Regulation of Ferritin Synthesis in Rat Liver*

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SUMMARY

Ferritin synthesis in rat liver is greatly increased by prior treatment of intact animals with iron salts. Liver slices from treated rats incorporate increased amounts of 14C-leucine into ferritin in vitro, with net synthesis of the protein. Slices of spleen, testis, intestine, and kidney also show increased incorporation following iron. The response of the liver to iron has been studied to determine whether increased transcription of genes and the formation of messenger RNA are involved, or whether control is at a later stage in the biosynthetic pathway. Three lines of evidence support the existence of transcriptional control. (a) Actinomycin D inhibits the response to iron. (b) Iron treatment stimulates incorporation of 14C-orotic acid into a rapidly labeled fraction of liver nuclear RNA. (c) Direct addition of iron to liver slices in vitro has failed to stimulate ferritin synthesis, although 56Fe penetrates liver cells.

Iron administered to intact rats markedly increases hepatic biosynthesis of the protein moiety of ferritin, as shown by studies in vivo (2, 3) and with liver slices incubated in vitro (4–6). The response is apparently adaptive, the ferritin providing an intracellular iron store which is nontoxic and available for metabolism. The mechanism involved in increasing the synthesis is unknown but of interest as an example of the control of protein synthesis, especially as ferritin is well characterized, readily isolated and estimated, and widely distributed in living cells. This report focuses on whether iron acts via gene activation and synthesis of messenger RNA or at subsequent stages in protein biosynthesis, e.g. translation of messenger RNA or stabilization of protein intermediates. A number of investigators have studied the effects of actinomycin D, an inhibitor of DNA-dependent synthesis of RNA (7), to answer this question. Yu and Feinberg (4) and Yoshino, Manis and Schachter (6) reported distinct inhibition of apoferritin synthesis in response to iron after administration of actinomycin D in doses, respectively, of 18 and 1 to 4 μg per g of rat. Drysdale and Munro (3), in contrast, observed no inhibition with 0.7 to 1.5 μg per g of rat. The disparity may result from insensitivity of the method used by the latter authors to study the effects of actinomycin D. They injected 14C-leucine in vivo and estimated the incorporation into ferritin after 2 hours. However, in their control experiments the incorporation was linear with time for less than 1 hour, so that over 50% inhibition of the initial rate of incorporation could remain undetected by estimations at the 2-hour point.

The present report describes further observations on the synthesis of apoferritin by liver slices in vitro and presents three lines of evidence to indicate that gene activation is involved in regulation of the synthesis. Inhibition of the response to iron administration by actinomycin D was confirmed by estimating net synthesis of hepatic ferritin in vivo. Iron administration was shown to stimulate the incorporation of 14C-orotic acid into the rapidly labeled fraction of liver nuclear RNA. Finally, direct addition of iron to liver slices in vitro, which might be expected to increase ferritin synthesis if iron influenced translation of messenger RNA, failed to stimulate synthesis despite uptake of Fe into the slices.

EXPERIMENTAL PROCEDURE

Incubation of Slices—The general methods described previously (6) were modified slightly. Albino male rats of the Sherman strain, 100 to 250 g, were fasted for 18 hours in metabolism cages before use. Iron preparations were injected intravenously under light ether anesthesia, and after appropriate intervals livers were removed from groups of four rats or more and sliced with a Stadie-Riggs microtome, and 1.0 g of pooled slices was incubated in 10.0 ml of medium containing 2.0 μC of 14C-1-leucine, at 37° with 95% O2-5% CO2 as gas phase. The incubation medium was similar to that described by Matioli and Eylar (8) except that
or rat liver ferritin by rabbit anti-horse spleen ferritin antiserum. Each reaction tube contained in a final volume of 3.1 ml the appropriate quantity of recrystallized ferritin and 0.1 ml of antiserum in 0.14 M NaCl containing 0.001 M Tris-HCl buffer, pH 7.5. Tubes were stored at 37° for 1 hour, and subsequently at 5° for 18 hours, and the precipitates were washed three times and then suspended in 1.0 ml of 0.1 N NaOH, heated at 90° to dissolve, and aliquots were taken for estimation of protein (10).

