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 diethyltriaminepentaacetic 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-
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, exsanguination 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 packed red cells/100 g, followed 2 days later by the intravenous (i.v.) injection of a trace amount of ferrous sulfate in pH 2.0 saline containing 200 μCi of 59Fe. The animals were sacrificed by exsanguination from the abdominal aorta 24 hr after injection of the radiolabel and the livers were removed for ferritin isolation. Half saturation with ammonium sulfate was used to precipitate the ferritin from the heat supernatant of a liver homogenate prepared in 0.25M acetate buffer (pH 4.8) and exposed to 73°C for 10 min. The ferritin precipitate was redissolved in acetate buffer and purified by ultracentrifugation (66,000 g for 2 hr) in a Beckman centrifuge (Model L). This was repeated on two occasions, the second being from a solution in phosphate-buffered saline in which the final precipitate was also dissolved. The ferritin iron concentration was determined after overnight incubation in 3N HCl at 37°C, using the colorimetric method described below. Further dilutions in phosphate-buffered saline in which the final precipitate was also dissolved. The ferritin iron concentration was determined after overnight incubation in 3N HCl at 37°C, using the colorimetric method described below. Further dilutions in phosphate-buffered saline in which the final precipitate was also dissolved. The ferritin iron concentration was determined after overnight incubation in 3N HCl at 37°C, using the colorimetric method described below. Further dilutions in phosphate-buffered saline in which the final precipitate was also dissolved. The ferritin iron concentration was determined after overnight incubation in 3N HCl at 37°C, using the colorimetric method described below.

**Processing of Samples for γ Counting**

Radioactivity in the liver, gut and contents, feces, blood, residual carcass, and urine, and in the bile cannulation experiments of bile, was determined using a Packard Autogamma Model 5330 scintillation spectrometer. The gut was divided into 6 separate counting tubes and the liver into 2. Tails and residual carcasses were counted separately in a Packard Model 3004 large volume counter with corrections for differences in geometry. The tail counts provided a check for the absence of any extravasation at the time of injection of the 59Fe ferritin. The percentage of the total injected counts present in each organ was standardized to a total recovery of 100%.

**Chemical Iron Determination**

Urine and bile iron content was measured colorimetrically using bathophenanthroline sulfonate. The thiglycolic, hydrochloric, and trihydroxyacetone reagent used in the ICSH recommended method14 for serum iron determination was used to reduce the iron of the chelator complex. In addition, any proteins present in the bile and occasional urine samples were precipitated by this reagent. After centrifugation to remove protein precipitant and/or urinary sediment, 2 aliquots of the supernatant were removed. The chromagen solution from the ICSH method (bathophenanthroline sulfonate in sodium acetate buffer) was added to 1 aliquot, and sodium acetate buffer alone (sample blank) to the other. The optical density of the sample, its blank, suitable standards, and a reagent blank were measured at 535 nm. Results using this method showed an excellent recovery (97.1% ± 7.1% SD) with respect to those obtained by using more cumbersome preparatory wet acid digestion. Liver nonheme iron was determined in tissue aliquots weighing 0.5–1 g using the modification of the HCl digestion method of Kaldor15 in which colorimetric assay was carried out using bathophenanthroline sulfonate.

**Preparation of Iron Chelators**

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-dihydroxymonoic acid (DHB) (obtained from Aldrich Chemical Co., Milwaukee, Wisc.) were injected at pH 8.0 and 7.1, respectively.

The standard assay procedure developed as a result of background studies is shown in Fig. 1. Groups of 6 normal female Sprague-Dawley rats weighing between 180 and 220 g with hepatic iron stores of 1.03 (±0.06 SEM) mg (n = 18) were fasted overnight. On the following morning the assay began with an injection in the tail vein of 7.5 μg of iron as 59Fe ferritin. Two hours later, 4 of the animals received an intramuscular (i.m.) injection of the test chelator in an amount equivalent to the iron binding capacity of 40 mg of DF. This dose was selected as the amount of i.m. DF that was just sufficient to give maximal chelation of hepatic radioiron in normal rats (see Results), but alternative routes and doses were used under special circumstances. DPTA was injected into the peritoneal cavity to avoid severe muscle swelling. In a comparison of 4 different routes of administration, a dose equivalent to 20 mg DF was used, since it was the maximum amount of this drug that was tolerated by intravenous (i.v.) injection. Two control rats were included in each assay. One received no chelator and allowed confirmation of the virtually exclusive liver uptake of the 59Fe-ferritin. The other received an i.m. injection of 40 mg DF 2 hr after the i.v. 59Fe-ferritin and provided a standard for comparison of chelator activity. Immediately after the injection of chelator, the animals were placed in individual metabolic cages prepared to minimize iron contamination as described above. The study was terminated 6 hr after the...
IRON-CHELATING AGENTS IN RATS

687

Fig. 2. Timing of availability of ferritin 59Fe to two different chelators. Desferrioxamine 40 mg i.m. (□—□), pyridoxal isonicotinoyl hydrazone 35 mg i.m. (○—○), or saline (□—□) were injected at intervals of 0–24 hr after i.v. 59Fe-ferritin. The animals were sacrificed 4 hr after the chelator or control injection. A separate group of 4 normal rats was used at each time interval and for each chelator and results are the mean ± 1 SEM.

