IRON INCORPORATION INTO ISOLATED RAT HEPATOCYTES

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SUMMARY

Isolated rat hepatocytes incorporated ferrous iron by a simple diffusion process, whereas the incorporation of ferric iron (complexed with citrate) and transferrin-bound iron followed Michaelis-Menten kinetics. Ferrous iron was incorporated at a rate 2-3 times that of ferric citrate, and 4 times that of transferrin-bound iron. When the hepatocytes were prepared using the soybean trypsin inhibitor in the perfusion medium, the rate of transferrin-bound iron incorporation was increased 3-fold. It is concluded that rat hepatocyte membranes contain an iron-transferrin receptor, which functions as part of the mechanism for iron uptake by the liver.

INTRODUCTION

Iron metabolism in the human organism has been studied for over 100 years. The major areas of concern have been the mode of iron absorption in the intestinal tract, the mechanism of its transport throughout the blood-stream, its incorporation into erythropoietic tissue, and the chemistry and biology of iron storage in the liver and other tissues. In all of these processes, the circulatory non-heme iron-binding protein called transferrin plays a crucial role (1). In the last few years, much attention has been focused upon the interaction of iron-transferrin with various types of cells that take up iron. In the rabbit reticulocyte system, transferrin carrying the iron first combines with a specific receptor on the reticulocyte surface, then, through an as yet poorly understood mechanism, releases the iron for the biosynthesis of heme. The specific trans-

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ferrin receptor has been purified and partially characterized (2, 3).

In addition to its transport into the erythropoietic cells, iron must also enter and exit into and from its principal storage sites in the liver cells. Several reports have dealt with the mechanisms whereby this may be accomplished, and it was proposed that a non-enzymatic process may be involved (4, 5, 6). Work in intact animals and perfused livers has shown that iron-containing transferrin can be taken up by the liver cells (7), that liver can take up both ferric and ferrous iron (8), and that ferrous iron was taken up by perfused liver cells faster than was ferric iron in combination with transferrin (9). Finally, liver cells maintained in tissue culture were also able to take up iron from iron-transferrin (10).

The work described in this paper deals with the mechanisms or iron incorporation into isolated rat hepatocytes prepared following the perfusion of liver with bacterial collagenase preparations.

MATERIALS AND METHODS

Animals. Male white Sprague-Dawley rats (200-300 g) were obtained from local suppliers, and were housed in light-cycled rooms with rat chow and water ad libitum.

Isolation of hepatocytes. Hepatocytes were isolated by a modification of the Bissel et al. procedure (11): under nembutal anesthesia, the peritoneal cavity was opened and the inferior vena cava ligated above the kidneys. The portal vein was then cannulated with a 16 ga. x 2" angiocatheter (Deseret Pharm. Co., Sandy, UT). The liver was flushed via the portal vein cannula with 5-10 ml of sterile pH 7.4 buffer solution (7.94 g citrate, 3.35 g NaCl, 1.41 g KCl, 2.3 g Na_2HPO_4, and 0.42 g NaH_2PO_4 per liter), then the cannula was closed. Next, the thoracic cavity was opened, and the superior vena cava was cannulated via the right atrium using a 14 ga. x 2" angiocatheter. After opening the portal vein cannula, some 20-30 ml of the above buffer were introduced via the superior vena cava cannula to exsanguinate and soften the organ. After this, the perfusion circuit was connected to the portal vein and superior vena cava cannulae, and approximately 200 ml of the perfusion solution (12) was circulated through the liver in the direction of blood flow. When circulation was well established, some 50 ml of the perfusion

medium containing 100 mg collagenase (Type II, Worthington Biochemicals, Freehold, NJ) was added to the circulating perfusion medium, and the pumping was continued until the hepatic tissue disintegrated and the perfusion fluid leaked out (usually less than 15 min). The liver was then excised, minced gently into 40 ml of the perfusion fluid containing 25 mg of the collagenase, and was agitated at 37° for 10-15 min. Connective tissue was then removed by filtration, total volume brought to 100 ml with the Seglen washing buffer (12), and the cells were permitted to settle under gravity for 10 min. After decanting the supernatant, the cells were washed twice with the above washing buffer with centrifugations at 50 x g for 2 min. The final cell preparation, suspended in 8-9 ml of the washing buffer, contained 3-5 x 10^6 cells/ml, with a "viability" of 66-90% as determined by the trypan blue dye exclusion technique (13). In certain experiments, the perfusion medium also contained 0.05% soybean trypsin inhibitor (Sigma Corp., St. Louis, MO).

Iron solutions. Ferric iron solutions were prepared as follows: to 5 ml of a $FeNH_4(SO_4)_2$ solution containing 64.4 ug Fe/ml in 0.1 M bicarbonate and stabilized with citrate were added 0.25 ml of the ^{59}Fe isotope solution (Mallincrodt Nuclear, St. Louis, MO, 22.6 mC/mg, 1.1 ug Fe/ml). Through this solution, O_2 was bubbled vigorously to ensure the oxidation of all ferrous iron. Various dilutions (up to 1:10) were then made from this solution using the Leibovitz tissue culture medium as diluent (14). Alternately, dilutions of the iron stock solution containing 64.4 ug Fe/ml were first made, then 0.025 ml of the isotope solution was added to each dilution. These were then treated with O_2 to ensure oxidation of all ferrous iron.

