

# Transferrin protein and iron uptake by cultured hepatocytes

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The binding and uptake of  $^{59}\text{Fe}$ -loaded  $^3\text{H}$ -labelled rat transferrin by cultured rat hepatocytes was investigated. At  $4^\circ\text{C}$ , there is no evidence for a specific binding of transferrin which could be related to the association of neo-synthesized transferrin with plasma membrane receptors. At  $37^\circ\text{C}$ , iron uptake is much more important than transferrin uptake; it proceeds linearly over the time of incubation, is largely proportional to the extracellular transferrin concentration, and is compatible with uptake by fluid phase endocytosis. The difference observed between iron and transferrin uptake implies the existence of a mechanism allowing the reutilization of transferrin after iron delivery.

*Transferrin      Iron      Uptake      Binding      Cultured hepatocytes*

## 1. INTRODUCTION

Hepatocytes play a key role in iron metabolism. They synthesize transferrin, the transport protein for iron in serum and extracellular fluids, as well as haptoglobin and haemopexin which recover haem after intravascular haemolysis [1]. They clear from the circulation the haptoglobin-haemoglobin complex by receptor-mediated endocytosis. This permits the release of iron for incorporation into hepatic ferritin [2], and the transfer of haptoglobin to the bile [3–5] or its delivery to lysosomes for digestion [4]. The haem-haemopexin complex is taken up by hepatocytes through receptor-mediated endocytosis with either recycling of haemopexin [6] or its digestion in lysosomes with recycling of iron to transferrin [7], incorporation of iron into ferritin, and secretion of oxidised haem in the bile. Hepatocytes also accumulate iron from transferrin [2,8,9] or from ferric citrate [9].

Several mechanisms have been proposed for transferrin iron uptake by different cell types. Once bound to a cell surface receptor [10], transferrin could release its iron at the plasma membrane and thereafter return to the ex-

tracellular medium while the iron could pass into the cytoplasm. Alternatively, in agreement with morphological observations [11,12], transferrin could be interiorised by endocytosis and release its iron within an intracellular acidic granule prior to its recycling and release in the extracellular medium. Such a mechanism seems to be involved in transferrin iron uptake by fibroblasts [13–15], teratocarcinoma cells [16], erythroblasts [17] and placental trophoblasts [18]. The mechanism of iron uptake by hepatocytes is not understood. On the basis of studies on the uptake of iron by rat hepatocytes in suspension, it has been proposed that after binding of transferrin to specific plasma membrane receptors, iron would be incorporated, the precise site of iron release being at present unknown [19]. It has been suggested that [9] the uptake of iron by isolated hepatocytes is related to the iron saturation of transferrin. At saturation levels of 30% and above, a non-specific uptake which is temperature-independent was observed.

We have investigated the uptake of iron from  $^{59}\text{Fe}$ -saturated  $^3\text{H}$ -labelled transferrin by cultured rat hepatocytes and here report results which show a largely non-specific binding of transferrin and

uptake of iron by the cells. We propose that this could be accounted for by the occupancy of putative plasma membrane receptors by neosynthesized transferrin secreted into the extracellular medium.

## 2. MATERIALS AND METHODS

Rat serum transferrin was isolated and labelled with  $^3\text{H}$  and  $^{59}\text{Fe}$  to a specific radioactivity of about 10 000 dpm/ $\mu\text{g}$  of protein as in [14]. Antibodies against rat serum transferrin were obtained as in [14]. They were purified by immunoadsorption using transferrin immobilised on Sepharose CL 4B (Pharmacia, Uppsala) and eluted as in [20]. [ $^3\text{H}$ ]Inulin (500 mCi/mmol) was from the Radiochemical Centre (Amersham).

Rat hepatocytes isolated and cultured as in [21] were incubated in 20 cm<sup>2</sup> gas permeable Petri dishes coated with collagen at 4°C or 37°C in 1 ml of culture medium (Dubelco Eagle H16) containing 15% foetal calf serum (Gibco Biocult, Paisley, Scotland). For the 4°C incubations, the medium was supplemented with 10 mM HEPES, buffered at pH 7.2. At the end of the incubations, cells were washed 4-times with 1 ml phosphate-buffered saline (PBS), once with culture medium, and twice with PBS. The cells were then solubilised with 1% (w/v) sodium deoxycholate adjusted to pH 11.3 with NaOH. The radioactivity accumulated by the cells was measured after dispersion in aqualuma cocktail (Lumac Systems, Basel) in a Tri Carb 460 CD scintillation counter (Packard Instruments, San Diego CA); all results were corrected for quenching.