59Fe-ferritin injection. Urine, collected from the metabolic cage after the bladder of the animal had been emptied by suprapubic pressure, was assayed for both gamma counts and total chemical iron. The rats were sacrificed by exsanguination from the abdominal aorta followed by perfusion with 20 ml of 0.9% saline. The liver and gastrointestinal tract (including its contents) were removed for gamma counting and, as shown in the results, provided a measure of chelator activity within hepatocytes and of biliary iron excretion.

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 (hrs) 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>.65 ± .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>
</tr>
</tbody>
</table>

Results are the mean ± 1SEM for groups of 4 normal rats sacrificed at hourly intervals after the chelator injection.

59Fe-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.
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 $39.3\% \pm 2.0\%$ 4 hr after DF. This was almost identical to the amount found in a 72-hr fecal collection ($38.1\% \pm 2.8\%$) in a parallel group of animals. The corresponding figures for a similar study using PIH (17.5 mg i.v.) were $38.0\% \pm 5.4\%$ in the gut and $33\% \pm 7.2\%$ 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 $6.6\% \pm 4.3\%$, $28.2\% \pm 3.6\%$, and $3.3\% \pm 1.0\%$ 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 $\mu$g 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 $0.85 \pm 0.06$ mg ($n = 26$) and of the iron-loaded rats $3.98 \pm 0.59$ 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/ug total iron) in iron-loaded rats ($0.11 \pm 0.007$) was conspicuously lower than in normal animals ($0.48 \pm 0.016$). The
Table 2. Activity of Five Known Iron Chelators in Rapid Screening Procedure

<table>
<thead>
<tr>
<th>Chelator*</th>
<th>Dose (mg)</th>
<th>Route</th>
<th>Liver</th>
<th>Gut and Contents</th>
<th>Urine</th>
<th>Total Iron in Urine (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>Controls</td>
<td></td>
<td>89.4 (± 1.5)</td>
<td>2.7 (± 0.2)</td>
<td>0</td>
<td>0.8 (± 0.2)</td>
</tr>
<tr>
<td>DF</td>
<td>40</td>
<td>i.m.</td>
<td>56.2 (± 1.9)</td>
<td>39.3 (± 2.0)</td>
<td>39.3 (± 2.0)</td>
<td>0.60 (± 0.05)</td>
</tr>
<tr>
<td>EDBHA</td>
<td>21</td>
<td>i.m.</td>
<td>37.3 (± 1.6)</td>
<td>53.5 (± 1.0)</td>
<td>53.5 (± 1.0)</td>
<td>0.38 (± 0.09)</td>
</tr>
<tr>
<td>DHB</td>
<td>10</td>
<td>i.m.</td>
<td>92.6 (± 0.6)</td>
<td>2.3 (± 0.4)</td>
<td>2.3 (± 0.4)</td>
<td>0</td>
</tr>
<tr>
<td>PIH</td>
<td>35</td>
<td>i.m.</td>
<td>44.3 (± 0.8)</td>
<td>50.9 (± 0.9)</td>
<td>50.9 (± 0.9)</td>
<td>0.08 (± 0.02)</td>
</tr>
<tr>
<td>DTPA</td>
<td>24</td>
<td>i.p.</td>
<td>92.2 (± 0.9)</td>
<td>1.4 (± 0.2)</td>
<td>1.4 (± 0.2)</td>
<td>0.22 (± 0.05)</td>
</tr>
<tr>
<td>DTPA</td>
<td>48</td>
<td>i.p.</td>
<td>88.9 (± 0.7)</td>
<td>2.7 (± 0.2)</td>
<td>2.7 (± 0.2)</td>
<td>0.49 (± 0.09)</td>
</tr>
</tbody>
</table>

*Abbreviations: DF, desferrioxamine; EDBHA, ethylenediaminebis[hydroxyphenylacetic acid]; DHB, 2,3-dihydroxybenzoic acid; PIH, pyridoxal isonicotinoyl hydrazone; DTPA, diethylenetriaminepentaacetic acid.

The doses, with one exception (†) represent equivalent iron binding capacities, assuming a stoichiometry of 1:1 for the iron complexes of DF, DTPA, EDBHA, and DHB and 2:1 for that of PIH, under physiologic conditions.
The latter was most easily measured by determining the decline in liver radioactivity provided a measure of iron excretion and with different chelating agents suggests current experiments, the S9Fe was chelated while in transit within the hepatocyte. The 59Fe was uniformly labeling a "physiologic" iron a measure of the amount excreted by this route. 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. 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 DF) could be identified in normal of the activity that it showed parenterally, whereas the DF was totally inactive by the oral route (Table 3).

**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.

Rat 59Fe-ferritin is cleared almost exclusively by rat liver parenchymal cells, 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-leucine 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.

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. 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 DF) could be identified in normal...
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.

REFERENCES

5. Graziano JH, Grady RW, Cerami A: The identification of 2,3-dihydroxybenzoic acid as a potentially useful iron-chelating drug. J Pharmacol Exp Ther 190:570, 1974
chelation in the hypertransfused rat: Definition of two alternative pathways of iron mobilisation. J Lab Clin Med 92:144, 1978
A rapid assay for evaluation of iron-chelating agents in rats

MJ Pippard, DK Johnson and CA Finch