Ferrous iron solutions were prepared immediately before their use as follows: 452 mg $Fe(NH_4)_2(SO_4)_2.6H_2O$, 528 mg ascorbic acid, and 294 mg of Na_3citrate.$2H_2O$ were diluted to 1 liter with water, and the pH was adjusted to 7.4 with solid $NaHCO_3$. This gave a solution containing 64.4 ug ferrous iron/ml, $3x10^{-3}$ M ascorbate, and $2x10^{-3}$ M citrate. the isotope solution (0.025 ml) was then added to various amounts of this stock solutions, and the volumes were made up to 1 ml with the Leibovitz medium where necessary.

In certain experiments, the iron was presented to the cells bound to transferrin (in the form of whole serum). Unhemolyzed rat serum was obtained from Pel-Freez Biologicals (Rogers, AR). It had an iron content of 220 ug/100 ml, and a total iron-binding capacity of 844 ug/100 ml (determined in a routine clinical laboratory). The tagged serum was prepared by mixing 8 ml thereof with 0.25 ml of the isotope solution, then letting it stand for 2 hours.

Incubation of hepatocytes with iron solutions. Hepatocytes were incubated in triplicate with solutions containing varying amounts of iron tagged with ^{59}Fe. A typical incubation mixture using ferric or ferrous citrate consisted of 2.5 ml of the L-15 Leibovitz tissue culture medium (14), 0.3 ml of the cell suspension, and 0.2 ml of the iron solution added at 0-time in a 25 ml Erlenmeyer flask. Where serum was used as the source of iron, there were 1.7 to 2.6 ml of the Leibovitz medium, 0.3 ml of the cell suspension, and 1 to 0.1 ml of the serum tagged with ^{59}Fe. The incubation was carried out in a Dubnoff shaker at 37° for 20 min, and the reaction was terminated by quick cooling in an ice bath. After adding 5 ml of ice-cold Leibovitz medium to each flask, the cells were sedimented at 2000 x g for 3 min, and washed

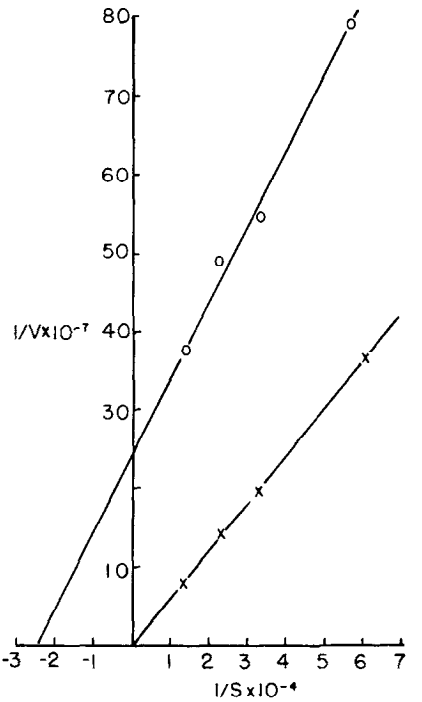

Fig. 1. A Lineweaver-Burk plot of iron incorporation into isolated rat hepatocytes: x-x-x represents ferrous iron, whereas o-o-o represents ferric iron. S is the initial iron concentration (in moles/l) in the incubation medium, whereas V is the velocity of iron incorporation into the cells. V is defined as moles of iron per liter incorporated into 10^6 cells per min.

twice with the same ice-cold medium. They were then dissolved in 1 N NaOH at 80° for 1 hr, and counted in a Biogamma counter (Beckman Instruments, Palo Alto, CA). Counting times depended on the amount of radioactivity present (1.5 to 10 times the background), so that the counting error did not exceed 3-5%.

The final concentration of iron in the incubation mixtures was between 0.37 and 3.7 ug/ml when the ferric and ferrous citrate complexes were used, and from 0,066 to 0.66 ug/ml when serum was used. Normal human serum iron levels are 2.5-4.4 ug/ml, whereas those for four strains of rats ranged from 1.3 to 3 ug/ml (16). In our experiments, therefore, iron levels offered to the cells were at or slightly below those of rat and human sera.

