A Rapid Assay for Evaluation of Iron-Chelating Agents in Rats

By Martin J. Pippard, David K. Johnson, and Clement A. Finch

The animal assay of potential new iron-chelating agents is at present dependent on cumbersome and imprecise iron balance studies in hypertransfused rodents. We report the development of a radioisotope assay in intact rats based on the transient labeling by ferritin Fe of the main source of chelatable iron within hepatocytes. The isotope was maximally available to chelators during the first 6 hr after its injection, nearly all the excretion being in the bile. The bile Fe/total iron ratio was independent of both the chelator and its dose. However, in iron-loaded rats, the ratio was reduced, and the isotope excretion was a less sensitive measure of intrahepatic chelation. In the proposed assay, test chelators were given to normal rats 2 hr after an intravenous injection of Fe-ferritin. Four hours later, the radioiron in the liver and in the gut gave a sensitive measure of the mobilization of hepatic ferritin to the bile. In addition, chemical iron determinations identified a small alternative source of urinary chelate with agents known to promote urine excretion in man. The assay gave a rapid and precise screen for chelators given by parenteral and oral routes.

The inability of man to excrete iron through physiologic mechanisms in the presence of toxic iron overload has stimulated a search for iron-chelating compounds for use in the treatment of iron-loading anemias. Only desferrioxamine (DF) and diethyleneetriaminepentaacetic acid (DTPA) have been used to any great extent in man. Toxicity discouraged the clinical use of DTPA while DF, though highly specific for iron, is expensive and has to be given by continuous parenteral infusion to be most effective. There is therefore a continuing search for alternatives, particularly agents effective when given by mouth.

In the investigation of potential iron chelators, a preliminary screen for biologic activity is required. In most studies, hypertransfused rats or mice have been used. Assessment of test compounds has involved time-consuming and inherently imprecise metabolic balance studies, combined in the mouse model with a measurement of tissue iron depletion. An alternative in vitro assay based on the inhibition of iron uptake and ferritin synthesis in cultures of Chang liver cells has been proposed as an initial screen. However, an in vivo model is required to establish the pharmacologic activity of the chelator, including absorption from the gut, in the intact organism.

A radioiron label for the source of chelatable iron could greatly simplify the monitoring of iron excretion, particularly when this is in the feces. Such a radioassay should reflect total iron mobilization and also take account of possible differences in the sources and routes of excretion of chelatable iron with different compounds. There is evidence that the hepatocyte is an important source of chelatable iron, since several agents (including DF) increase excretion of hepatocyte radioiron into the feces. However, this is not seen with DTPA, and it has been suggested that an alternative source of chelatable iron, excreted in the urine after both DTPA and DF, may be available only in hypertransfused rats. This raises the further question of whether the experimental animals need preparatory iron loading in order to reflect more closely the behavior of iron-loaded patients.

The present studies explore these questions by comparing the action of a variety of chelating agents with that of DF. After ferritin radioiron labeling of the hepatocyte, radioiron chelation and its relationship to total iron excretion in the bile and urine has been determined in both normal and iron-loaded rats. This has led to the development of a simple and rapid assay procedure that has proved effective in monitoring the ability of various chelators to mobilize iron.

MATERIALS AND METHODS

Female Sprague-Dawley rats weighing between 180 and 220 g were used. "Normal" rats were fed a regular Purina rat chow diet containing 380 mg of iron/kg, and "iron-loaded" rats were produced by feeding a diet containing 10 g of iron/kg in the form of ferrous sulfate. This iron-fortified diet was fed 5 days in every 7 for a period of 5-6 wk. A regular diet was given on 2 days of each week to permit a normal growth pattern. In all studies, food was removed during the night before each experiment, although free access to water was permitted. Stainless steel individual metabolic cages washed with iron-free water and sprayed with Teflon were used for urine and feces collections.

In some studies, bile cannulation was carried out under Innovar...
Flot a anesthesia using PE 50 tubing inserted into the bile duct above the pancreas. During collection of bile, Innovar anesthesia was maintained and fluid replacement equal to the bile drain was given by subcutaneous injection of Ringer’s saline. At sacrifice, evisceration from the abdominal aorta was followed by perfusion with 20 ml of normal saline before removal of organs for gamma counting.

**Ferritin Preparation**

This was based on the method of Bjorklid and Helgeland. Male rats of approximately 400 g were given a single intraperitoneal injection of 12 mg iron dextran. After 1 wk, they received 2 ml of a 0.1 mlof the diluted Innovar as necessary.

