first three rats (treated with iron but not with actinomycin) averaged more than twice the uptake of the earlier iron-treated rats, but Experiment 5 was conducted with somewhat different animals, dosage, time, and incubation conditions. The administration of actinomycin prior to the iron (last three rats) produced a marked inhibition (85%) of the uptake of label into ferritin in the slices incubated.

**DISCUSSION**

**Ferritin Synthesis**—The low amount of label in the ferritin isolated from slices from uninjected control animals compared with that from iron-treated animals suggests that there is no significant contamination by the large excess of radioleucine with which the slices were incubated. The inhibition by actinomycin supports the contention that the label represents protein and the stimulation by iron lends strong support to the belief that the label resides primarily in ferritin and not in any contaminating labeled proteins.

Although the fraction of the administered leucine incorporated into the ferritin was small, the absolute rate of incorporation appears to be of the order of magnitude to be expected in a living animal. Taking a value of 16% for the leucine content (12) as well as for N content (13) of apo-ferritin, the average rate of ferritin synthesis computed for the liver slices from the three (iron-stimulated) control rats in Experiment 5 is 1.2 μg of ferritin N per g of liver (wet weight) per hour, even without correcting for losses of ferritin during isolation. This value is about one-half of the average rate of iron-stimulated hepatic ferritin synthesis estimated in vivo in infant guinea pigs (2) which came to a little over 2 μg of N per g per hour. The present calculation rests on the assumptions that the specific activity of the intracellular leucine rapidly attained the level of that in the medium and that the initial level in the medium was maintained during the incubation. If either criterion were not satisfied, the rate of synthesis would be underestimated. The data from Experiment 5 were employed in the calculation because in the other experiments the leucine concentrations were lower and less likely to “saturate” the intracellular leucine pool with label. Evidence for this likelihood resides in the absence of any marked trend toward decreased uptake (as fraction of dose) with increasing dose.

The response to injected iron was not only similar in degree to that previously observed in intact guinea pigs (2), but was also similar in the variability from one animal to another. One of the advantages to be expected from the liver slice technique is the avoidance of this variation in future studies, if slices from the same liver are used. A further advantage is closer control of cell environment than is possible in vivo.

During the preparation of this report, another paper appeared (14) describing ferritin synthesis in rat liver slices and stimulation by the prior injection of iron.

**Effect of Actinomycin**—By blocking DNA-dependent RNA synthesis (15), actinomycin inhibits the synthesis of any proteins which depend on the continuous replacement of unstable messenger RNA. The apparent inhibition by actinomycin of the stimulatory effect of iron on ferritin synthesis suggests that iron exerts its control at the level of the gene, presumably by derepression, a mechanism of control of inducible proteins extensively investigated in microorganisms (16). In the case of many mammalian proteins, evidence is accumulating for a more or less permanent repression of the genetic control of synthesis upon maturation of the cell (17, 18), with subsequent control being exerted primarily at lower levels, the synthesis proceeding with more or less stable messenger RNA (19).

The dose of actinomycin in the present experiment was about 22 times the LD<sub>50</sub> reported (20) for the subcutaneous route. The lethal effects, however, are attributable not to direct poisoning of vital processes in cells generally, but rather to the indirect, delayed effects of the interruption of cell replacement in tissues of high cell turnover such as intestinal epithelium. At the time that the animals were killed (44 hours) in the present experiment, their external appearance and behavior were quite normal. Much of the dose was still visible in the edematous subcutaneous injection sites by its yellow color. On the assumptions that half was absorbed, retained in the body, and distributed evenly in the total body fluid, the concentration bathing the liver cells would be about 15 μg per ml. Such a dose applied to animal cells in vitro, while markedly inhibiting RNA synthesis (21), is not believed to inhibit the subsequent steps in protein synthesis. Indeed, when Seed and Goldberg (18) incubated sheep thyroid slices for 20 hours in actinomycin at a level of 10 μg per ml, they found that thyroglobulin synthesis continued at almost normal rates.

Should more exhaustive further experiments verify the indicated short term genetic control of ferritin synthesis by iron, then this storage protein would seem to fall in with a group of mammalian enzymes adapting drastically to nutritional fluctuations, in which control at the DNA level is also apparently employed (e.g. 19).

**SUMMARY**

Some properties of rat liver ferritin pertinent to its isolation were studied. The isoelectric point was about pH 4.1. The presence of chloride ion was necessary for crystallization in high yield from cadmium sulfate solutions. The protein was more susceptible to heat denaturation than horse ferritin. A procedure for small scale isolation of ferritin from rat liver slices was evolved based mainly on immunoprecipitation following diethylaminoethyl cellulose chromatography.

Rat liver slices incubated with radioleucine incorporated the label into ferritin. Slices from iron-treated rats averaged 5-fold higher uptakes. Rates of incorporation of label by the slices were comparable with rates of hepatic ferritin synthesis in vivo estimated elsewhere. Uptake in slices from rats given actinomycin before iron was 85% less than controls without the antibiotic, suggesting that the stimulation of ferritin synthesis by iron was mediated by ribonucleic acid synthesis.
REFERENCES

Biosynthesis of Ferritin by Rat Liver Slices
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