RESULTS

The incorporation of iron into isolated rat hepatocytes was measured as a function of iron concentration in the medium for a period of 20 min. It was expected that such experiments would yield data that could be handled via the principles of Michaelis-

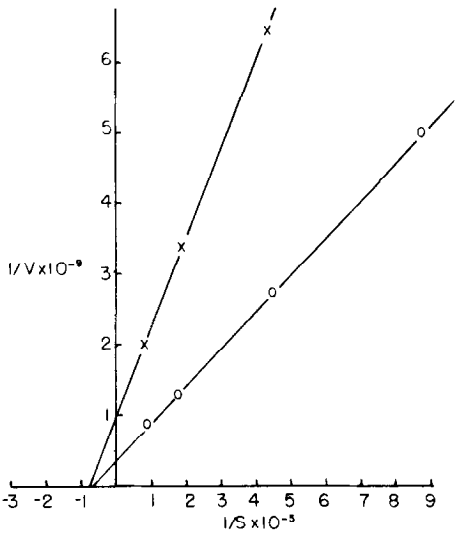

Fig. 2 A Lineweaver-Burk plot of iron incorporation into isolated rat hepatocytes from rat serum: x-x-x represents cells from liver perfused with collagenase in the absence of soybean trypsin inhibitor, whereas o-o-o represents cells obtained from a liver perfused in the presence of the trypsin inhibitor. The meaning of S and V are the same as those in Fig. 1.

Menten theory. The results obtained with ferrous and ferric iron are shown in Fig. 1. It is seen that the ferrous iron was absorbed faster than was ferric iron, and, in addition, did not exhibit saturation kinetics. Ferric iron, on the other hand, was absorbed slower, but did exhibit saturation kinetics with definite K_M- and V_{max}-values. When iron was offered to hepatocytes in combination with transferrin, there was observed a relatively low degree of incorporation that also showed saturation kinetics (Fig. 2).

Iron is present in the circulation almost entirely in the form of ferric iron-transferrin complex. It has been proposed that prior to its transfer into the hepatocyte, transferrin-bound iron may be reduced to the ferrous state by ascorbate, or possibly by a ferroreductase (15), is thereby released from transferrin (which does not bind ferrous iron), ans then incorporated as an

ATP or citrate comple diffusion process (4, 6). It is more likely, however, that a mechanism involving a transferrin and an iron receptor on the hepatocyte membrane surface is operative under physio logical conditions, similar to the situation observed with immature red cells (2, 3). Since the uptake of iron from transferrin in our system was relatively slow, it was reasoned that the collagenase preparation used to perfuse the rat livers might contain proteases that may be capable of destroying such possible receptors. Consequently, soybean trypsin inhibitor was incorporated into the perfusion medium, and the results involving transferrin-bound iron are depicted in Fig. 2. It was indeed observed that the rate of iron incorporation into hepatocytes was increased under these conditions, though the K_M-values remained unchanged.

The results obtained from all experiments described above are summarized in Table 1. It may be concluded that the rate of ferrous iron incorporation into isolated rat hepatocytes without the trypsin inhibitor was 2-3 times that of ferric iron, and 4 times that of transferrin-bound iron. When the trypsin inhibitor was used in preparing the cells, the rate of ferric iron uptake was increased somewhat, however, that of transferrin-bound iron exceeded that of ferric iron and approached that of ferrous iron.

DISCUSSION

The work cited above confirms qualitatively the results of Zimelman *et al*. (9) and those of Hoy and Harrison (8). Our results further indicate that the ferrous iron is taken up well by a simple diffusion process, apparently requiring no enzymatic mechanism for its entry into the hepatocyte. On the other hand, ferric iron and transferrin-bound iron utilize a mechanism that may involve receptors on the hepatocyte membrane surface, and possibly other enzymatic steps.

Table 1. Kinetic parameters of iron incorporation into isolated rat hepatocytes[1].

Form of iron	$K_m \times 10^5$	$V_{max} \times 10^9$	Initial dv/dS $\times 10^4$
Ferrous-citrate	-	-	2.4
Ferric-citrate	5.9	5.9	0.94
In serum (ferric)	1.2	0.80	0.66
Ferric-citrate (with trypsin inhibitor in perfusion medium)	1.0	3.2	1.2
In serum (ferric with trypsin inhibitor in perfusion medium)	1.3	2.5	1.7

[1] Average of three determinations in each case, except for ferric-citrate, which is an average of six determinations. Velocities (v) are expressed in moles of iron incorporated into cells/liter min. 10^6 cells. dv/dS is the rate of incorporation of iron into hepatocytes as a function of iron (S) concentration in the reaction mixture.

The fact that the cells prepared in the presence of the trypsin inhibitor incorporated iron at a rate almost 3 times that observed in the absence of such an inhibitor indicates that there may be a specific receptor for the iron-transferrin complex on the hepatocyte membrane surface, which is susceptible to a trypsin-like enzyme digestion. Significantly, the K_M-values observed with both types of cells remained the same with respect to transferrin-bound iron. This indicates that both types of cells had an identical receptor, though the cells prepared without the trypsin inhibitor had fewer such receptors on their membrane surfaces.

It may also be proposed that a receptor exists on the hepatocyte membrane surface that can combine with ferric iron. Judging from the K_M-values observed in the two types of cells (1.0 and 5.0×10^{-5}M), it may be surmised that this receptor was in some way altered by the trypsin-like enzyme(s) present in the collagenase preparation, rather than being destroyed as was the case with the transferrin receptor.

In conclusion, it may be proposed that under physiological conditions, the incorporation of iron into rat hepatocytes takes place via a mechanism not dissimilar to that observed with rabbit reticulocytes and other hemopoietic cells.

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