Serum Requirement for Apoferritin Induction in Rat Liver Slices

By Lester Smith and R. A. Fineberg

In studies of ferritin induction, liver slices failed to respond favorably to iron added directly to the suspending medium unless serum was added. The addition of 5 μg Fe/ml to slices in whole serum accelerated the incorporation of radiouleucine into ferritin 2.5-fold. The favorable effect of serum on iron-induced ferritin synthesis was correlated with its attenuation of the toxic effects of the iron on protein synthesis; the serum was also found to diminish the uptake of iron by the liver cells.

Transferrin, purified from the serum by ion exchange, failed to mediate the iron induction of apoferritin while albumin, completely freed from transferrin as monitored with radioiron, was effective. Albumin, apparently by limiting the uptake of excess iron by the liver cells, modulates the nonspecific toxic effects of iron on protein synthesis and thereby permits the specific induction by iron of the biosynthesis of apoferritin.

ADMINISTERED IRON ACCELERATES manyfold the synthesis of apoferritin in the liver of intact animals. Accelerated apoferritin synthesis has also been demonstrated in liver slices from iron-pretreated rats, but rat liver slices failed to respond favorably to iron added directly to the incubation medium. Iron added directly in vitro has been effective in reticulocytes, HeLa cells, and in preliminary work with liver microsomes, but the response has been less than a doubling of the rate. We have found that iron was effective on liver slices if it were simply added to normal serum. The identity of the active principle in the serum was investigated by testing the activity of serum fractions, and the mechanism was partially elucidated by studying the effects on iron uptake.

MATERIALS AND METHODS

Male Long-Evans rats, age 21 (weaning)-26 days, were fasted 17 hr before sacrifice. Human serum was obtained from volunteers and rat serum from adult rats. Anti-rabbit ferritin rabbit antiserum was prepared as previously described. Carrier ferritin was partially purified from the livers of rats that had received intraperitoneal iron dextran (0.1 g Fe/kg); a saline extract was heated at 65°C for 10 min, and the ferritin-containing fraction precipitated three times from 2 M ammonium sulfate. DL-[1-14C] leucine was purchased from New England Nuclear, 59FeCl₃ from International Chemical and Nuclear, crystalline serum albumin from Pentex, and ferric ammonium citrate (green scales) from J. T. Baker. Deferoxamine (Desferal) was kindly donated by Ciba.

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Fig. 1. Apoferritin synthesis in rat liver slices. Effect of iron concentration and of incubation medium on rate of incorporation of radioleucine into ferritin. Circle, serum; open square, Tyrode's solution. Slices in either serum or Tyrode's solution were first incubated with or without ferric ammonium citrate for 30 min, and then for 2 or 3 hr after addition of radioleucine. Ordinate is amount of radioleucine incorporated into ferritin in presence of added iron, relative to that incorporated into iron-free controls. In many of the controls, deferoxamine (10⁻⁴ M) was added. Since the effect was slight, data were pooled. Some of serum samples were human and some rat. Since results were similar, data were pooled. Vertical lines span 2 SEM, and numbers of samples are shown.

Assay for Synthesis of Apoferritin and Mixed Proteins

The rate of incorporation of radioleucine into the protein moiety of ferritin during incubation of rat liver slices in the presence and absence of added iron was determined by analyzing the radioactivity of ferritin isolated immunochemically from the slices.

Liver slices (0.35 mm) were prepared with a Mickle chopper. Portions (400 mg) were placed in 1.5 ml of medium under 5% CO₂ and O₂. Either iron (ferric ammonium citrate) iron-chelating agent, (deferoxamine), or only incubation medium was added, and the vials were incubated with shaking at 37°C for 30 min. Then [¹⁴C] leucine was added (3 µCi, 1.4 mM final), and the incubation continued for 2 or 3 hr. Finally, 3 ml of 50 mM carrier leucine in 0.9% NaCl was added ice cold, and the slices were centrifuged and washed.

Labeled ferritin was extracted by a modification of the method of Yu and Fineberg. The slices were homogenized with 2.5 ml of carrier ferritin (about 80 µg Fe) in the carrier leucine solution, heated 7 min at 65°C, and adjusted to pH 5.5 with acetic acid. Ferritin was precipitated from the supernatant fluid with 2.2 M ammonium sulfate, dialyzed against 5 mM sodium acetate (pH 6.0), centrifuged to remove a small, nonferritin precipitate, and made isotonic with NaCl. Antiserum was added and incubated 20 min at 37°C. The precipitate (including both apoferritin and ferritin) was washed twice, dissolved in Hyamine, and counted in dioxane-based scintillation fluid.