10.0 ml of medium contained 2.0 ml of normal rat serum in place of serum albumin and transferrin (6). After incubation the slices were either homogenized in their incubation medium or removed from the medium, weighed, and homogenized in 10 volumes of 0.01 M NaCl. The tissue homogenates and where appropriate, aliquots of the incubation medium were heated to 70° for 10 min and precipitated proteins were removed by centrifugation at 15,000 × g for 20 min. Duplicate aliquots of the supernatant solutions were assayed with antiferritin antiserum as described below. In each experiment two control aliquots of liver slices were homogenized immediately with incubation medium containing 14C-leucine, without prior incubation, and the homogenates were assayed as just described. The 14C estimated in immune precipitates from nonincubated controls averaged less than 10% of the radioactivity in incubated samples and was subtracted as a blank value.

Precipitin Reaction—Ferritin in the samples was precipitated with rabbit anti-horse spleen ferritin antiserum, prepared as previously described (9), and assayed by precipitin reaction with crystalline ferritin from horse spleen and rat liver. Fig. 1 shows that under the standard assay conditions the quantity of immune precipitate obtained by reaction with 0.1 ml of antiserum was directly proportional to the quantity of rat liver ferritin added up to 100 μg. Accordingly, in all assays aliquots containing 50 μg of ferritin or less were added for immunological precipitation. In earlier experiments 40 μg of carrier rat liver or horse spleen ferritin were added routinely (6), but comparable results were subsequently obtained with no carrier added. The immune precipitates were centrifuged after 18 hours at 5°, washed three times with 12 ml of 0.9% NaCl, and dissolved in 1.0 ml of 0.1 N NaOH by heating at 90° for 20 min, and portions were taken for estimation of protein (10) and of radioactivity in Bray's solution (11) in a liquid scintillation spectrometer. In each experiment 25 and 50 μg of horse spleen ferritin were treated with antisera as reference standards and values for rat liver ferritin were calculated from the curves shown in Fig. 1.

Separation of Iron-poor and Iron-rich Ferritin—Recrystallized rat liver and horse spleen ferritin containing, respectively, 24.5 and 20.0% iron, by weight, were dialyzed against sodium hydroxide (1) until the dialysate contained no iron as indicated by reaction with α,α′-dipyridyl and, finally, dialyzed against 0.01 M sodium acetate, pH 4.6. The final preparations of iron-poor ferritin contained less than 0.15% iron by weight. A convenient method was developed to separate iron-rich and iron-poor ferritins for experiments described below. When dissolved in 0.15 M NaCl, 2.4 × 10⁻⁴ M potassium phosphate at pH 7.0 and centrifuged at 105,000 × g for 2 hours in the Spinco model L2 ultracentrifuge, 96.6 and 98.7%, respectively, of rat liver and horse spleen iron-rich ferritin was found in the pellet. Under identical conditions 66.3 and 58.8%, respectively, of the rat and horse iron-poor ferritin was found in the supernatant. Application of this separation is described further under “Results.”

Other Methods and Materials—Nuclear and cytoplasmic, phenol purified RNA was prepared by the method of Dingman and Sporn (12), modified slightly as follows. Recrystallized bentonite was added to the sucrose solutions to a final concentration of 0.5% potassium acetate, pH 5.0, were precipitated by the addition of 2 volumes of absolute ethanol and storage at -10° for 18 hours. The RNA precipitate obtained on centrifugation was washed twice with cold 70% ethanol before further assay. Methods for preparation of crystalline rat liver ferritin and rabbit antihorse spleen ferritin antiserum were described previously (9). 56FeSO₄ (specific activity >10 mCi per mg of iron) was purchased from Abbott Laboratories. 56Fe was counted in Bray's solution in a liquid scintillation spectrometer. Iron dextran (Imferon) was purchased from Lakeside Laboratories (Milwaukee, Wisconsin), 14C-orotic acid (specific activity >2 mCi per mmole) and 14C-1-leucine (specific activity >200 mCi per mmole) were from New England Nuclear, and human serum protein fractions were from Nutritional Biochemicals.

