Uptake and subcellular processing of ^{59}Fe - ^{125}I -labelled transferrin by rat liver

Evan H. MORGAN,* Geoffrey D. SMITH and Timothy J. PETERS

Division of Clinical Cell Biology, M.R.C. Clinical Research Centre, Watford Road, Harrow, Middx. HA1 3UJ, U.K.

The uptake of transferrin and iron by the rat liver was studied after intravenous injection or perfusion *in vitro* with diferric rat transferrin labelled with ^{125}I and ^{59}Fe . It was shown by subcellular fractionation on sucrose density gradients that ^{125}I -transferrin was predominantly associated with a low-density membrane fraction, of similar density to the Golgi-membrane marker galactosyltransferase. Electron-microscope autoradiography demonstrated that most of the ^{125}I -transferrin was located in hepatocytes. The ^{59}Fe had a bimodal distribution, with a larger peak at a similar low density to that of labelled transferrin and a smaller peak at higher density coincident with the mitochondrial enzyme succinate dehydrogenase. Approx. 50% of the ^{59}Fe in the low-density peak was precipitated with anti-(rat ferritin) serum. Uptake of transferrin into the low-density fraction was rapid, reaching a maximal level after 5–10 min. When livers were perfused with various concentrations of transferrin the total uptakes of both iron and transferrin and incorporation into their subcellular fractions were curvilinear, increasing with transferrin concentrations up to at least $10\ \mu\text{M}$. Analysis of the transferrin-uptake data indicated the presence of specific transferrin receptors with an association constant of approx. $5 \times 10^8\ \text{M}^{-1}$, with some non-specific binding. Neither rat nor bovine serum albumin was taken up into the low-density fractions of the liver. Chase experiments with the perfused liver showed that most of the ^{125}I -transferrin was rapidly released from the liver, predominantly in an undegraded form, as indicated by precipitation with trichloroacetic acid. Approx. 40% of the ^{59}Fe was also released. It is concluded that the uptake of transferrin-bound iron by the liver of the rat results from endocytosis by hepatocytes of the iron-transferrin complex into low-density vesicles followed by release of iron from the transferrin and recycling of the transferrin to the extracellular medium. The iron is rapidly incorporated into mitochondria and cytosolic ferritin.

INTRODUCTION

The liver has a high capacity for iron exchange with plasma transferrin. Only the erythroid marrow and the chorioallantoic placenta are more active (Morgan, 1981). In the rat 10–20% of the plasma iron turnover is directed to the liver (Mazur *et al.*, 1960; Cheney *et al.*, 1967; Gardiner & Morgan, 1974). Most or all of this is due to uptake by the hepatocytes (Hershko *et al.*, 1973). Despite the quantitative importance of hepatocytes in iron metabolism, the mechanism by which they acquire iron from plasma transferrin is poorly understood. There is evidence to support two types of processes, namely (1) a specific receptor-mediated process and (2) non-specific processes.

The fenestrated nature of liver sinusoid allows ready entry of plasma proteins into the space of Disse with the possibility of direct interaction between transferrin and the hepatocyte cell membrane. Several investigators have reported that transferrin binds to a limited number of specific high-affinity binding sites (receptors) on the membranes of cells present in rat liver-cell suspensions, the number of sites varying from 25000 to 162000 per cell in the different reports (Grohlich *et al.*, 1977; Young & Aisen, 1980; Cole & Glass, 1983; Morley & Bezkorovainy, 1983; Thorstensen & Romslo, 1984a; Tei *et al.*, 1984). However, other workers were unable to obtain evidence for receptors in cell suspensions prepared in a similar manner (Sibille *et al.*, 1982; Soda & Tavassoli, 1984).

Hence the question of the presence of transferrin receptors on hepatocytes is open to debate, and, if they exist, the mechanism by which they mediate iron exchange is uncertain. It is possible that this is by receptor-mediated endocytosis, as in developing erythroid cells (Morgan, 1981) and in several transformed cell lines in culture, including hepatoma cells (Ciechanover *et al.*, 1983). However, evidence for this in normal hepatocytes is lacking. Previous studies using subcellular-fractionation techniques failed to provide any evidence for endocytosis of transferrin (Milsom & Batey, 1979; Batey *et al.*, 1980).

The possibility that iron uptake from transferrin is mediated by non-specific processes as well as by transferrin receptors is supported by experiments with liver-cell suspensions or cultured hepatocytes in which transferrin and iron uptake by the cells were found to increase linearly as the transferrin concentration was raised above that required to saturate the transferrin receptors (Sibille *et al.*, 1982; Cole & Glass, 1983; Page *et al.*, 1984). Two mechanisms for this effect have been proposed, namely fluid-phase endocytosis and mediation by low- M_r chelators. The first undoubtedly occurs, but can account for less than 10% of the observed iron uptake (Cole & Glass, 1983; Page *et al.*, 1984). The second is supported by observations that liver cells rapidly take up iron complexed to citrate or other chelators (Mazur *et al.*, 1960; Charley *et al.*, 1960; Grohlich *et al.*, 1977, 1979; Zimelman *et al.*, 1977). Whether or not similar

* Present address: Department of Physiology, University of Western Australia, Nedlands, Western Australia 6009, Australia.

mechanisms are involved in the uptake of transferrin-bound iron is unknown.

The aim of the present research was to investigate the mechanism of iron exchange between transferrin and the liver in the rat by using a subcellular-fractionation procedure to monitor the binding, endocytosis and subcellular distribution of iron and transferrin. The livers were fractionated after either intravenous injection or short-term perfusion with transferrin labelled with ^{59}Fe and ^{125}I or ^{131}I . The results show that transferrin is endocytosed into a low-density-vesicle fraction of the cells and that the uptake of transferrin and iron by the cells is dependent on two specific processes, one saturable at low concentrations of transferrin and the other non-saturable up to transferrin concentrations of at least $10\text{ }\mu\text{M}$. Both processes involve endocytosis of transferrin.

METHODS

Materials

Rat transferrin was isolated from plasma as previously described (Morgan, 1979). It was a mixture of the 'fast' and 'slow' forms, which have been shown to donate iron equally to the liver and to other tissues in the rat (Huebers *et al.*, 1978). Rat liver ferritin was isolated by the method of Huebers *et al.* (1976). Rat serum albumin and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and pig insulin (Monotard MC) was from Novo (Basingstoke, Hants., U.K.). Chloroquine, methylamine and digitonin were from Sigma Chemical Co. The radioactive isotopes (^{59}Fe as FeCl_3 , ^{125}I and ^{131}I as NaI) were obtained from Amersham International (Amersham, Bucks., U.K.). Anti-(rat transferrin) serum and anti-(rat ferritin) serum were obtained from rabbits given three intramuscular injections of rat transferrin or rat ferritin emulsified with Freund's complete adjuvant at 2-week intervals.