Mixed liver proteins were isolated from the precipitate obtained from the heated and acid-treated homogenate. The pellet was homogenized with 5% trichloroacetic acid (TCA) in carrier leucine solution; an aliquot was washed in TCA, heated to hydrolyze RNA, defatted, and finally dissolved in Hyamine and counted in scintillation fluid.

RESULTS

Effect of Iron on Apoferritin Synthesis in Presence and Absence of Serum

The inhibitory effect of iron on apoferritin synthesis in liver slices incubated in Tyrode's solution is shown as a function of concentration of added iron in the lower curve in Fig. 1. On the average, inhibition was first manifest at a dose of about 1 µg Fe/ml, was half-complete at 4, and was almost complete at 100.
Apoferitin synthesis could be accelerated by added iron if the slices were suspended in serum (upper curve). The inductive effect was strongly dependent on the concentration of iron. The response started abruptly at about 3 μg Fe/ml, and was maximal at 5 μg Fe/ml, at which the average rate of incorporation of radioleucine was 2.5 ± 0.1 (SE) times that of the control slices incubated in the absence of added iron. High concentrations of iron were markedly inhibitory even in the presence of serum. It may be estimated from the curve that there was some degree of net stimulation over the range of about 2–14 μg Fe/ml, and a half-maximal or greater effect over the range of 3–8 μg/ml.

Mixed Protein Synthesis

The simultaneous uptake of labeled leucine into nonferritin proteins was studied as a control for possible nonspecific effects of iron (Fig. 2). The synthesis of mixed proteins was more susceptible than apoferritin to iron poisoning. With slices immersed in Tyrode’s solution (lower curve), the 50% inhibitory concentration was little more than 1 μg Fe/ml. When the slices were incubated in serum, the incorporation of radioleucine was unaffected (upper curve), until the concentration of added iron exceeded about 2 μg/ml. At 5 μg Fe/ml, the optimum dose for induction of ferritin, the synthesis of general proteins was diminished by about 25%. Much higher doses of iron were severely inhibitory in spite of the presence of serum.

In the absence of added iron, Tyrode’s solution supported protein synthesis in the liver slices just as well as did the serum; the averages of the raw data from the various separate experiments showed no statistically significant differences in the level of leucine incorporation from the two incubation media.

Activity of Serum Protein Fractions

Since serum ultrafiltrates were inactive and the total protein fraction (from Sephadex G-25) was active, the serum proteins were further fractionated essentially by the methods of Gellote et al.9 Radioiron (2 μg Fe/ml) was first added to serum to label the transferrin. The 59Fe was found only in one sym-
metrical peak in the Sephadex G-200 gel filtration, centering about 0.03 column-volumes ahead of the main (albumin) absorbency peak. To effect a complete separation of these two components, the later-eluting two-thirds of the proteins were put on a column of DEAE-Sephadex (A-50) equilibrated with 0.1 M Tris-HCl (pH 7.4). After eluting some nonradioactive material with the same buffer, the transferritin-containing fraction was completely eluted by a stepwise increase in the concentration of eluting buffer (0.15 M). Then the albumin-containing fraction was eluted by another stepwise increase (0.3 M). The fractions pooled for transferrin and albumin comprised about 70% of the uv-absorbing material eluted.

The transferrin-containing fraction, as examined by polyacrylamide gel electrophoresis by the procedure of Davis, contained one appreciable contaminant with a mobility similar to albumin and with a stain density perhaps 30% of that of the main band. The main band was identified as transferrin by its radioiron content. The albumin-containing fraction itself was essentially homogeneous by gel electrophoresis.

The fractions were adjusted approximately to the original concentration of the respective protein fraction in serum, and the salts of Tyrode’s solution were added. Liver slices were suspended in the various fractions, incubated with radiolabeled leucine with and without iron, and analyzed for the amount of label in the isolated ferritin and in the mixed proteins.

The albumin fraction, completely cleared of transferrin, significantly increased the incorporation of radiolabeled leucine into ferritin (Table 1, row 3) relative to the iron-free controls. Crystalline human serum albumin (Pentex) also supported a significant, iron-induced increase in apoferritin synthesis (Table 1, row 4). The apparent differences in the degree of the albumin-supported inductions as compared with whole serum (Table 1, row 2) are not statistically significant. Furthermore, a lower dose of iron was added with albumin (2 µg/ml) than with serum (5 µg/ml), because of the absence of transferrin in the former (see below). The albumin samples were also apparently effective, like serum, in moderating the degree of inhibition of mixed protein synthesis.