RESULTS

Time Course of Synthesis in Vitro—Rats were given 45 μg per g of body weight of iron as iron dextran, intravenously, and 16 hours later groups of four treated and untreated animals were killed and pooled liver slices were prepared for each group and incubated at 37° in the standard medium containing 14C-leucine. At the onset and at the intervals thereafter shown in Fig. 2 duplicate flasks were removed, slices and media were separated by filtration through a single layer of coarse filter paper, and ferritin content and incorporation of 14C into ferritin were estimated in each sample immunologically as described under “Experimental Procedure.” The results of three experiments were similar and the values, therefore, were averaged and plotted in Fig. 2. Incorporation of 14C-leucine into apoferritin increased linearly with time to 3 hours of incubation, and the rate of incorporation was 13 times higher in the iron-treated as compared to the control group. Net synthesis of apoferritin in vitro was observed only in the iron-treated group in the 1st hour of incubation. In 10 comparable experiments the values for net synthesis in vitro varied from 16 to 81 (mean 41) μg per g of liver slices. The broad range of values probably results partially from tech-
The linear incorporation of \(^{14}\text{C}\)-leucine as contrasted with negligible net synthesis in the 1- to 3-hour interval illustrated in Fig. 2 suggested the possibility of turnover of ferritin in vitro. Direct evidence was obtained in three experiments in which groups of six rats were given the prior dose of iron dextran and 19 hours later each animal received 5 \(\mu\)C of \(^{14}\text{C}\)-leucine intravenously. Four hours later liver slices were prepared and incubated in the foregoing medium modified to contain 10 mM \(^{14}\text{C}\)-leucine and no radioactive leucine. In the 1st hour of incubation the specific radioactivity of the isolated \(^{14}\text{C}\)-ferritin increased slightly, by 5.4 to 20.9\% (mean 15.8), suggesting the completion of molecules partially synthesized at the time of onset of incubation. Over the next 3 hours the specific radioactivity decreased by 11.8 to 28.3\% (mean 19.8), indicating a slow turnover of radioactive ferritin in vitro.

Effects of Iron Dose—The effects of four dose levels of iron dextran, varying from 10 to 88 \(\mu\)g of iron per g of rat, were tested in each of two experiments. Each dose was administered intravenously to a group of four rats, and 18 hours later liver slices were prepared and tested in vitro as described in the preceding section. As shown in Fig. 3, incorporation of \(^{14}\text{C}\)-leucine into apoferritin increased linearly with the dose to approximately 40 \(\mu\)g of iron per g of rat, with proportionately less response thereafter. Net synthesis of apoferritin in vitro also increased with the dose, with distinct synthesis observed in the dose range 40 to 88 \(\mu\)g of iron per g of rat.

Effects of Various Iron Salts—Groups of four rats were given 10 \(\mu\)g of iron per g of rat intravenously of one of several iron salts and 18 hours thereafter liver slices were prepared and tested in vitro for incorporation of \(^{14}\text{C}\)-leucine into apoferritin. Table I indicates that, whereas all of the iron salts markedly increased incorporation, ferrous sulfate and ferrous lactate were about twice as effective as iron dextran \((p < 0.01)\). FeCl\(_3\) was not significantly more effective than iron dextran. Dextran alone, 0.5 \(\mu\)g per g of rat, was administered similarly. Control rats treated with vehicle alone and treated animals were killed after 18 hours and liver slices tested as described under "Experimental Procedure." Differences between iron dextran and the two ferrous salts were statistically significantly different, \(p < 0.01\).