**Iron Determination**

Suitable dilutions of all chelators were made in 0.9% sodium chloride to give a final dose volume of 0.5 ml. DF (desferrioxamine mesylate) was provided in 500 mg ampules as a lyophilized powder (CIBA Pharmaceuticals, Summit, N.J.) Solutions of pyridoxal isonicotinoyl hydrazone (PIH) were prepared using 1 M sodium hydroxide in 0.9% sodium chloride and adjusted to pH 10 with 1 M hydrochloric acid prior to the injection. Ethylenediaminebis (hydroxyphenylactic acid), (EDBHA)* was dissolved in the same manner and injected at pH 8. Diethylenetriaminepentaacetic acid (DTPA) and 2,3-dihydroxybenzoic acid (DHB) (obtained from Aldrich Chemical Co., Milwaukee, Wisc.) were injected at pH 8.0 and 7.1, respectively.

*Innovar-Vet (0.4 mg fentanyl) and 20 mg droperidol/ml, supplied by Pitman-Moore, Inc., Washington Crossing, N.J.) 0.1 ml of a 1:10 dilution in water/100 g given 1 M, with repeated injections of 0.1 ml of the diluted Innovar as necessary.
Fe-Ferritin Labeling of the Hepatocyte

The rate of disappearance of 59Fe-ferritin injected into the lateral tail vein of groups of 4 normal rats was measured by serial sampling of 0.1 ml of blood from a different tail vein. The clearance was found to be dose dependent. With doses of ferritin iron of 1.5, 7.5, 15, and 50 μg, the amounts remaining in circulation at 20 min were 1.6% ± .2%, 18.1% ± 1.5%, 31.3% ± 4.5%, and 56.9% ± 2.6%, respectively. With the 7.5-μg dose there was no identifiable radioactivity in circulation at 2 hr, when 94.2% ± 1.2% of the label was in the liver. There was only a 6% spontaneous loss of hepatic radioactivity over the following 24 hr (Fig. 2). In subsequent studies, the dose of 7.5 μg of ferritin radioiron was used since it was cleared rapidly and provided sufficient radioactivity to monitor chelator induced excretion.

Excretion of Ferritin 59Fe After DF

Five groups each of 4 normal rats were given i.v. 59Fe ferritin and 2 hr later an i.m. injection of 40 mg (200 mg/kg) of DF. Groups were sacrificed serially from 0 to 4 hr after the DF and the radioactivity in the liver, gut and contents, and urine, together with total iron in the urine, was determined (Table 1). Very little further mobilization of 59Fe from the liver was seen after 3 hr when 40% of the injected counts were in the gut. Both total and radioiron excretion in the urine continued to increase during the fourth hour.

In order to clarify the meaning of the gut radioactivity, 2 additional groups of animals were anesthetized with Innovar and given 40 mg i.m. DF at 2 hr after i.v. 59Fe-ferritin. In one group, gut radioactivity was determined at 6 hr as 49% ± 1.7% of the injected counts. In the other group, bile cannulation showed bile 59Fe excretion to be almost identical at 51.9% ± 1.4%. As well as indicating the validity of radioactivity in the gut as a measure of bile radioiron excretion, this study shows an enhanced excretion of 59Fe with Innovar anesthesia. This was associated with a prolongation of the time DF was detectable in bile and blood (unpublished data) and is probably the result of a delay in release from the i.m. injection site.

RESULTS

Values for groups of rats are expressed as the mean ± 1 SEM.

Table 1. Duration of Action of i.m. Desferrioxamine (40 mg) Given 2 hr After i.v. 59Fe-Ferritin.

<table>
<thead>
<tr>
<th>Time (h) After Desferrioxamine</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>59Fe (% of Injected Counts)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>94.2 ± 1.2</td>
<td>75.7 ± .9</td>
<td>60.7 ± 1.2</td>
<td>55.3 ± 2.7</td>
<td>53.9 ± 2.12</td>
</tr>
<tr>
<td>Gut and contents</td>
<td>1.6 ± .2</td>
<td>21.0 ± 1.0</td>
<td>34.9 ± 1.1</td>
<td>40.4 ± 2.8</td>
<td>41.1 ± 1.39</td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td>.20 ± .03</td>
<td>.63 ± .03</td>
<td>.75 ± .06</td>
<td>.98 ± .07</td>
</tr>
<tr>
<td>Total iron (μg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td>1.7 ± .1</td>
<td>6.8 ± 1.5</td>
<td>7.7 ± .6</td>
<td>9.3 ± .9</td>
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</table>