Labelling of proteins

Transferrin was saturated with iron and labelled with ^{59}Fe as in previous experiments (Hemmaplardh & Morgan, 1974) before the labelling with radioiodine. The proteins (transferrin, rat serum albumin and bovine serum albumin) were labelled with ^{125}I or ^{131}I by the ICI method (McFarlane, 1963) to a maximum extent of 1 iodine atom per protein molecule. Unbound radioiodine was removed by passage through an anion-exchange column (IRA 410) followed by dialysis against three changes of 0.15 M-NaCl . Previous studies have shown that rat transferrin and albumin labelled by these methods are not denatured and show no evidence of increased rates of catabolism when injected into rats (Morgan, 1966). Insulin was labelled with ^{125}I by the lactoperoxidase method, and the A14-labelled derivative was isolated by h.p.l.c. as previously described (Christensen *et al.*, 1985).

Intravenous injections

In some experiments rats were injected intravenously with 0.8 mg of ^{59}Fe - ^{125}I -transferrin at given times before the livers were removed and fractionated as described below. The injections were usually performed via a lateral tail vein with the animals under light anaesthesia induced with Penthrane (Abbott Laboratories, Queenborough, Kent, U.K.). In one experiment the injection was made

into the portal vein with the rat anaesthetized with an intraperitoneal injection of sodium pentobarbitone. For removal of the livers the rats were anaesthetized with sodium pentobarbitone and each liver was perfused through the portal vein with ice-cold phosphate-buffered saline ($150\text{ mM-NaCl}/10\text{ mM-sodium phosphate buffer}$, pH 7.4) for 2.5 min. The liver was then removed and weighed, and a sample was homogenized and fractionated by sucrose-density-gradient centrifugation as described below.

Perfusion studies

Rat livers were perfused in a non-circulating system with Krebs-Henseleit bicarbonate Ringer solution containing glucose (50 mM) and bovine serum albumin ($0.5\text{ g}/100\text{ ml}$) as previously described (Smith *et al.*, 1980). After a 'pre-perfusion' period of 2 min the perfusion solution was changed to one containing radio-labelled transferrin and the perfusion was continued for various times. The liver was then 'post-perfused' with the Krebs-Ringer medium alone for 2.5 min unless otherwise specified. The perfusates were saturated with O_2/CO_2 (19:1). The temperature of the perfusate was 37°C , except for one perfusion, when a temperature of 4°C was used. The effluent perfusion fluid was collected over 15 s or 30 s intervals during the perfusion and post-perfusion periods.

Liver fractionation

Portions of the liver weighing 2.0 g were homogenized with 10.0 ml of ice-cold 0.25 M-sucrose in $20\text{ mM-ethanol}/10\text{ mM-Hepes}/\text{NaOH}$ buffer, pH 7.4, with ten strokes of pestle A and ten strokes of pestle B in a 15 ml Dounce homogenizer (Kontes Glass Co., Vineland, NJ, U.S.A.). The homogenate was filtered through a $50\text{ }\mu\text{m}$ nylon mesh. A 5 ml portion was then fractionated by analytical density-gradient centrifugation on a linear sucrose gradient in a Beaufay automatic zonal rotor (Smith & Peters, 1980). From this 15 or 16 fractions were collected, weighed and their densities determined indirectly by refractometry. Samples from each fraction were assayed for enzyme activities, total protein and transferrin concentrations, and for trichloroacetic acid-soluble and -precipitable radioactivity. Radioactivity measurements were also made on the plasma (in the intravenous injection experiments) or the perfusate and effluent perfusion solutions (in the liver perfusion experiments).

Autoradiography

The cellular distribution of diferric ^{125}I -transferrin in the liver was examined by electron-microscope autoradiography in two experiments, one in a perfused liver and one *in vivo*. In the first, a liver was perfused with ^{125}I -transferrin ($0.25\text{ }\mu\text{M}$) for 8 min followed by post-perfusion for 2.5 min, and, in the second, liver samples were taken 1, 4, 9 and 20 min after intravenous injections of 0.5 mg of labelled transferrin. Small samples of the livers were fixed in 2.5% (w/v) glutaraldehyde in $0.1\text{ M-cacodylate buffer}$, pH 7.2, for 2 h at 4°C and post-fixed with 1% (w/v) OsO_4 in the same buffer for 1 h at room temperature. The tissue was then prepared for electron-microscope autoradiography as previously described (Iacopetta & Morgan, 1983). Sections were examined with a Philips 300 electron microscope. The autoradiographic grains were scored as lying over

hepatocytes, sinusoidal endothelial cells, Kupffer cells or the sinusoid lumen plus space of Disse. If any part of a grain impinged on the plasma membrane or lay within the confines of the plasma membrane of an endothelial or Kupffer cell it was scored as lying over that type of cell. Grains over the microvilli of the hepatocyte sinusoidal surface were scored as being in the sinusoidal lumen plus space of Disse.

Analytical methods

Marker enzymes were assayed as described by Smith & Peters (1980). Protein was measured by a modified Lowry procedure (Shacterle & Pollack, 1973), with bovine serum albumin as standard. Transferrin was determined by radial immunodiffusion (Mancini *et al.*, 1965) after treatment of homogenates or fractions with Triton X-100 (0.5%, v/v) to release the protein from cellular organelles. Trichloroacetic acid-precipitable radioactivity was determined by precipitation with an equal volume of ice-cold 20% (w/v) trichloroacetic acid followed by centrifugation at 1500 *g* for 30 min. Radioactivity was counted in a two-channel γ -radiation scintillation counter (LKB Wallac).

Computation

Frequency-density histograms were constructed by the method of Leighton *et al.* (1968). Frequency is defined as the ratio of the activity in a fraction to the total activity in the gradient divided by the density span of that fraction. Relative frequency is defined as the product of the frequency and the relative specific activity of the activities in the total homogenates. The data were also processed by a suitable computer program to calculate the activity between defined density boundaries.