Serum Requirement—Optimal incorporation of \(^{14}\text{C}\)-leucine into apoferritin in vitro was found to require whole rat serum at a level of approximately 20\% (v/v) in the ambient medium. Table II summarizes the results of three experiments which show that serum increased the incorporation approximately 2-fold, and crystalline rabbit albumin failed to replace whole serum. Additional experiments showed that dialyzed rat serum was equally effective as whole serum, human serum was about 60\%
TABLE II
Requirement for serum for optimal incorporation of \( _{14}C \)-leucine into ferritin by rat liver slices in vitro

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>( ^{14}C )-leucine incorporated into ferritin (cpm/( g ) slices)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>1195</td>
</tr>
<tr>
<td>Minus serum</td>
<td>412</td>
</tr>
<tr>
<td>Minus serum, minus amino acids</td>
<td>528</td>
</tr>
<tr>
<td>Minus serum, plus rabbit albumin</td>
<td>479</td>
</tr>
</tbody>
</table>

![Diagram showing incorporation of \( _{14}C \)-leucine into ferritin in various tissues](image)

Fig. 4. Effect of iron dextran on capacity of various tissue slices to incorporate \( _{14}C \)-leucine into ferritin in vitro. Groups of four rats were treated with 40 \( \mu g \) of iron per g of rat intravenously, and 18 hours thereafter tissue slices were prepared and tested as described under "Experimental Procedure." Incubations were at 37°C for 3 hours. Values shown above for liver, spleen, duodenum, and kidney are average values of five experiments and are representative of each experiment. Values for testis are means of two experiments.

as effective as rat serum, and the following purified, human serum protein fractions (prepared by Cohn fractionation and purchased from Nutritional Biochemicals) could not substitute individually for whole serum: albumin, \( \alpha \) globulin, \( \beta \) globulin, \( \gamma \) globulin, transferrin. The results indicate that one or more non-dialyzable constituents of serum are necessary for the incorporation in \( \text{in vitro} \), and their identity is under investigation.

Other Tissues—Slices of various tissues were prepared from untreated and iron-treated rats (40 \( \mu g \) of iron per g of rat, intravenously, 18 hours before death) and tested in \( \text{in vitro} \) as described above. Prior iron increased incorporation of \( _{14}C \)-leucine into apoferititin in a number of tissues as indicated in Fig. 4. Spleen responded as well as liver and testis responded almost as well, whereas with slices of duodenum and kidney the increments in incorporation were only approximately 10 to 15% of that observed with liver. In additional experiments rat heart muscle showed no effect of prior iron.

Effects of Actinomycin D—Prior evidence shows that when actinomycin D is administered before iron the usual response of increased incorporation of \( _{14}C \)-leucine into apoferititin by liver slices in \( \text{in vitro} \) is inhibited (4, 6). The following studies indicate that actinomycin D also decreases net synthesis of apoferititin in \( \text{in vivo} \) following iron. In each experiment a group of four rats were given either 2 or 4 \( \mu g \) per g of rat of actinomycin D intravenously followed 30 min later by 40 \( \mu g \) of iron per g of rat as iron dextran, intravenously. Additional groups of four rats received either vehicle alone or iron dextran at the appropriate times. Four hours after iron the liver was removed, homogenized, and assayed immunochemically for ferritin as described under "Experimental Procedure." The apoferititin content of rat liver was increased by iron dextran in each of the five experiments summarized in Table III, and actinomycin D inhibited this response by 39.9 to 83.7% (mean 55.1). To determine whether actinomycin D in the dosage used acted indirectly via stimulation of the adrenal cortex, rats were subjected to bilateral adrenalectomy and the preceding experiment was repeated 24 hours thereafter. The results were comparable to those observed with intact animals.