Results are the mean ± 1SEM for groups of 4 normal rats sacrificed at hourly intervals after the chelator injection.
The absence of any significant gastrointestinal reabsorption of bile $^{59}$Fe was shown in a further study. The radioactivity in the gut and contents of a group of 5 animals was 39.3% ± 2.0% 4 hr after DF. This was almost identical to the amount found in a 72-hr fecal collection (38.1% ± 2.8%) in a parallel group of animals. The corresponding figures for a similar study using PIH (17.5 mg i.v.) were 38.0% ± 5.4% in the gut and 33% ± 7.2% in the feces. No counts appeared in the feces during the first 4 hr after DF or PIH. In the periods 0–24, 24–48, and 48–72 hr after DF, the respective amounts of fecal radioiron excretion were 6.6% ± 4.3%, 28.2% ± 3.6%, and 3.3% ± 1.0% of injected counts.

**Time Relationship Between $^{59}$Fe-Ferritin and Chelator Injection**

At variable time intervals from 0 to 24 hr after the i.v. injection of 7.5 μg of $^{59}$Fe-ferritin, different groups of animals were given injections of 40 mg of DF, 35 mg of PIH, or saline as a control. The chelator doses represented equivalent iron binding capacities. The animals were sacrificed 4 hr after the chelator injection, by which time chelating activity would be complete. A separation of 1–2 hr between ferritin and chelator injections gave maximum availability of the $^{59}$Fe label to both chelators (Fig. 2). The increase in gut counts was accompanied by a decrease in liver counts of equal magnitude. The maximum percentage available to PIH (70%) was greater than the maximum available to DF (40%). At no time did urinary $^{59}$Fe exceed 1% of the injected counts. By 6 hr, relatively little iron was available to either chelator.

**Effect of Iron Stores**

The effect of differences in liver iron stores on the chelation or radioiron was examined in normal and iron-loaded rats prepared with i.v. $^{59}$Fe-ferritin (Fig. 3). The mean liver iron content of the normal rats was $0.85 ± 0.06$ mg ($n = 26$) and of the iron-loaded rats $3.98 ± 0.59$ mg ($n = 18$). Separate groups of animals received varying doses of i.m. DF (5–160 mg) at the interval of 2 hr already shown to be the time of maximum availability of the ferritin $^{59}$Fe. Mobilization of hepatic $^{59}$Fe to the gut was monitored 3 hr later. At the lower DF doses, $^{59}$Fe excretion was much greater in normal than in iron-loaded animals. However, the normal animals reached a maximum level of excretion (43%) at a dose of 40 mg DF, whereas in iron-loaded animals the excretion continued to increase to a maximum of 34% at a dose of 80 mg DF. Radioiron excretion was therefore less sensitive as an indicator of chelator activity within hepatocytes in iron-loaded compared with normal animals.

In order to investigate the mechanism of this phenomenon further, a similar experiment was carried out under Innovar anesthesia in which a comparison was made between total iron and $^{59}$Fe excretion in the bile (Fig. 4). Within each group of normal and iron-loaded animals, the ratio between $^{59}$Fe and total iron excretion (the specific activity) was constant, despite the wide range of total iron excretion produced by the different doses of DF. However, the mean of the individual specific activities (% $^{59}$Fe/μg total iron) in iron-loaded rats (0.11 ± 0.007) was conspicuously lower than in normal animals (0.48 ± 0.016). The
Fig. 5. Biliary excretion of total and radioiron in response to 3 different chelators. DF (desferrioxamine, 40 mg), EDBHA (ethylenediaminebis[hydroxyphenylacetic acid], 21 mg), and PIH (pyridoxal isonicotinoyl hydrazone, 35 mg) were given by i.m. injection 2 hr after i.v. $^{59}$Fe-ferritin at 0 hr. Results are the mean ± 1SEM for 5 normal rats in each group.

inverse relationship between bile-specific activity and liver iron content suggests that the ferritin $^{59}$Fe was uniformly labeling a tissue-chelatable iron pool the size of which was dependent on liver iron stores.

Isotope Versus Total Biliary Iron Using Different Chelators

Studies were carried out employing three different chelators in which the radioactivity of hourly fractions of bile was compared with the total iron content (Fig. 5). An i.m. injection of the chelator was given to anesthetized normal rats 2 hr after the injection of $^{59}$Fe-ferritin. The patterns of $^{59}$Fe and total iron excretion were similar, though the specific activity of the bile declined with each succeeding fraction, consistent with the decreasing availability of $^{59}$Fe with time already documented. Despite large differences in the total iron excretions, the specific activities of the first postchelator bile fractions were $0.49\% ± 0.08\%$, $0.47\% ± 0.09\%$, and $0.41\% ± 0.15\%$ $^{59}$Fe/$\mu$g of total iron using DF, EDBHA, and PIH, respectively, there being no significant differences between these values.