The transferrin-containing fraction (4 mg/ml) definitely inhibited the incorporation of radiolabeled leucine into mixed proteins, and the uptake of leucine into apoferritin was decreased (Table 1, row 5). The dose of added iron averaged 4 µg/ml.

The “large serum protein” fraction, the first third of the serum proteins eluted from Sephadex G-200 (Table 1, row 6) was essentially inactive in that it failed to support the induction of ferritin by added iron (5 µg/ml) and permitted an inhibition of mixed protein synthesis that was just as severe as that with Tyrode’s solution alone.

Iron Uptake

The kinetic data on the accumulation of iron by liver slices are plotted for different doses of iron in Fig. 3. In either the presence or absence of serum the curves were hyperbolic, reaching a plateau in time. The initial rate of iron uptake was roughly proportional to the log of the dose of iron; the final level increased linearly with the dose, except for the two high doses of Tyrode’s
Table 1. Effect of Serum Fractions on Induction of Apoferritin Synthesis by Iron Added Directly to Rat Liver Slices*

<table>
<thead>
<tr>
<th>Incubation Medium</th>
<th>Number of Samples</th>
<th>Incorporation of[^14C] Leucine (Relative to iron-free Controls)</th>
<th>Ferritin</th>
<th>Mixed Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrode's solution</td>
<td>8</td>
<td>%</td>
<td>p</td>
<td>%</td>
</tr>
<tr>
<td>Whole serum</td>
<td>78</td>
<td>31</td>
<td>&lt; 0.01</td>
<td>20</td>
</tr>
<tr>
<td>Albumin fraction</td>
<td>6</td>
<td>143</td>
<td>&lt; 0.01</td>
<td>77</td>
</tr>
<tr>
<td>Crystalline albumin</td>
<td>12</td>
<td>212</td>
<td>&lt; 0.01</td>
<td>91</td>
</tr>
<tr>
<td>Transferrin fraction</td>
<td>24</td>
<td>78†</td>
<td>&lt; 0.01</td>
<td>26</td>
</tr>
<tr>
<td>Large serum proteins</td>
<td>6</td>
<td>65</td>
<td>0.12</td>
<td>19</td>
</tr>
</tbody>
</table>

*Serum fractions were reconstituted to their approximate serum concentration. Half of the slices were incubated with and half without iron in these media, all containing radioleucine. Ferritin and mixed proteins were then isolated from slices. Average radioactivity incorporated in proteins from iron-treated slices is here recorded as percent of that from noniron-treated controls. Number of samples includes both iron-treated and iron-free. Probability (p) of no significant iron effect was computed from Student's one-tailed t test. Dose of added iron was not the same in all cases (see Results).

†Radioactivity corrected for ^59Fe (from the transferrin) by dual channel counting.

solution. At all three lower doses of iron, the uptake from Tyrode's solution greatly exceeded that from serum. At the optimum dose for ferritin induction (5 μg/ml), there was almost a threefold difference in uptake from the two media. At the highly toxic dose of 30 μg/ml, the uptake from Tyrode's solution was practically the same as from serum, and at 100 μg, the uptake was no higher than at 30 μg/ml and was much lower than its counterpart in serum.

The radioactivity of the slices and of the suspending medium were measured in other experiments after incubation of slices to essentially maximum uptake with radioiron in serum and in Tyrode's solution. At all iron doses from 1 to 125 μg/ml, essentially all of the added radioiron was recovered either in the incubation medium or in the washed slices. In serum, there was negligible uptake at 1 μg of added Fe/ml, which is within the binding capacity of the serum transferrin. At all of the other iron concentrations (beginning at 3 μg/ml) the uptake was about 15% of the total added; the ratio of concentration of radioiron in tissue and medium was 3.5. In Tyrode's solution, the distribution was much different. At low levels of iron, including 1 μg/ml, liver slices took up about 40% of the dose of iron, concentrating the radioiron 14-fold above that in the medium. At doses of iron beyond 10 μg/ml, however, the slices in Tyrode's solution took up progressively smaller proportions of the radioiron, falling well below the uptake in serum after about 30 μg Fe/ml in the medium. In another experiment, the reversibility of the iron uptake from serum was tested. About one-half of the radioactivity that had been taken up in 1 hr was lost during a chase incubation with nonlabeled iron; that portion that was exchanged had a half time of about 15 min.