The effects of actinomycin D administered after iron dextran were also studied. Rats were dosed with iron dextran as described in the preceding paragraph, and 14 or 18 hours thereafter actinomycin D, 4 \( \mu g \) per g of rat, were administered intravenously to half of the group in each experiment. Actinomycin treated and untreated animals were killed in groups of four at intervals thereafter, and liver slices were prepared and tested for \( _{14}C \)-leucine incorporation into apoferititin in \( \text{in vitro} \). The values for actinomycin-untreated animals shown in Fig. 5 indicate that the stimulatory effect of iron dextran ended at approximately 16 to 18 hours, in confirmation of prior results (6). Thereafter, the capacity to incorporate \( _{14}C \)-leucine into apoferititin decreased exponentially with a half-life of approximately 12 hours. Following actinomycin, in contrast, the capacity to incorporate in \( \text{in vitro} \) decreased promptly and exponentially with a half-life of approx...
approximately 5.6 hours (Fig. 5). The results suggest several conclusions. First, inasmuch as cellular uptake of iron dextran and the stimulation of ferritin synthesis has reached its peak by 18 hours after intravenous injection, the effect of actinomycin D subsequent to this time suggests that the inhibitor does not act merely by blocking iron uptake. Second, the exponential fall in capacity to incorporate \(^{14}\text{C}\)-leucine following 4 \(\mu\text{g}\) per g of body weight of actinomycin D suggests the gradual depletion of some essential component in the biosynthetic pathway, rather than general, nonspecific inhibition of protein synthesis (3) which would decrease the synthetic capacity abruptly. It is reasonable to suspect that messenger RNA is the rate-limiting component depleted after actinomycin, and that the messenger RNA for apoferritin may have a half-life of approximately 6 to 6 hours.

Although the studies with actinomycin D are in accord with the hypothesis that iron stimulates apoferritin synthesis via increased formation of messenger RNA, definitive proof is not provided. Clearly one cannot exclude actions of the inhibitor in addition to the well established block in DNA-dependent synthesis of RNA. Accordingly, the effects of iron on incorporation of \(\text{erotic acid into rapidly labeled RNA were examined.}^{14}\text{C\)-Orotic Acid Incorporation into Rapidly Labeled RNA—}\)

Groups of four rats were given either iron dextran (40 \(\mu\text{g}\) of iron per g of body weight) or, in one experiment, ferrous sulfate (10 \(\mu\text{g}\) of iron per g of body weight) intravenously. Four and one-half or 18 hours thereafter untreated controls and iron-treated animals were injected intravenously with \(^{14}\text{C\)-orotic acid (5 \(\mu\text{C} / 100 \text{ g of body weight)}, and 10 min later the livers were removed and nuclear and cytoplasmic RNA were prepared by the method of Dingman and Sporn (12). Final preparations were assayed for RNA with the orcinol method (13), and aliquots in 0.1 N NaOH were added to Bray’s solution for liquid scintillation spectrometry. The values for incorporated \(^{14}\text{C per mg of RNA in six experiments are listed in Table IV. No reproducible effect of iron was observed on pulse-labeled RNA isolated from the cytoplasm. In contrast, iron increased the incorporation into nuclear RNA in each of the experiments, and the increments ranged from 3.7 to 106.0\% (mean 52.6).}

Other Effects of Iron—Although the preceding results support the hypothesis that iron stimulates apoferritin synthesis via increased formation of messenger RNA, additional effects of iron are not excluded. Drysdale and Munro (3), for example, have proposed that iron acts primarily by stabilizing some intermediate in the biosynthesis subsequent to the synthesis of messenger RNA. To test the latter hypothesis we have attempted to show stimulation of ferritin synthesis by direct addition of iron to liver slices in vitro. If the biosynthesis in vitro is similar to that in vivo, and if liver cells take up iron from an ambient medium, direct addition should increase ferritin formation according to the stabilization hypothesis. Additional evidence that ferritin synthesis in vitro is qualitatively similar to that in vivo was obtained by estimating the incorporation of \(^{14}\text{C\)-leucine into an iron-poor and an iron-rich pool of ferritin. In intact rats iron administration stimulates the synthesis of ferritin molecules of relatively low iron content (2, 3), and a similar result was obtained for the synthesis in vitro. Liver slices prepared from groups of four control and iron-treated rats were tested for incorporation of \(^{14}\text{C\)-leucine into total ferritin as described previously. In addition, aliquots of the supernatant solutions obtained after treatment at 70\° for 10 min (see “Experimental Procedure”) were mixed with 1.0 mg of horse apoferritin as carrier and centrifuged as described under “Experimental Procedure” to separate the iron-poor (supernatant) and iron-rich (pellet) fractions. Ferritin in the supernatant solutions was precipitated immunochemically, the \(^{14}\text{C was estimated, and values for incorporation into iron-poor ferritin were calculated from the known distribution of iron