RESULTS

Subcellular fractionation after liver perfusion

Transferrin, ^{125}I , ^{59}Fe and several marker enzymes were determined in the sucrose-density-gradient fractions obtained after fractionation of livers that had been perfused with ^{125}I - ^{59}Fe -transferrin for 5 min. The mean results from six gradients, plotted as frequency-distribution histograms, are presented in Fig. 1. Both ^{125}I and ^{59}Fe have bimodal distributions. Approx. 70% of the ^{125}I is in a low-density peak (1.13 g/cm³), which is coincident with that of the Golgi-membrane marker galactosyltransferase (Fig. 1) and also with latent phosphodiesterase (results not shown). A smaller ^{125}I peak is discernible at 1.18–1.19 g/cm³, the same density as that of the plasma-membrane marker 5'-nucleotidase. More than 97% of the ^{125}I in the fractions of density 1.10 g/cm³ and greater was precipitated with trichloroacetic acid, but in the less-dense fractions 5–10% of the ^{125}I was soluble in trichloroacetic acid. The distribution of ^{125}I -transferrin in the gradient differs from that of immunologically assayed endogenous transferrin, which was broadly distributed between 1.15 and 1.25 g/cm³, corresponding to the density of the endoplasmic-reticulum marker enzyme neutral α -glucosidase. The larger of the two ^{59}Fe peaks has a modal density similar to that of the major ^{125}I peak, but the smaller ^{59}Fe peak has a greater density (1.22 g/cm³) than the minor ^{125}I peak. The distribution of this peak is similar to that of succinate dehydrogenase and *N*-acetyl- β -glucosaminidase.

Fig. 1. Distribution of ^{125}I -transferrin, ^{59}Fe , total immunoprecipitable transferrin (Tf) and marker enzymes in density-gradient fractions from rat liver

The results are averages of six gradients from livers that had been perfused with radiolabelled transferrin for 5 min at transferrin concentrations of 0.1–0.25 μM . The vertical lines indicate 1 s.d. Key to marker enzymes: Gal-Trans., galactosyltransferase; SDH, succinate dehydrogenase; *n*- α -Glu, neutral α -glucosidase; *N*-Ac- β -Glu, *N*-acetyl- β -glucosaminidase; 5'NT, 5'-nucleotidase.

The results in Fig. 1 are the means for six perfusions. When the individual gradients were examined it was found that the ^{59}Fe distribution was more closely mirrored by succinate dehydrogenase than by *N*-acetyl- β -glucosaminidase. This is illustrated in Fig. 2(a). In every gradient succinate dehydrogenase and the smaller ^{59}Fe peak had almost identical distributions, while the more broadly distributed *N*-acetyl- β -glucosaminidase extended into a more-dense region of the gradient. Also shown in Fig. 2 (Fig. 2b) are the results expressed as the ratio of pmol of iron to pmol of transferrin found in each fraction. The pattern is typical of that observed with the other five perfusions. The Fe/transferrin molar ratio was greater than 2:1 (the ratio in the perfusion solution) throughout most of the gradient, but was consistently found to be less than 2:1 (1.4–1.9:1) in the low-density peak of ^{125}I -transferrin activity.

The ^{59}Fe in the gradient fractions differed from that in the perfusion solution with respect to its precipitability by trichloroacetic acid and with anti-(rat transferrin) and anti-(rat ferritin) sera. The ^{59}Fe in the perfusion solution was precipitated by anti-transferrin serum but not by

Fig. 2. ^{59}Fe and ^{125}I -transferrin in density-gradient fractions of rat liver: (a) distribution of iron uptake (●) and activities of succinate dehydrogenase (■) and *N*-acetyl- β -glucosaminidase (▲); (b) distribution of the iron/transferrin uptake ratio (●)

The results are from one gradient and show iron uptake in terms of pmol/ml of each subcellular fraction, calculated from the radioactivity of the fractions and the specific radioactivity of the iron in the perfusion solution. This solution contained $0.1\ \mu\text{M}$ diferric transferrin.

Table 1. Precipitation of ^{59}Fe with anti-transferrin serum, anti-ferritin serum and trichloroacetic acid

Sucrose-density-gradient fractions and perfusion solution from experiments in which the liver was perfused for 5 min with radiolabelled transferrin were treated with antisera to transferrin and ferritin and with 10% trichloroacetic acid as described in the text. The results are expressed as the percentage of ^{59}Fe present in the samples that was precipitated by these procedures. Each value is the mean $\pm$ S.E.M. for six separate perfusions.

Sample	^{59}Fe in precipitate (% of total radioactivity)		
	Anti-transferrin serum	Anti-ferritin serum	Trichloroacetic acid
Density-gradient fractions with $\rho = 1.11\text{--}1.14\ \text{g/cm}^3$	20.5 ± 2.73	47.5 ± 1.93	82.0 ± 2.66
Density-gradient fractions with $\rho = 1.20\text{--}1.23\ \text{g/cm}^3$	23.0 ± 2.99	6.3 ± 1.24	88.9 ± 2.32
Perfusate	98.3 ± 0.45	—	3.6 ± 0.75

anti-ferritin serum, and was about 95% soluble in trichloroacetic acid (Table 1). In contrast, only about 20% of the radioactivity in either of the two ^{59}Fe gradient peaks was precipitated with anti-transferrin serum whereas 85% was precipitated with trichloroacetic acid. Approx. 50% of the radioactivity in the lower-density ^{59}Fe peak, but only 6% in the more-dense peak, was precipitated with anti-ferritin serum. Hence most of the ^{59}Fe in the liver-cell fractions was free of transferrin and approx. 50% of that in the lower-density peak had been incorporated into ferritin during the 7.5 min perfusion plus post-perfusion period used in these studies.

Effect of digitonin on the density of transferrin-rich fractions

A liver was perfused with ^{59}Fe - ^{125}I -transferrin ($0.25\ \mu\text{M}$) for 5 min and post-perfused for 2.5 min, and portions were homogenized in the usual $0.25\ \text{M}$ -sucrose/Hepes solution or in the same solution containing $1.0\ \text{mg}$ of

digitonin/ml. As shown in Fig. 3, the digitonin caused a shift in the ^{59}I -transferrin peak to a more-dense region of the gradient (median density $1.20\ \text{g/cm}^3$). This was associated with a smaller shift in the density of the Golgi-membrane marker enzyme galactosyltransferase, so that it was no longer coincident with the distribution of ^{125}I -transferrin.