Total uptake is plotted in Fig. 4 against final concentration of iron in the medium, for all cases of low iron dosage. Data points derived from Fig. 3 were supplemented by additional experiments. The iron uptake from Tyrode's solu-
SERUM REQUIREMENT FOR APOFERRITIN INDUCTION

Fig. 3. Kinetics of uptake of different doses of iron into rat liver slices, from human serum and from Tyrode's solution. Rat liver slices (50 mg) were incubated in 1 ml of either serum or Tyrode's solution. \(^{55}\)Fe (as ferric chloride) was equilibrated with carrier ferric ammonium citrate prior to its addition to incubation medium; specific activity of added radioiron was the same in each experiment. After incubation, slices were washed twice in the cold with 3 ml of carrier iron of a concentration proportional to that in incubation medium and counted in a solid scintillation counter. Numbers after curves indicate concentration (µg/ml) of iron added to incubation medium. Ordinate scale (iron uptake) is contracted fivefold for two highest doses of iron (upper graphs).

Uptake was directly proportional to the concentration of iron at these doses. In serum, the uptake was nil until the level of iron exceeded 2 µg/ml, which corresponds with the average normal human value for the latent iron-binding capacity of serum transferrin.\(^{11}\) The dotted line drawn parallel to the curve for uptake from Tyrode's solution makes allowance for the amount of iron that serum transferrin should bind and thus represents the uptake curve to be expected with serum if transferrin were the only serum factor inhibiting cellular uptake of iron. The actual inhibition was much more than could be accounted for by transferrin-binding alone.

Fig. 4. Total uptake of iron into rat liver slices as function of iron concentration in serum and in Tyrode's solution. Circles, serum; squares, Tyrode's solution. Liver slices (50 mg) were incubated in 1 ml of either medium for 90 or 120 min with radioiron of constant specific activity and treated as in Fig. 3. Numbers indicate number of samples. Abscissa (iron concentration) has been corrected to final concentration in medium on basis of data obtained in a separate experiment. Broken line is drawn parallel to line for Tyrode's solution and through the intercept for serum (see text).
The Effect of Serum Proteins on Iron Uptake

The effect of the total serum protein fraction from G-25 Sephadex was compared directly with that of whole serum and was found to behave identically to serum in retarding both the initial rate and total uptake of iron by rat liver slices, when compared to uptake from Tyrode's solution. The iron uptake from either human or bovine commercial crystalline serum albumin at the concentration normally found in serum was about one-half the iron uptake from whole human serum. In another experiment various dilutions of whole serum and dilutions of a normal concentration of crystalline bovine serum albumin were prepared in Tyrode's solution and tested for inhibition of iron uptake, as compared to Tyrode's solution alone. In each case, a tenfold dilution was required in order to reduce the degree of inhibition by half.

DISCUSSION

The data clearly establish the induction of the biosynthesis of apoferritin by iron added directly to the incubation medium containing rat liver slices. The stimulation of synthesis 2.5-fold over the "resting" level, although less than the results with liver in vivo, is better than the stimulation reported in other systems involving the direct addition of iron in vitro.

The apoferritin induction was successfully demonstrated only in the presence of serum; iron inhibited apoferritin synthesis in the absence of serum. Serum is known to be essential for the growth of many mammalian cells in vitro, a protein in serum stimulates the synthesis of RNA and protein in 3T3 cells in culture, and a serum macromolecule enhances the cortisol-induced synthesis of tyrosine transaminase in hepatic tumor cells in culture. However, the simplest explanation for the action of serum on the induction of apoferritin synthesis by iron would appear to be merely an effect on the absorption of iron by the liver.

The liver slices absorbed iron avidly, as compared for example with HeLa cells. Tested over a wide range of doses of added iron in the absence of serum, HeLa cells took up less than one-third as much iron per mg of tissue as did liver slices in comparable circumstances. Furthermore, HeLa cells were very tolerant of high doses of iron; as much as 100 μg Fe/ml caused only a 15% inhibition of leucine incorporation into mixed proteins in cells suspended in a 10% serum medium. Protein synthesis in liver slices, in sharp contrast (Fig. 2), was very sensitive to added iron.