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Iron compound</th>
<th>Time after iron</th>
<th>(^{14}\text{C Incorporation into RNA}</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>18</td>
<td>0.5</td>
<td>0.7 (cpm x 10^6/mg)</td>
</tr>
<tr>
<td>2</td>
<td>Iron dextran</td>
<td>18</td>
<td>0.5</td>
<td>0.6 (cpm x 10^6/mg)</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>18</td>
<td>0.8</td>
<td>0.6 (cpm x 10^6/mg)</td>
</tr>
<tr>
<td>4</td>
<td>Iron dextran</td>
<td>18</td>
<td>1.0</td>
<td>0.9 (cpm x 10^6/mg)</td>
</tr>
<tr>
<td>5</td>
<td>None</td>
<td>18</td>
<td>1.6</td>
<td>1.0 (cpm x 10^6/mg)</td>
</tr>
<tr>
<td>6</td>
<td>Iron dextran</td>
<td>4(|$</td>
<td>0.3</td>
<td>0.5 (cpm x 10^6/mg)</td>
</tr>
</tbody>
</table>

**Table IV**

Effect of iron administration on incorporation of \(^{14}\text{C\)-orotic acid into rapidly labeled liver RNA

Groups of four iron-treated and control rats were used in each experiment. Livers were removed 10 min after intravenous injection of \(^{14}\text{C\)-orotic acid and pooled in each group, and RNA prepared and assayed as described in the text. FeSO\(_4\), was administered in a vehicle of 50\% rat serum-50\% isotonic saline.

![Fig. 5. Effect of actinomycin D administered 14 or 18 hours after iron dextran on the capacity of liver slices in vitro to incorporate \(^{14}\text{C\)-leucine into ferritin. At 14 or 18 hours after iron dextran, groups of four rats were given either actinomycin D (\(\bullet\)) or vehicle alone (\(\bigcirc\)) and the livers were tested at various times after the latter injection. Values for \(^{14}\text{C\)-leucine incorporation into ferritin by liver slices as a function of time are plotted semilogarithmically. Results in the actinomycin-treated groups were independent of when iron dextran was given and are plotted together. Results in the groups not treated with actinomycin are plotted separately for rats given iron dextran 14 hours (Curve A) or 18 hours (Curve B) before the experiment.](http://www.jbc.org/content/291/36/2915)
poor rat ferritin ("Experimental Procedure"). Table V lists the results of six experiments. Prior iron treatment increased the pool of iron-poor ferritin labeled in vitro from 3- to 20-fold, and the increment in this pool accounted for 39.9 to 72.3% (mean 53.5) of the increase in total ferritin labeled in vitro.

Evidence that liver slices take up iron in vitro has been provided by prior investigators (14, 15) and confirmed in the present studies. FeSO₄ was added to the ambient medium, and following incubation washed liver slices contained radioactivity. Moreover, in three experiments identical with those described in the preceding paragraph, FeSO₄ and carrier FeSO₄ were added to liver slices in the usual ambient medium (final concentrations of iron were 10 and 40 mmaries per ml in one and two experiments, respectively). Iron-poor and total ferritin were isolated immunochemically following 3 hours of incubation, and the ⁵⁹Fe content was estimated. From 1.3 to 20% of the iron added was recovered in the isolated ferritin, with 7 to 8% of this in the iron-poor fraction and the remainder in the iron-rich moiety. The results indicate, therefore, that iron is taken up by liver slices and incorporated into ferritin.