Cell protein was determined as in [22] using bovine serum albumin as standard. The results are expressed as dpm/mg cell protein.

Methylamine was used at a final concentration of 20 mM (at this concentration, no toxic effect was detected as judged by observation under phase contrast microscopy throughout the incubation time). For some experiments, the collected media was assayed for degradation products after precipitation of proteins by 15% (w/v) trichloroacetic acid, followed by 30 min at 1500  $\times$  g and 4°C; the trichloroacetic acid supernatants were assayed for radioactivity as above. The secretion of transferrin in the medium was measured by a Particle Counting Immuno Assay (PACIA) technique [23] using a monospecific anti-

transferrin antiserum and purified transferrin as standard.

## 3. RESULTS AND DISCUSSION

### 3.1. Uptake of $^{59}\text{Fe}$ -saturated $^3\text{H}$ -labelled transferrin

That cultured rat hepatocytes accumulate transferrin iron and protein is apparent in fig. 1. At 37°C, iron uptake proceeds linearly over the entire period of incubation studied (26 h). In contrast, accumulation of  $^3\text{H}$ -labelled material increases linearly up to 7 h and then reaches a plateau. At this time, most of the  $^{59}\text{Fe}$  is associated with cytosol ferritin since about 90% of the intracellular  $^{59}\text{Fe}$  reacts with anti-ferritin IgG immobilised on Sepharose-protein A as in [14].

Since the initial specific radioactivities in  $^{59}\text{Fe}$  and  $^3\text{H}$  are similar, it appears that after 26 h incubation, the hepatocytes have accumulated about 16-times more iron than transferrin. Under these conditions, no significant amounts of degradation products soluble in trichloroacetic acid are detected in the culture medium. Thus the difference between the accumulation of iron and protein cannot be explained by the digestion of

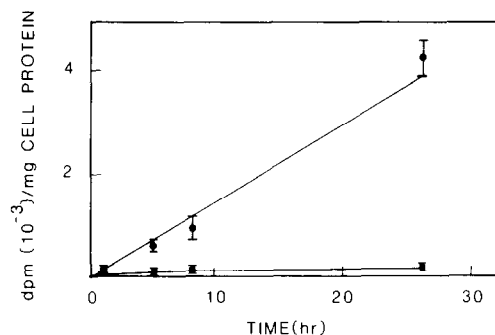


Fig. 1. Kinetics of accumulation of iron-saturated transferrin by cultured hepatocytes. Cells (~2.5 mg protein) were incubated for various times at 37°C in 20 cm<sup>2</sup> Petri dishes, in 1 ml of culture medium containing 10  $\mu\text{g}/\text{ml}$  of (●)  $^{59}\text{Fe}$ -loaded (■)  $^3\text{H}$ -labelled transferrin (spec. act. 10 000 dpm/ $\mu\text{g}$  for both isotopes). At the end of the incubation, the cells were washed 4-times with PBS, dissolved in 1% sodium deoxycholate adjusted to pH 11.3 with NaOH, and analysed for radioactivity and protein. Mean results  $\pm$  SD of 3 independent expts are given.

transferrin and the release of degradation products in the extracellular medium, and there must be a mechanism which assures the reutilisation of transferrin after iron delivery.

A few years ago, we proposed a model for transferrin iron uptake which permits the reutilisation of iron depleted transferrin [13–15]. In this model, after binding of transferrin to specific plasma membrane receptors, the protein is interiorised by receptor mediated endocytosis; during the fusion of the resulting endocytic vesicle with a lysosome or any other prelysosomal acidic compartment [24], iron is released from transferrin as a consequence of the acidic pH prevailing in these granules. Iron depleted transferrin, still bound to its receptor, escapes lysosomal degradation and is recycled back to the cell surface. This model was supported by the effect of lysosomotropic agents such as methylamine, which increases the intralysosomal pH and greatly inhibits iron uptake from transferrin [15].

A comparable model could be envisaged to explain transferrin iron uptake by cultured hepatocytes if we could establish the presence of specific receptors for transferrin at the plasma membrane of hepatocytes, and if it could be shown that lysosomotropic drugs decrease iron uptake.

### 3.2. Low temperature binding of transferrin

Cultured hepatocytes were incubated for 1 h at 4°C in the presence of increasing concentrations of  $^3\text{H}$ -labelled transferrin. As shown in fig. 2, the binding of  $^3\text{H}$ -labelled material is essentially proportional to the extracellular concentration, indicating that the binding is largely non-specific. However, at very low concentration (0.5–5  $\mu\text{g Tf/ml}$ ) the existence of a specific interaction cannot be excluded.