Assay Results

Utilizing the assay procedure outlined under Materials and Methods, a group of chelators already known to have variable effects in respect to iron excretion were assayed in doses equivalent to 40 mg DF. Fifteen to 20-fold increases in gut $^{59}$Fe excretion were obtained with DF, EDBHA, and PIH, with only small variations between animals in each group (Table 2). These changes were accompanied by a reciprocal decrease in liver radioactivity. These three chelators also produced 5–10-fold increases over control levels in urinary chemical iron excretion. DTPA was active in promoting urinary excretion but did not affect $^{59}$Fe excretion into the gut. DHB had no detectable activity by either route. The maximum urinary radioiron excretion was $0.6\%$ with DF. The mean urine specific activities with DF, EDBHA, and PIH were $0.056\%$, $0.072\%$, and $0.016\%$ $^{59}$Fe/$\mu$g of total iron, respectively, much less than those previously found in the bile after the same chelators (see above).

A second study was carried out in which different routes of chelator administration were compared using DF and PIH in doses equivalent to 20 mg of DF. With PIH all parenteral routes were equally effective, whereas with DF, the i.v. route gave less excretion. Of particular interest, however, was the demonstration that when given by stomach tube, PIH had about 50%
The latter was most easily measured by determining decline in liver radioactivity provided a measure of excretion and with different chelating agents suggests current experiments, the S9Fe was chelated while in over the following 24 hr in normal rats. However, this developed in which iron chelation within parenchymal chelatable iron in the rat using 59Fe-ferritin. As a result, a rapid screen for chelator activity has been developed in which iron chelation within parenchymal liver cells is sensitively detected by the mobilization of radioiron.

Rat 59Fe-ferritin is cleared almost exclusively by rat liver parenchymal cells,12,16 and in the present studies, virtually no release of 59Fe to other tissues occurred over the following 24 hr in normal rats. However, this radioiron was highly susceptible to chelator action when the time relationship between ferritin and chelator injections was optimal. The “time window” of maximal availability to chelator was shown to be between the second and sixth hour after 59Fe-ferritin injection. Previous work using ferritin dually labeled by 59Fe and 14C-leucine16 indicated that by 4 hr after its injection, over 80% of the 59Fe taken up by the liver had been separated from the protein shell. The 59Fe was subsequently rapidly incorporated into endogenous ferritin. It therefore seems probable that in the current experiments, the 59Fe was chelated while in transit within the hepatocyte and that after its storage in endogenous ferritin it became relatively unavailable.

The close relationship between bile radioiron and total iron excretion over a wide range of amounts of excretion and with different chelating agents suggests that the 59Fe was uniformly labeling a “physiologic” pool of chelatable iron within the hepatocyte. The decline in liver radioactivity provided a measure of total iron mobilized from this pool, and the bile radioiron a measure of the amount excreted by this route. The latter was most easily measured by determining the radioactivity in the gut and contents with which there was close agreement. A comparison of the DF-induced excretion with that seen after PIH does suggest that there may be important differences in the degree of penetration of the physiologic pool by different chelators. The 59Fe (and total iron) excretion with 35 mg PIH was considerably greater than the maximum obtainable with DF even when the latter was given in the very large doses shown in Fig. 3. One possible reason for this effect might be that DF is less evenly distributed within the hepatocyte than PIH and that certain parts of the chelatable pool are consequently unavailable to the drug. An alternative possibility is that the concept of “iron in transit” really represents a graded phenomenon, such that iron from ferritin becomes steadily more available during the process of ferritin catabolism before declining in availability as it is reincorporated in new ferritin molecules. PIH might then be active over a wider range of the spectrum of transit iron, giving rise to greater chelation with this agent than with DF during each of the time frames shown in Fig. 2.