The effects of adding iron directly to the medium on incorporation of ¹⁴C-leucine into ferritin were studied in 12 experiments. Iron was added as ferrous ascorbate, FeCl₂, or iron dextran in amounts yielding 0.5 to 25 μg of iron per ml; liver and spleen slices were tested; incubations were carried out as long as 5 hours. No consistent increase in ferritin synthesis was observed.

**DISCUSSION**

The genes involved in ferritin synthesis and its regulation appeared relatively early in evolutionary history, for proteins of the ferritin type have been found in such diverse organisms as urodeles (16), plants (17), mollusks (18), and mammals (19). Synthesis of the protein moieties of these compounds is generally stimulated by iron in the environment and apparently represents an adaptive response necessary to store the mineral in a non-toxic form. The present studies indicate that stimulation of this synthesis in rat liver is via increased formation of messenger RNA during a 10-min pulse is significantly increased by iron. Confirmatory evidence has come from experiments with actinomycin D which significantly inhibits the response to iron as tested either by incorporation of ¹⁴C-leucine into ferritin by liver slices in vitro or by estimations of liver ferritin content in vivo. Drysdale and Munro (3) have objected to the use of actinomycin D, particularly in doses exceeding 1.5 μg per g of body weight, because it may have nonspecific inhibitory effects on protein synthesis. Nonspecificity of action is a generally valid objection to inhibitor studies. In the present experiments, on the other hand, the effect of actinomycin was observed with as little as 1 μg per g (6). Moreover, failure of the prior investigators (3) to detect inhibition could well have been due to insensitivity in their detection method. When administered 14 to 18 hours after iron in the present studies, actinomycin D exponentially decreased the capacity of liver slices to synthesize labeled ferritin. The observation seems best explained by the gradual depletion of an intermediate required in the biosynthesis, most likely messenger RNA.

The three lines of evidence presented above, i.e. the effects of actinomycin D, the stimulation of incorporation of ¹⁴C-orotic acid, and the failure to observe increased ferritin synthesis on addition of iron in vitro, taken together support the conclusion that gene activation is involved in the stimulation of ferritin synthesis by iron. Nonetheless, the conclusion remains a working hypothesis, for alternative explanations could be offered for each line of evidence. Thus actinomycin D might act indirectly in vivo, although the present studies indicate that an indirect effect would not be mediated by the adrenal glands. The enhancement of incorporation of ¹⁴C-orotic acid could be nonspecific. Finally, failure to observe an effect of iron added in vitro might simply result from improper experimental conditions. More definitive proof awaits the development of a satisfactory method in vitro for studying the stimulation of ferritin synthesis by iron. The present evidence also does not exclude additional effects of iron, e.g. on the translation of messenger RNA or on the stability of subunit intermediates in the biosynthesis, as previously suggested (3). Such mechanisms to account for the stimulatory effects of iron seem less likely, however, in view of the failure thus far to observe stimulation by addition of iron in vitro, although it appears that some iron penetrates the liver cells. One simple hypothesis seems definitely excluded: that iron increases net synthesis merely by converting iron-poor to iron-rich ferritin. Prior studies in vivo (2, 3) and the present observations in vitro show that iron markedly increases the pool of iron-poor ferritin.

A number of features indicate that ferritin formation provides a particularly useful pathway for the study of the regulation of protein synthesis. The synthesis is readily increased by many iron compounds, the end product is a well characterized protein, and the process can be studied, partially at least, in vitro, in which both net synthesis and incorporation of a labeled amino acid are observed. The present results indicate that the regulation involves gene activation or derepression and support the expectation that further studies may lead to molecular descriptions of these fundamental mechanisms.

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