To further investigate whether transferrin iron could be taken up by a non-specific mechanism (e.g., fluid phase endocytosis), or whether it could also result from a receptor-mediated process, we compared the uptake of transferrin iron to that of [ $^3\text{H}$ ]inulin, a marker of fluid phase endocytosis [25] and studied the effect of methylamine, a substance known to interact with endocytosis [26] and iron uptake [15].

### 3.3. Uptake of [ $^3\text{H}$ ]inulin and $^{59}\text{Fe}$ -labelled transferrin

When cultured hepatocytes are incubated for 1 h

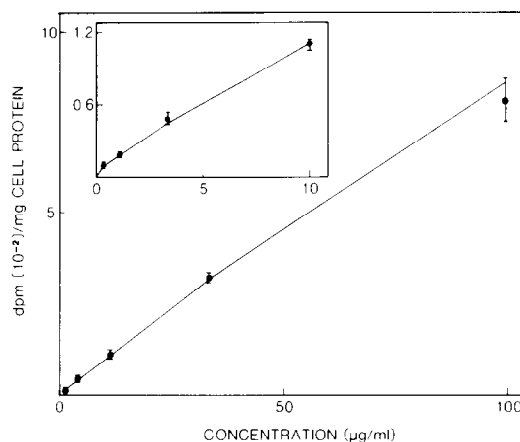


Fig. 2. Low-temperature binding of transferrin. Cells ( $\sim 2.5$  mg protein) were incubated for 1 h at 4°C in 1 ml of culture medium containing increasing concentrations of  $^3\text{H}$ -labelled transferrin (spec. act. as in fig. 1). After the incubation, the cells were washed, dissolved and analysed as in fig. 1. Mean results  $\pm$  SD of 3 different expts are given.

at 37°C in the presence of increasing concentrations of  $^3\text{H}$ -labelled inulin, the uptake of  $^3\text{H}$  is proportional to the extracellular concentration of the polysaccharide. This uptake corresponds to the interiorisation of 0.2  $\mu\text{l}$  of extracellular medium. mg cell protein $^{-1}$ .h $^{-1}$ . Under the same conditions, when incubated in the presence of increasing concentrations of  $^{59}\text{Fe}$ -labelled transferrin, hepatocytes accumulate  $^{59}\text{Fe}$  largely as a function of the external concentration with a clearance of 0.3  $\mu\text{l}$ . mg cell protein $^{-1}$ .h $^{-1}$  (fig. 3A).

### 3.4. Effect of methylamine

Methylamine is a weak base which accumulates in lysosomes, increases the lysosomal pH [27], inhibits lysosomal digestion [26] and decreases the rate at which the membrane of endocytic vesicles is recycled back to the plasma membrane [26]. Its effect on iron and inulin accumulation in hepatocytes is illustrated in fig. 3B. At low external concentrations of transferrin (1–30  $\mu\text{g/ml}$ ) 20 mM methylamine reduces  $^{59}\text{Fe}$  accumulation without any significant effect on inulin uptake. These results suggest that while the accumulation of  $^3\text{H}$ -labelled inulin and  $^{59}\text{Fe}$  are comparable at high concentrations and could result mainly from