Injection of ^{59}Fe - ^{125}I -transferrin *in vivo*

In preliminary studies it was found that the uptake and distribution of ^{59}Fe and ^{125}I in the liver subcellular fractions were similar when the labelled transferrin was injected by the portal vein or by a tail vein. Moreover, the subcellular distributions of the labels were similar to that observed after perfusions *in vitro*. Hence the perfusion technique was used to investigate uptake during the first 15 min and injection by a tail vein was used in an experiment aimed at studying the uptake of transferrin and iron over time periods from 5 to 135 min.

Fig. 3. Effect of digitonin on the distribution of ^{125}I -transferrin and galactosyltransferase activity in density-gradient fractions from rat liver

A liver was perfused with $0.25\ \mu\text{M}$ radiolabelled rat transferrin for 5 min, then post-perfused for 2.5 min, and portions were homogenized in the presence (fine line) or in the absence (bold line) of digitonin (1 mg/ml) before fractionation by sucrose-density-gradient centrifugation.

Fig. 4. Changes in plasma and liver ^{125}I and ^{59}Fe after intravenous injection of $0.8\ \text{mg}$ of ^{125}I - ^{59}Fe -transferrin

The results for liver ^{125}I show total radioactivity as percentage of injected dose in uncorrected form (●) and after correction for the fall in plasma ^{125}I radioactivity (■). The results for ^{59}Fe show total radioactivity (○) and also the radioactivity in the fractions with density boundaries of $1.10\text{--}1.16\ \text{g/cm}^3$ (△) and $1.16\text{--}1.25\ \text{g/cm}^3$ (◆).

Time course of transferrin and iron uptake

As shown in Fig. 4, the plasma concentration of ^{59}Fe declined rapidly after intravenous injection of ^{59}Fe - ^{125}I -transferrin whereas that of ^{125}I decreased more slowly. Uptake of ^{125}I by the liver was rapid, reaching maximal values within 5–15 min. In contrast, ^{59}Fe uptake continued in an approximately linear fashion for the duration of the experiment (135 min) and by then amounted to 13% of the injected amount of the label. The relative distribution of ^{125}I between the subcellular

fractions did not change with time. However, with ^{59}Fe there was a more rapid increase in the radioactivity in the lower-density peak than in the higher-density peak after the 15 min time point (Fig. 4).

Perfusion studies for times varying from 2 to 15 min confirmed the rapid uptake of transferrin by the liver and its equally rapid appearance in the subcellular fractions. By 5 min near-maximal values were found for total uptake of ^{125}I -transferrin and for ^{125}I -transferrin in the low-density peak. As in the investigation conducted *in vivo*, total ^{59}Fe uptake increased in a linear manner with

Fig. 5. Effect of transferrin concentration on transferrin and iron uptake by the perfused rat liver

The livers were perfused for 5 min with diferric transferrin at the indicated concentrations. The Figure shows total uptake (●) and uptake into the fractions with density boundaries of 1.10–1.16 g/cm³ (○) and 1.16–1.25 g/cm³ (■). The inset shows iron uptake into these two fractions at transferrin concentrations up to 1.25 μM.

time but there was little change in the relative distribution of the ⁵⁹Fe between its two peaks.

Effect of transferrin concentration

Livers were perfused for 5 min with radiolabelled diferric transferrin at concentrations ranging from 0.025 to 10 μM. As the concentration was raised the uptake of both transferrin and iron increased rapidly in a curvilinear fashion until the transferrin concentration reached about 1 μM and thereafter more slowly in a linear manner (Fig. 5). The amount of transferrin uptake into the low-density peak showed a similar pattern of increase, although the percentage of total uptake found in this peak decreased progressively as the transferrin concentration was raised, from 70% at 0.025 μM-transferrin to 53% when the concentration was 10 μM. Nevertheless, transferrin uptake into the low-density peak increased 2.5-fold as the transferrin concentration was raised from 1.0 to 10 μM. Over the same concentration range iron uptake increased 2.8-fold. At transferrin concentrations below 0.5 μM relatively more of the iron uptake was directed to the lower-density ⁵⁹Fe peak, but at higher transferrin concentrations iron incorporation into the higher-density peak predominated (Fig. 5 inset).

The total transferrin-uptake data in Fig. 5 were analysed by a non-linear curve-fitting program (Munson & Rodbard, 1980) to assess the affinity and number of binding sites for transferrin in the liver. The experimental

results best fitted a model based on a single species of specific binding sites plus some non-specific binding. By using this model the apparent binding constant of association between transferrin and its receptors was calculated to be $5.2 \times 10^6 \pm 1.9 \times 10^6 \text{ M}^{-1}$ and the receptor-site concentration to be $57 \pm 13 \text{ pmol/g}$ (means $\pm$ S.E.M.). The ratio of non-specifically bound to free transferrin was $1.44 \times 10^{-5} \pm 0.43 \times 10^{-5}:1$.

Specificity of transferrin uptake

The above results suggested that transferrin uptake by the liver, including translocation to the lower-density membrane fraction, is dependent on a non-specific process as well as specific uptake mediated by a limited number of high-affinity binding sites. The specificity of these uptake processes was assessed in another manner, by simultaneous perfusion of the liver with radiolabelled transferrin plus another labelled protein, namely rat serum albumin, bovine serum albumin or insulin. The results with ¹³¹I-transferrin plus ¹²⁵I-labelled rat serum albumin, and ¹³¹I-transferrin plus A14-tyrosine-¹²⁵I-labelled pig insulin are illustrated in Fig. 6. With rat albumin there was very little uptake into the low-density membrane fractions of the liver, which contained most of the transferrin. However, relatively more albumin than transferrin was found in the fractions with density greater than 1.19 g/cm³. Simultaneous perfusion with insulin and transferrin led to a different picture (Fig. 6b). The distribution patterns of the two proteins in the density gradient were very similar, both being found in maximal concentrations in the same low-density membrane fractions.

Effect of perfusion at 4 °C

When a 5 min perfusion was performed with the medium and perfusion chamber at 4 °C transferrin and iron uptakes by the liver were markedly decreased compared with perfusion at 37 °C, there was negligible uptake into the fractions of density 1.10–1.15 g/cm³, but some transferrin and iron were associated with a more-dense region of the gradient from 1.15 to 1.21 g/cm³ (Fig. 7a). This corresponds to the distribution of 5'-nucleotidase (Fig. 1).