With the chelators tested, nearly all the 59Fe was excreted via the bile. However, it is conceivable that a future test compound might chelate iron from the 59Fe-labeled pool with subsequent excretion in the urine. For this reason the 4-hr urine radioactivity was included as a measure in the proposed assay procedure. The simultaneous measurement of the total chemical iron excretion in the urine identified a relatively small additional source for chelatable iron that was not labeled by the 59Fe. There are a number of possible candidates for this additional source. Previous experiments with radioiron-labeled heat-damaged red cells suggested the reticuloendothelial (RE) cells as one possibility.10,17 However, there are considerable problems in interpreting these studies due to the evidence of redistribution to parenchymal tissues during the time of exposure to the chelators. Other possibilities include a second form of transit iron related to transferrin iron exchange and hemoglobin catabolism in the hepatocyte (unpublished data) and the kidney itself. However, for the purposes of the assay, the important feature is that those agents known to produce urinary excretion in man (DTPA, EDBHA, and DF18-20) could be identified in normal

### DISCUSSION

These studies have demonstrated the possibility of transiently labeling the main intrahepatocyte source of chelatable iron in the rat using 59Fe-ferritin. As a result, a rapid screen for chelator activity has been developed in which iron chelation within parenchymal liver cells is sensitively detected by the mobilization of radioiron.

<table>
<thead>
<tr>
<th>Route of Administration</th>
<th>DF</th>
<th>PIH</th>
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<tr>
<td></td>
<td>59Fe (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>Gut and Contents</td>
</tr>
<tr>
<td>i.v.</td>
<td>71.3 (±0.9)</td>
<td>21.3 (±0.7)</td>
</tr>
<tr>
<td>i.m.</td>
<td>62.4 (±1.4)</td>
<td>33.9 (±1.1)</td>
</tr>
<tr>
<td>i.p.</td>
<td>63.0 (±1.4)</td>
<td>33.0 (±1.4)</td>
</tr>
<tr>
<td>p.o.</td>
<td>93.9 (±0.5)</td>
<td>2.6 (±0.2)</td>
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</tbody>
</table>
rats without the necessity for preparatory iron loading.

In currently available assays it has been generally assumed that iron-loaded animals are essential in order to produce a model similar to that of iron overload in man. However, even with hypertransfusion there remain important differences, of which the most striking is the much greater proportion of iron mobilized by DF to the stool in rodents (80%–90%) compared with iron-loaded man (30%–50%). Part of this difference may relate to the particular pathophysiology underlying the iron loading in man, where the proportion excreted in the urine is directly related to the level of erythropoiesis, and where in the absence of any increase in plasma iron turnover, the fecal excretion can exceed that in the urine by as much as 3 times (unpublished data). In man, increased parenchymal iron stores and not RE stores are correlated with both tissue iron toxicity and an increase in chelatable iron. Since dietary iron loading carried out in this study produces a more direct increase in hepatic parenchymal iron stores than do red cell transfusions, it might be expected to produce a more satisfactory model for human iron overload. However, with the proposed isotope assay, enlarging the intrahepatic chelatable iron pool reduces its specific activity, and thus the amount of isotope excreted by a given dose of chelator (Fig. 3). We therefore believe that animals with normal stores are most satisfactory as well as more convenient to use in the proposed assay. In order to circumvent the effects of unsuspected variations in iron stores of test animals, DF controls may be employed using a standard dose for comparison with the test compound.

The usefulness of the assay is probably best judged from the results obtained in evaluating chelators of known effectiveness. The marked increases in both gut radioactivity and urine iron with DF, EDBHA, and PIH are consistent with their known ability to mobilize iron in man or in existing models. DTPA was without effect on biliary excretion. Its effect on fecal iron in man is not known, but it does produce a considerable increase in urine excretion in iron-loaded patients. It was therefore of interest that at 2 dose levels DTPA produced fourfold and 17-fold increases in urine iron in the present assay. The latter figure compares favorably with the 2–3-fold increase seen in the hypertransfused mouse at a comparable dose (200 mg/kg). One of the few chelators tested in all the available assays is DHB. It was totally inactive in the present assay, the hypertransfused mouse, and in Chang cells unless previously incubated with a liver homogenate. Though DHB consistently increased urine iron excretion in the hypertransfused rat, it has been disappointing in clinical use where it produces only a marginal increase in fecal excretion. Of particular interest was the ninefold increase in gut radioactivity with oral PIH, confirming the findings of a previous balance study and indicating the potential use of the assay in the screening of orally active agents.

Compared with this assay, existing models using hypertransfused rodents have a number of disadvantages in addition to those inherent in prolonged iron balance studies and the time needed to prepare the test animals. There is considerable variation in the absolute amounts of fecal iron excretion between different groups of animals. In addition, increases in stool iron of only 20% over a baseline control period have been considered significant. These considerations allow at best only a semiquantitative grading of activity of each chelator. A further variable is that the level of iron excretion observed may be dependent on the timing of drug administration in relation to the transfusion schedule. The isotope model is much more sensitive and reproducible, suggesting that in addition to the advantages of simplicity and speed it may allow more precise correlations to be made between structure and function within the main groups of iron-chelating agents.

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