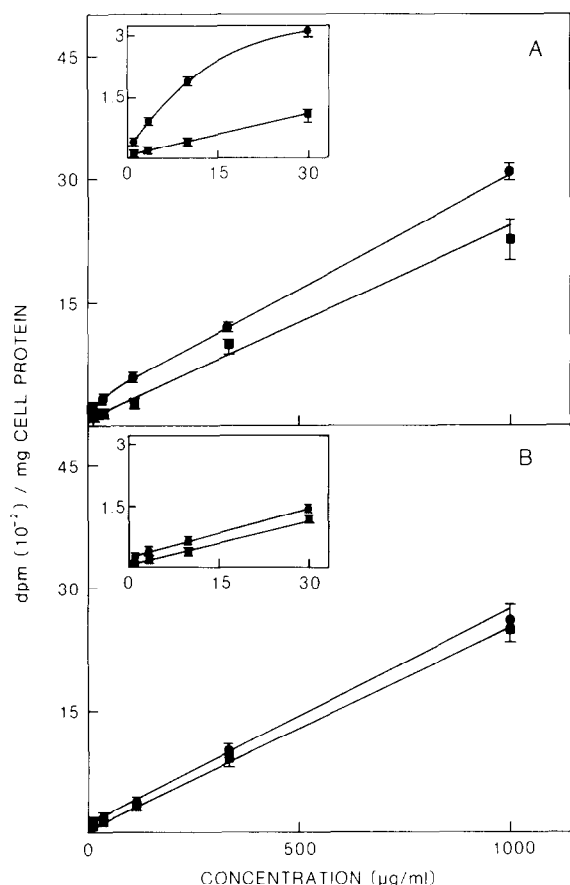


Fig. 3. Accumulation of transferrin iron and inulin by cultured hepatocytes. Cells ( $\sim 2.5$  mg protein) were incubated for 1 h at  $37^\circ\text{C}$  in 1 ml of culture medium containing increasing concentrations of ( $\bullet$ )  $^{59}\text{Fe}$ -loaded transferrin or ( $\blacksquare$ )  $^3\text{H}$ -labelled inulin (spec. act. as in fig. 1) in the absence (A) or the presence (B) of 20 mM methylamine. At the end of the incubation, the cells were washed, dissolved and analysed as in fig. 1.

fluid phase endocytosis, a process which is not affected by methylamine, at low concentrations (1–30  $\mu\text{g/ml}$ ) transferrin iron may be taken up by a more specific mechanism which is affected by the drug, such as receptor-mediated endocytosis. To reconcile this result with the low temperature-binding studies which suggested the absence of a specific receptor, we have considered the possibility that transferrin receptors might be occupied by transferrin neo-synthesized by cultured hepatocytes.

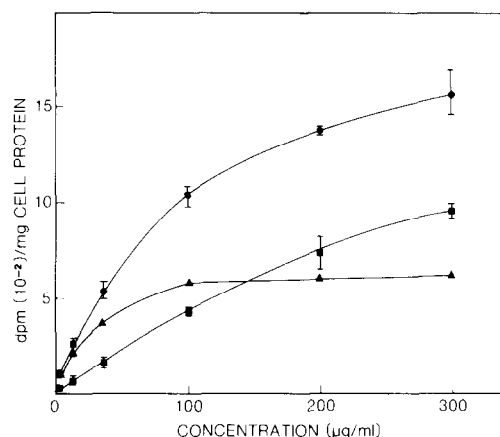


Fig. 4. Binding of anti-transferrin IgG to cultured hepatocytes. Cells ( $\sim 2.5$  mg protein) were incubated in 20  $\text{cm}^2$  Petri dishes for 1 h at  $4^\circ\text{C}$  culture medium containing increasing concentrations of  $^3\text{H}$ -labelled anti-transferrin IgG ( $\bullet$ ) or  $^3\text{H}$ -labelled non-specific IgG ( $\blacksquare$ ) (spec. act. as in fig. 1). Mean results  $\pm$  SD of 3 independent expts are given.  $\blacktriangle$  represents the specific binding.

### 3.5. The secretion of transferrin by cultured hepatocytes

Since one of the physiological roles of hepatocytes is the synthesis and the secretion of transferrin, the appearance of rat transferrin in the extracellular medium of cultured hepatocytes was investigated. Our results indicate that every h, 0.45  $\mu\text{g}$  transferrin/mg cell protein appear in the culture medium. Thus, if there are specific receptors for transferrin at the plasma membrane, they would be occupied by the secreted transferrin. In order to confirm this hypothesis, we measured the binding of anti-transferrin IgG to cultured hepatocytes.

### 3.6. Binding of anti-transferrin IgG to cultured hepatocytes

Cultured rat hepatocytes were incubated for 1 h at  $4^\circ\text{C}$  in the presence of increasing concentrations of purified  $^3\text{H}$ -labelled anti-transferrin IgG. The same experiment was carried out with non-specific IgG. The results presented in fig. 4 indicate that the difference between the binding of anti-transferrin IgG and that of non-specific IgG results from a saturable process indicating a specific binding of anti-transferrin IgG probably as a conse-

quence of the presence of transferrin at the plasma membrane.

#### 4. CONCLUSIONS

Our results indicate that the binding of transferrin to cultured hepatocytes as well as transferrin iron uptake seem to be largely non-specific. However, the difference observed at 37°C between the accumulation of <sup>59</sup>Fe and that of <sup>3</sup>H-labelled transferrin implies the existence of a mechanism allowing the reutilisation of transferrin after iron delivery.

The effect of methylamine reported here might suggest the existence of such a mechanism responsible for transferrin iron uptake by cultured hepatocytes. Whether the apparently non-specific character of transferrin binding is related to the presence of neo-synthesized transferrin at the plasma membrane associated with a specific receptor is currently under investigation.

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