Release of transferrin and iron from the liver

In order to investigate whether the transferrin and iron taken up during a 5 min perfusion period could be released from the liver, four perfusions were performed with a 20 min post-perfusion period instead of the standard 2.5 min. Non-radioactive diferric transferrin was added to the post-perfusion solution at a concentration of 1.25 μM in two of these perfusions but was not included in the other two. The results from one each of these two types of perfusions are illustrated in Figs. 7(b) and 8. The other perfusions gave similar results. The concentrations of ⁵⁹Fe and ¹²⁵I in the post-perfusion fluids fell rapidly during the first 2 min as the radioactive perfusate was washed out of the liver (Fig. 8). After that time there were differences with respect to the two radioisotopes and to whether or not transferrin was added to the post-perfusion solution. In each case the concentration of ¹²⁵I in this solution was greater than that of ⁵⁹Fe, the difference being greater when non-radioactive transferrin was added to the solution. After post-perfusion for 8–10 min without added transferrin the concentrations of ⁵⁹Fe and ¹²⁵I in the solutions stabilized and thereafter

Fig. 6. Distribution of radiolabelled rat serum albumin and transferrin (a) and of insulin and transferrin (b) in density-gradient fractions from rat liver

The livers were perfused for 5 min with each pair of proteins. Transferrin (bold line) was labelled with ^{131}I , and the other proteins (fine line) were labelled with ^{125}I . Transferrin and albumin concentrations were $0.25\ \mu\text{M}$, and the insulin concentration was $48\ \mu\text{M}$.

fell very slowly. In contrast, when added transferrin was present the concentrations of the two radioisotopes continued to decline throughout the period of the experiment, initially being greater than when no transferrin was added but subsequently falling to lower values. Approx. 95% of the ^{125}I in each post-perfusion solution was precipitated with 10% trichloroacetic acid (mean 95.2%, range 93.4–96.3%), compared with 98% in the original radioactive perfusion solution (mean 98.1%, range 98.6–97.7%). The percentage of ^{125}I precipitated with trichloroacetic acid did not increase during the post-perfusion period.

Subcellular fractionation of the liver after 20 min post-perfusion showed that there had been a marked decline in hepatic ^{125}I and a lesser decline in ^{59}Fe (Fig. 7b). The total ^{125}I values fell to 46 and 18% and the ^{59}Fe values to 70 and 61% of the control values with transferrin-free and transferrin-added post-perfusate solutions respectively.

Release of transferrin from the low-density-vesicle fraction

Portions of the liver from the experiment described above were homogenized in the standard manner and a low-density-vesicle fraction was prepared by centrifugation in a discontinuous sucrose density gradient as described previously (Pease *et al.*, 1984). The vesicle fraction was suspended in $0.25\ \text{M}$ -sucrose/Hepes, pH 7.4, and was divided into four aliquots. The pH of two aliquots was adjusted to 5.2 by the addition of $0.1\ \text{M}$ -HCl, and the detergent Brij 35 (Sigma Chemical Co.) was added to a concentration of $1\ \text{mg/ml}$ to one aliquot at each pH value (7.4 and 5.2). The four samples were then centrifuged at $100\,000\ g$ for 1 h. Radioactivity was

counted in the supernatant and sedimented fractions. The entire procedure was performed at $4\ ^\circ\text{C}$. At pH 7.4 the addition of Brij 35 caused an increase in the release of ^{125}I -transferrin into the supernatant fraction from 20% to 61%. However, at pH 5.2 transferrin release in the absence of Brij was decreased to about 13%, and to an even lower value in its presence (7.1%).

Autoradiography

In both experiments the majority of the autoradiography grains were found to lie over hepatocytes, with few over endothelial cells and virtually none on Kupfer cells. Examination of the autoradiographs from the liver that had been perfused with ^{125}I -transferrin for 8 min showed 127 grains in 114 hepatocytes but only three grains in 53 endothelial cells. The distribution of grains in the experiment conducted *in vivo* is summarized in Table 2. As early as 1 min after the injection the majority of the grains were over hepatocytes and at no time were more than 10% found over endothelial cells. Furthermore, many of the grains scored as being in the sinusoids and space of Disse were over microvilli on the sinusoidal membrane of hepatocytes and probably indicate the location of transferrin molecules in the process of being taken up by hepatocytes.

DISCUSSION

Radioiodinated transferrin was rapidly taken up by the liver and was localized mainly into a low-density membrane fraction of the cells. This fraction has been shown to contain large and small vesicles and tubular elements, many of which have the appearance of being

Fig. 7. Distribution of ^{125}I -transferrin and ^{59}Fe in density-gradient fractions from rat liver after perfusion at 37 °C or 4 °C (a) and after perfusion at 37 °C followed by 2.5–20 min post-perfusion (b)

In (a) the perfusions were performed for 5 min followed by 2.5 min post-perfusion at 37 °C (bold line) or 4 °C (fine line) with 25 μM diferric transferrin. (b) shows the results for a control perfusion (bold line) and for a liver of the perfusion illustrated in Fig. 8, after 20 min post-perfusion, with the addition of unlabelled transferrin to the post-perfusion solution (fine line). The results obtained after 20 min post-perfusion in the absence of added transferrin were intermediate between those shown in the Figure.

derived from the Golgi complex (Pease *et al.*, 1984). The fractions enriched with ^{125}I -transferrin contained the Golgi-membrane marker enzyme galactosyltransferase, but could be partly differentiated from that enzyme marker by treatment with digitonin. These properties are similar to those attributed to ligandosomes, membranes implicated in the intracellular processing of insulin, glucagon and prolactin (Smith *et al.*, 1980; Smith & Peters, 1982; Bassett *et al.*, 1984). However, it is not known whether transferrin is translocated to exactly the same membranes as the polypeptide hormones, although the density distributions of transferrin and insulin were found to be extremely similar. The ultimate fates of transferrin and the hormones are very different, transferrin being released from the cells with little evidence of degradation, whereas insulin is extensively degraded.

In the present work the ^{125}I -transferrin-rich fractions are referred to as 'low-density vesicles'.