Serum markedly retarded the uptake of iron by the liver slices and concomitantly protected protein synthesis from iron poisoning. Although it may seem paradoxical that serum could favor the iron-stimulated synthesis of apoferritin by retarding the uptake of iron, the explanation would appear to lie in the assumption of two different actions of iron in apoferritin synthesis. One is inhibitory, poisoning the synthesis of mixed liver proteins, and is non-specific, affecting some factor required for the synthesis of all proteins, including apoferritin. The other site of action of iron is stimulatory and is specific for the induction of apoferritin. According to current evidence, the specific
site of action of iron is at a post-transcriptional stage of transmission of the message for apoferritin synthesis. To produce a net stimulation of apoferritin synthesis, the iron must presumably be delivered at the rate, place, or in the chemical form that is sufficient for the ferritin-specific stimulation but not enough to inhibit too strongly the nonspecific processes of protein synthesis. The observed sharp peak of ferritin induction as a function of concentration of iron (Fig. 1) would appear to be the resultant of the stimulatory and inhibitory actions of iron, the range of favorable iron dosage consequently being restricted.

If moderation of iron uptake is the only action of serum, it should be possible to stimulate apoferritin synthesis in the absence of serum with a properly chosen low dose of iron. However, such a demonstration might be technically difficult without the presumed “buffering” by the serum; indeed, the available data at low doses show considerable scatter (Fig. 2). The reported failure of induction of ferritin even in the presence of serum may have depended simply on the concentration of iron. In the 20% serum used by those authors, the iron tolerance of liver was probably even more restricted than in the present experiments.

Transferrin, known to mediate the absorption of plasma iron by reticulocytes and to serve as a source of ferritin iron in liver slices, at least in trace amounts, might have been suspected a priori to be the serum factor mediating the induction of ferritin by iron. However, the minimum stimulatory dose of iron exceeded the unsaturated iron-binding capacity of the transferrin; the isolated transferrin even though fully saturated with iron failed to induce apoferritin synthesis; and the isolated albumin completely freed of transferrin did support the iron-induction of apoferritin synthesis. Iron bound to serum transferrin was much less rapidly and extensively taken up by the liver slices than was the excess iron. For example, Fig. 4 shows a negligible uptake of iron at doses below the threshold of saturation of transferrin. Thus, the effects on protein synthesis in the present experiments are attributable to the nontransferrin iron. The results further show that serum retards the uptake of this excess iron.

The minimal, apoferritin-inducing dose of iron in the present experiments appears to be just beyond the transferrin capacity. Although this is still higher than the levels normally observed in the peripheral blood, the question may be raised whether the iron in the portal vein blood ever transiently exceeds the capacity of the transferrin, the excess being then cleared in the passage of the blood through the liver. The maximally effective intraperitoneal dose (6 μg/g) injected into rats by Drysdale and Munro corresponds roughly to our observed optimal dose (5 μg/ml) of iron added to serum in vitro. This was stimulatory in the presence of serum and inhibitory in its absence. In vivo, the Kupffer cells are exposed directly to the whole plasma, as are also the liver parenchymal cells through the sinusoidal fenestrae.

The iron uptake of liver slices suspended in serum leveled off with time, suggesting an equilibration, a notion fortified both by the finding of a linear relation of total uptake to concentration of iron added and by the demonstrated release of previously absorbed iron. These observations in the presence of
serum are similar to those of Saltman et al. in the absence of serum.\textsuperscript{17} We have neither determined which cell types in the slices are involved, nor what proportion of the radioiron uptake may represent nonspecific external absorption. Nor can the data distinguish among possible mechanisms of membrane transport; pinocytosis, for example, is possible, since the excess iron could be in the form of colloidal-sized aggregates.\textsuperscript{18} Whatever the mechanism of iron uptake, the fourfold lesser concentration gradient of radioiron between slices and medium that we found in serum, as compared to Tyrode's solution, could well be the result of iron-binding by the serum.

The albumin-containing fraction was the only serum fraction tested that clearly supported the induction of apoferritin synthesis by iron. Albumin is known to bind iron loosely.\textsuperscript{19} The simplest explanation of the present data is that albumin iron binding decreases the avid hepatic uptake of excess plasma iron, minimizing the nonspecific poisoning of protein synthesis, and permitting the specific stimulation of synthesis of apoferritin. Whether this mechanism functions in vivo remains to be determined. As a purely technical matter, the addition of albumin will render the liver slice system more useful in further studies of the mechanism of apoferritin induction by iron.

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