The results of the autoradiographic studies show that most of the transferrin taken up by the liver was in hepatocytes, not in Kupffer cells or sinusoidal endothelial cells. This is compatible with previous work that demonstrated that transferrin-bound iron taken up by the liver is incorporated almost exclusively into hepatocytes (Hershko *et al.*, 1973) but is in disagreement with a report that transferrin binds only to endothelial cells (Soda & Tavassoli, 1984). The latter conclusion was based on studies with suspensions of isolated rat liver cells. At least two explanations can be advanced for the apparent conflict between the results obtained in that work and in the present investigation. Firstly, transferrin may bind to endothelial cells in the intact liver and be transported to

Fig. 8. Efflux of ^{59}Fe and ^{125}I from two rat livers that had been perfused with ^{59}Fe - ^{125}I -transferrin

The initial perfusion was for 5 min with 0.25 mM diferric radiolabelled transferrin. This was followed by 20 min post-perfusion with oxygenated Krebs-Henseleit Ringer solution either containing no added transferrin (● and ○) or containing 1.25 μM non-radioactive diferric transferrin (■, □). The Figure shows the concentrations of ^{125}I (● and □) and ^{59}Fe (○ and ■) in the post-perfusion effluent solution expressed as percentages of the concentrations in the initial perfusate.

the space of Disse, from which it can be taken up by hepatocytes. Alternatively, the procedures used in the preparation of the suspensions of isolated liver cells may have damaged the hepatocytes so that they could no longer bind transferrin. For the first explanation to be correct it would be necessary to assume that transport through the endothelial cells is very rapid, since ^{125}I -transferrin was found predominantly in hepatocytes as early as 1 min after intravenous injection, and that it leads to a modification of the transferrin molecule so that it becomes able to bind to hepatocytes. The alternative explanation requires that the transferrin receptors on endothelial cells are more resistant to damage than are those on hepatocytes or that the observed binding of transferrin to the endothelial cells is non-specific in nature. With respect to the latter possibility it may be noted that hepatic endothelial cells have been shown to bind non-specifically and endocytose many ligands (Ghitescu & Fixman, 1984).

Several lines of evidence indicate that transferrin was

Table 2. Distribution of autoradiography grains in thin sections of the liver of rats after intravenous injection of ^{125}I -transferrin

Electron-microscope autoradiography was performed as described in the text on samples of liver removed 1, 4, 9 and 20 min after the intravenous injection of 0.5 mg of diferric ^{125}I -transferrin.

Time (min)	No. of autoradiographic grains			
	Sinusoids and space of Disse	Hepatocytes	Endothelial cells	Kupffer cells
1	44	87	14	2
4	96	128	11	0
9	24	173	4	1
20	24	100	3	0

incorporated into the low-density vesicles by receptor-mediated endocytosis. The density distribution of transferrin was the same as that of insulin and that described for glucagon (Smith *et al.*, 1980), both of which have been shown by electron-microscope autoradiography to be taken into hepatocytes by receptor-mediated endocytosis (Bergeron *et al.*, 1977, 1979; Carpentier *et al.*, 1979). The time course of incorporation of transferrin into the low-density vesicles at 37 °C and inhibition of this process at 4 °C are characteristic properties of receptor-mediated endocytosis of many ligands by a variety of cells (Neville & Chang, 1978), including transferrin endocytosis by immature erythroid cells (Morgan & Appleton, 1969; Iacopetta & Morgan, 1983).

In addition to the ^{125}I -transferrin present in the low-density vesicle fractions, smaller amounts were found in the soluble fraction of the cell and in fractions corresponding to the plasma-membrane marker. The former probably represents transferrin released from membrane components during homogenization, and the latter probably represents transferrin bound to receptors present on the plasma membrane. Very little ^{125}I -transferrin was found in the lysosome-containing fractions, and the proportion of cell-associated labelled transferrin in these fractions did not increase with time. Hence it may be concluded that little or none of the endocytosed transferrin is translocated to lysosomes. This may account for the very low degree of transferrin degradation.

The intracellular distribution of endocytosed transferrin was different from that of endogenous transferrin. The latter is synthesized and secreted by the hepatocytes and has been shown to accumulate in the microsomal fraction of the liver (Morgan & Peters, 1971). The present results indicate that it is mainly in the endoplasmic reticulum, with little in the Golgi complex.

Iron was released from transferrin and accumulated by the liver into two broad peaks, probably corresponding to ferritin and mitochondria. The identity of the first of these peaks is indicated by the precipitability of a large proportion of the ^{59}Fe with anti-ferritin serum and the fact that its density distribution is the same as that observed for soluble ferritin added to liver homogenates (Selden *et al.*, 1980). The close correspondence between the density distribution of succinate dehydrogenase and

the second ^{59}Fe peak supports the suggestion that it is due to iron accumulation in mitochondria, although further work is required to confirm this. When the molar ratio of iron and transferrin uptake into the different density fractions was examined it was consistently found to be lower than 2:1 in the low-density membrane fractions. Hence it is probable that iron is released from transferrin while in the low-density vesicles. Possible this is due to acidification of the vesicles, as has been shown to be the case in immature erythroid cells (Paterson *et al.*, 1984) and transformed cells (van Renswoude *et al.*, 1982).

The effects of increasing concentrations of diferric transferrin in the perfusion solution on transferrin and iron uptake by the liver suggest that two processes are involved, one saturable at transferrin concentrations of 0.5–1.0 μM and the other non-saturable within the range of concentrations tested. Similar conclusions have been reached in studies with liver-cell suspensions and cultured hepatocytes (Cole & Glass, 1983; Page *et al.*, 1984).

Both the saturable and the non-saturable iron-uptake processes led to transferrin incorporation into low-density vesicles from which rat serum albumin and bovine serum albumin were excluded. Hence both of the transferrin-uptake processes show specificity, probably due to interaction with specific binding sites or receptors on the cell membrane. Also, both led to iron accumulation by the cells. Analysis of the transferrin-uptake data by a non-linear curve-fitting program indicated that they best fitted a model based on transferrin binding to a limited number of high-affinity receptors plus 'non-specific' binding. The latter component of the model is due to the non-saturable binding of transferrin at concentrations above 1 μM . However, the fact that transferrin uptake into the low-density vesicles and iron uptake increased more than 2-fold as the transferrin concentration was raised from 1 to 10 μM , although albumin was excluded from the vesicles, indicates that the term 'non-specific' is not applicable. It would be better to refer to this component of the uptake process as low-affinity specific uptake. Whether or not it is due to a finite number of binding sites that could be saturated at very high transferrin concentrations is unknown. However, because of the apparent lack of saturability it is not possible to calculate the affinity and number of binding sites involved in the low-affinity uptake of transferrin.

The apparent association constant of binding of transferrin to the high-affinity binding sites was calculated to be $5.2 \times 10^6 \text{ M}^{-1}$ and the number of sites was 57 pmol/g of liver. The properties of these receptors are similar to those of immature erythroid cells (Ecarot-Charrier *et al.*, 1980; Iacopetta *et al.*, 1982; Morgan, 1983), in that binding occurs with approximately the same association constant and appears to be more stable at pH 5.2 than at 7.4. The number of binding sites corresponds to approx. 270000 univalent receptors per cell, assuming that transferrin binding is confined to hepatocytes and that the number of hepatocytes per g of liver is 128×10^6 (Seglen, 1973). These results should be compared with reported values for the binding affinity of 1.0×10^7 – $15 \times 10^7 \text{ M}^{-1}$ and receptor numbers varying from zero to 162000 per cell for liver-cell suspensions (Young & Aisen, 1980; Cole & Glass, 1983; Morley & Bezkorovainy, 1983; Thorstensen & Romslo, 1984a; Tei *et al.*, 1984). The reasons for the differences between the present results and those in the literature are uncertain. They may reflect the different methods employed, but this is unlikely to

account for the great differences observed, especially in the number of binding sites. One possible explanation is the same as that mentioned above, namely that during preparation of liver-cell suspensions the plasma membranes became damaged with loss and/or alteration of transferrin receptors.

Thorstensen & Romslo (1984b) published evidence for the presence of high-affinity and low-affinity transferrin receptors on rat liver cells in suspension. Binding to the low-affinity sites could be prevented by inclusion of bovine serum albumin in the incubation medium at concentrations of 0.4–0.6 g/100 ml. Since bovine serum albumin was included in the perfusion solution used in the present work in this concentration range it is unlikely that the low-affinity binding is of the same nature as that described by Thorstensen & Romslo (1984b).

Radioiodinated transferrin and a smaller proportion of the ^{59}Fe were both released from the liver during post-infusion. After 20 min perfusion with a medium containing non-radioactive transferrin about 90% of the ^{125}I had been released, mostly in a trichloroacetic acid- and anti-transferrin-precipitable form. Hence most of the transferrin taken up by the liver appears to be recycled to the perfusion solution in a non-degraded form but with lower iron content. However, there was an increase in the percentage solubility of the ^{125}I in trichloroacetic acid from 2% in the perfusion solution to 3–10% in the liver-cell fractions and 5% in the post-perfusion solution, suggesting that a low degree of transferrin degradation occurs in its cycle through the liver cells. This is in keeping with previous observations on the rate of transferrin degradation by the perfused rat liver (Hoffenberg *et al.*, 1970).

Less transferrin and iron were released from the liver when the post-perfusion solution was free of added transferrin than when it was present. This may be due to rebinding and recycling of transferrin in the absence of added unlabelled transferrin to compete for the receptor sites.

The fact that some ^{59}Fe was released from the liver is indicative of the reversible nature of iron exchange between this organ and plasma transferrin. It may also be due to incomplete release of iron from transferrin as the transport protein cycles through the low-density vesicles. As a result of previous studies with the perfused liver (Baker *et al.*, 1980) and isolated hepatocytes (Baker *et al.*, 1981, 1985) it was concluded that iron released from transferrin enters a labile compartment in the liver from which it can proceed to ferritin or other intracellular iron compounds, or be released from the cells to be rebound by transferrin. The results of the present investigation support this conclusion and indicate that iron in the labile pool may very quickly be incorporated into ferritin or into mitochondria.

Evidence for transferrin endocytosis by normal liver cells has not previously been reported, although some authors have assumed, without evidence, that they were dealing with such a process for studying isolated liver cells (Sibille *et al.*, 1982; Young *et al.*, 1983). Previous studies using the subcellular-fractionation technique after intravenous injection of radiolabelled transferrin failed to provide any evidence for transferrin endocytosis or iron transport to mitochondria (Milsom & Batey, 1979; Batey *et al.*, 1980). The reasons for the discrepancies between these and the present results probably lie in the fractionation techniques that were employed. However,

the pattern of results described in the present work was found in every fractionation of liver performed after perfusion with radiolabelled transferrin at 37 °C or after intravenous injections. Moreover, the effects of temperature, of time and of post-perfusion and comparison with insulin and albumin all combine to indicate the validity of the present experiments and the conclusions based on the results obtained.

On the basis of the present investigation it may be concluded that the uptake of the transferrin-bound iron by the liver involves receptor-mediated endocytosis of the iron-transferrin complex to a low-density vesicle compartment of hepatocytes where the iron is released from transferrin. This is followed by recycling the transferrin to the cell membrane and its release into the perfusing solution or blood. There are both high-affinity and low-affinity transferrin receptors, the latter being present in very large numbers. Transferrin endocytosis and iron uptake follow binding to either type of receptor. As a consequence the rate of iron uptake by the liver increases as the iron-transferrin concentration is raised. This is probably the reason for the high degree of hepatic deposition of iron and the susceptibility of the liver to iron overload in conditions associated with high concentrations of serum iron (Zimelman *et al.*, 1977; Bothwell *et al.*, 1979).

REFERENCES

- Baker, E., Morton, A. & Tavill, A. S. (1980) *Br. J. Haematol.* **45**, 607–620
- Baker, E., Vicary, F. R. & Huchns, E. R. (1981) *Br. J. Haematol.* **47**, 493–504
- Baker, E., Page, M. & Morgan, E. H. (1985) *Br. J. Haematol.* **47**, 493–504
- Bassett, M., Smith, G. D., Pease, R. & Peters, T. J. (1984) *Biochim. Biophys. Acta* **769**, 79–84
- Batey, R. G., Williams, K. & Milsom, J. P. (1980) *Am. J. Physiol.* **238**, G30–G33
- Bergeron, J. J. M., Levine, G., Sikstrom, R., Kopriwa, B., Nadler, N. J. & Posner, B. I. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5051–5055
- Bergeron, J. J. M., Sikstrom, R., Hand, A. R. & Posner, B. I. (1979) *J. Cell. Physiol.* **80**, 427–443
- Bothwell, T. H., Charlton, R. W., Cook, J. H. & Finch, C. A. (1979) *Iron Metabolism in Man*, p. 334, Blackwell, Oxford
- Carpentier, J.-L., Gorden, P., Barazzoni, P., Freychet, P., Le Cam, A. & Orci, L. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2803–2807
- Charley, P., Rosenstein, M., Shore, E. Y. & Saltman, P. (1960) *Arch. Biochem. Biophys.* **88**, 222–226
- Cheney, B. A., Lothe, K., Morgan, E. H., Sood, S. K. & Finch, C. A. (1967) *Am. J. Physiol.* **212**, 376–380
- Christensen, J. R., Smith, G. D. & Peters, T. J. (1985) *Cell Biochem. Funct.* **3**, 13–19
- Ciechanover, A., Schwartz, A. L., Dautay-Varsat, A. & Lodish, H. F. (1983) *J. Biol. Chem.* **258**, 9681–9689
- Cole, E. S. & Glass, J. (1983) *Biochim. Biophys. Acta* **762**, 102–110
- Ecarot-Charrier, B., Grey, V. L., Wilczynska, A. & Schulman, H. M. (1980) *Can. J. Biochem.* **58**, 418–426
- Gardiner, M. E. & Morgan, E. H. (1974) *Aust. J. Exp. Biol. Med. Sci.* **52**, 723–736
- Ghitescu, L. & Fixman, A. (1984) *J. Cell Biol.* **99**, 639–647
- Grohlich, D., Morley, C. G. D., Miller, R. J. & Bezkorovainy, A. (1977) *Biochem. Biophys. Res. Commun.* **76**, 682–690
- Grohlich, D., Morley, C. G. & Bezkorovainy, A. (1979) *Int. J. Biochem.* **10**, 797–802
- Hemmaplardh, D. & Morgan, E. H. (1974) *Biochim. Biophys. Acta* **373**, 84–99
- Hershko, C., Cook, J. D. & Finch, C. A. (1973) *J. Lab. Clin. Med.* **81**, 876–886
- Hoffenberg, R., Gordon, A. H., Black, E. G. & Louis, L. N. (1970) *Biochem. J.* **118**, 401–404
- Huebers, H., Huebers, E., Rummel, W. & Crichton, R. R. (1976) *Eur. J. Biochem.* **66**, 447–455
- Huebers, H., Heubers, E., Csiba, E. & Finch, C. A. (1978) *J. Clin. Invest.* **62**, 944–951
- Iacopetta, B. J. & Morgan, E. H. (1983) *J. Biol. Chem.* **258**, 9108–9115
- Iacopetta, B. J., Morgan, E. H. & Yeoh, G. C. T. (1982) *Biochim. Biophys. Acta* **687**, 204–210
- Leighton, F., Poole, B., Beaufray, H., Baudhuin, P., Coffey, J. W., Fowler, S. & de Duve, C. (1968) *J. Cell Biol.* **37**, 482–513
- Mancini, G., Carbonara, A. O. & Heremans, J. F. (1965) *Immunocytochemistry* **2**, 235–254
- Mazur, A., Green, S. & Carleton, A. (1960) *J. Biol. Chem.* **235**, 595–603
- McFarlane, A. S. (1963) *J. Clin. Invest.* **42**, 346–361
- Milsom, J. P. & Batey, R. G. (1979) *Biochem. J.* **182**, 117–125
- Morgan, E. H. (1966) *Am. J. Physiol.* **211**, 1486–1494
- Morgan, E. H. (1979) *Biochim. Biophys. Acta* **580**, 312–326
- Morgan, E. H. (1981) *Mol. Aspects Med.* **4**, 1–122
- Morgan, E. H. (1983) *Biochim. Biophys. Acta* **762**, 498–502
- Morgan, E. H. & Appleton, T. C. (1969) *Nature (London)* **223**, 1371–1372
- Morgan, E. H. & Peters, T. (1971) *J. Biol. Chem.* **246**, 3508–3511
- Morley, C. G. D. & Bezkorovainy, A. (1983) *Clin. Physiol. Biochem.* **1**, 318–328
- Munson, P. J. & Rodbard, D. (1980) *Anal. Biochem.* **107**, 220–239
- Neville, D. M. & Chang, T.-M. (1978) *Curr. Top. Membr. Transp.* **10**, 66–150
- Page, M. A., Baker, E. & Morgan, E. H. (1984) *Am. J. Physiol.* **246**, G26–G33
- Paterson, S., Armstrong, N. J., Iacopetta, B. J., McArdle, H. J. & Morgan, E. H. (1984) *J. Cell. Physiol.* **120**, 225–232
- Pease, R. J., Sharp, G. A., Smith, G. D. & Peters, T. J. (1984) *Biochim. Biophys. Acta* **774**, 56–66
- Seglen, P. O. (1973) *Exp. Cell Res.* **82**, 391–398
- Selden, C., Owen, H., Hopkins, J. M. P. & Peters, T. J. (1980) *Br. J. Haematol.* **44**, 593–603
- Shacterle, G. L. & Pollock, R. L. (1973) *Anal. Biochem.* **51**, 654–655
- Sibille, J.-C., Octave, J.-N., Schneider, Y.-J., Trouet, A. & Crichton, R. R. (1982) *FEBS Lett.* **150**, 365–369
- Smith, G. D. & Peters, T. J. (1980) *Eur. J. Biochem.* **104**, 305–311
- Smith, G. D. & Peters, T. J. (1982) *Biochim. Biophys. Acta* **716**, 24–30
- Smith, G. D., Evans, W. H. & Peters, T. J. (1980) *FEBS Lett.* **120**, 104–106
- Soda, R. & Tavassoli, M. (1984) *Blood* **63**, 270–276
- Tei, I., Makino, Y., Kadofuku, T., Kanamaru, I. & Konno, K. (1984) *Biochem. Biophys. Res. Commun.* **121**, 717–721
- Thorstensen, K. & Romslo, I. (1984a) *Biochim. Biophys. Acta* **804**, 200–208
- Thorstensen, K. & Romslo, I. (1984b) *Biochim. Biophys. Acta* **804**, 393–397
- van Renswoude, J., Bridges, K. R., Harford, J. B. & Klausner, R. D. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6186–6190
- Young, S. P. & Aisen, P. (1980) *Biochim. Biophys. Acta* **633**, 145–153
- Young, S. P., Bamford, A. & Williams, R. (1983) *J. Biol. Chem.* **258**, 4972–4976
- Zimelman, A. P., Zimmerman, H. J., McLean, R. & Weintraub, L. R. (1977) *Gastroenterology* **72**, 129–131