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Haem Transport to the Liver by Haemopexin

RECEPTOR-MEDIATED UPTAKE WITH RECYCLING OF THE PROTEIN

By Ann SMITH and William T. MORGAN
Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, CA 92037, *U.S.A.*

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Rat [^{59}Fe]haem–^{125}I-labelled haemopexin complexes (700pmol/rat) associate rapidly and exclusively with the liver after intravenous injection into anaesthetized rats. The two isotopes exhibit different patterns of accumulation. Liver ^{125}I-labelled haemopexin is maximum 10min after injection (20±4.9pmol/g of liver) and then declines by 2h to the low values (about 3pmol/g of liver) seen after injection of the apoprotein. In contrast, [^{59}Fe]haem accumulates in the liver for at least 2h. Haemopexin undergoes no extensive proteolysis during 2h of haem transport as shown by precipitation with acid (98%) and specific antiserum (92%) and by electrophoresis. Moreover, only 1–2% of the dose is located in extrahepatic tissues, and there is no significant urinary excretion of either ^{125}I or ^{59}Fe. Hepatic uptake at 10min is saturable, reaching 200pmol of haemopexin/g of liver and 350pmol of haem/g of liver at a dose of 9nmol/rat, whereas uptake of the apoprotein is 3–5% of the dose. This suggests that the interaction of haem–haemopexin with the liver is a specific receptor-mediated process. The complex probably interacts via the protein moiety, since the haem analogues mesohaem and deuterohaem do not affect association of the protein with the liver but the species of haemopexin does. Increasing amounts of protein are associated with the liver 5min after injection in the order: human > rabbit > rat, and haem uptake is consistently increased. For both rat and rabbit haemopexin saturation is reached at the same concentration of protein, i.e. 180–200pmol/g of liver, indicating that the different protein species bind to a common receptor. We propose that haemopexin transports haem to the liver by a specific receptor-mediated process and then returns to the circulation.

Three serum transport proteins, haptoglobin, haemopexin and albumin, are involved in the catabolism of the haem moiety of haemoglobin. In haemolytic states when haptoglobin becomes depleted, haem dissociated from circulating methaemoglobin is bound by albumin and haemopexin and is transported to the liver as the haem–haemopexin complex. Association of both haemopexin and haem with liver parenchymal cells has been shown qualitatively by radioautography (Muller-Eberhard *et al.*, 1970; Liem *et al.*, 1975*a*) and semi-quantitatively (Liem, 1974) after injection of labelled haem or haemopexin. However, basic questions, such as whether, after interaction at the cell surface, haem and haemopexin enter the cell simultaneously or whether the protein recycles, were not directly addressed.

A conformational change in haemopexin on binding haem (Morgan & Muller-Eberhard, 1972) has been proposed to be necessary for recognition of the haem–protein complex by a receptor on the liver cell surface (Morgan, 1976). We have been investigating haemopexin-mediated haem uptake by the liver, considering among other things the time course, capacity and saturation of the process. The work described here using [^{59}Fe]haem bound to rat, rabbit or human ^{125}I-labelled haemopexin extends our previous studies (Smith & Morgan, 1978) in defining the role of haemopexin in haem transport and haem uptake by the liver.

We present evidence for a rapid receptor-mediated interaction of equimolar haem–haemopexin complexes with the liver, after which haem enters the cells and haemopexin returns intact to the circulation.

Materials and Methods

Protein purification, iodination and characterization

Haemopexin was purified from rabbit serum as previously described (Hrkal & Muller-Eberhard, 1971) by using $HClO_4$ precipitation, followed by chromatography on DEAE-cellulose (Whatman DE-52) and from human and rat serum by poly(ethylene glycol) precipitation, and chromatography on DEAE-cellulose and on wheat-germ lectin–Sepharose (Pharmacia) (Vretblad & Hjorth, 1977).

The purity of haemopexin preparations was assessed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Laemmli, 1970) and immunological analysis with antisera to rabbit, human or rat haemopexin and serum. Concentrations of apo-haemopexin and haem–haemopexin solutions were determined spectrophotometrically by using published absorption coefficients for the human and rabbit proteins (Seery *et al.*; 1972). The absorption coefficients used for rat haemopexin were 110mM·cm^{-1} at 280nm for the apoprotein and 120mM·cm^{-1} at 280nm and 110mM·cm^{-1} at 414nm for the haem protein. For storage, the protein solutions were quickly frozen in liquid N_2 and kept at −20°C.

Proteins were labelled with ^{125}I by using 0.5–1.0mg/ml protein solutions and 20μg of chloramine-T and 500μCi of ^{125}I per mg of protein (McConahey & Dixon, 1966); they were then dialysed for 24h against phosphate-buffered saline [10mM-sodium phosphate (pH7.4)/0.15M-NaCl]. At least 95% of the ^{125}I was precipitable with 12.5% (w/v, final concentration) trichloroacetic acid, with 1mg of bovine serum albumin/ml used as a co-precipitant. No evidence for altered immunological, biological or haem-binding properties of ^{125}I-labelled haemopexin was found. In some experiments ^{125}I-labelled haemopexin was mixed with unlabelled haemopexin. Specific radioactivities (c.p.m./mol) of apo-haemopexin and haem–^{125}I-labelled haemopexin were calculated from the absorbance and radioactivity of each solution. ^{125}I and ^{59}Fe (see below) were measured in a Nuclear-Chicago model 1185B well-type γ-counter. Quantitative immunoprecipitation of ^{125}I-labelled haemopexin was carried out as previously described (Kida & Muller-Eberhard, 1975).

Asialofetuin was prepared from fetuin (Calbiochem, La Jolla, CA, U.S.A.) as described by Conway *et al.* (1975). Asialofetuin, fetuin and human serum albumin (Behringwerke A.G., Marburg, W. Germany) were labelled with ^{125}I by the chloramine-T method (McConahey & Dixon, 1966).

Preparation of [^{59}Fe]haem and [^{59}Fe]haem–^{125}I-labelled haemopexin

^{59}Fe (ICN, Irvine, CA, U.S.A.; sp. radioactivity 10–30Ci/g) was inserted into protoporphyrin-IX (Porphyrin Products, Logan, UT, U.S.A.) under refluxing in dimethylformamide (Adler *et al.*, 1970). The concentration of the [^{59}Fe]haem was measured in dimethyl sulphoxide (Brown & Lantzke, 1969).

[^{59}Fe]haem–^{125}I-labelled haemopexin complexes were prepared by mixing 1.3equiv. of haem with ^{125}I-labelled haemopexin and incubating at ambient temperature for 1h. Excess haem and iron were removed by adsorbing the haem–haemopexin on to DEAE-cellulose equilibrated with 15mM-sodium phosphate buffer, pH7.4, and eluting with phosphate-buffered saline. The absorbance and radioactivity of the eluted haem protein were determined. The saturation of haemopexin (>90%) with haem was assessed, and the integrity of the complex was confirmed by the characteristic absorption spectrum of haem–haemopexin (Seery *et al.*, 1972). Unlabelled haem–^{125}I-labelled haemopexin was prepared similarly.

Unlabelled haem (defined here as iron-protoporphyrin-IX) was from Eastman Organic Chemicals, Rochester, NY, U.S.A.; deutero- and meso-haem were from Porphyrin Products. The concentrations of haem solutions were determined in dimethyl sulphoxide (Brown & Lantzke, 1969) or with pyridine haemochrome (Falk, 1964).

Animals

Male inbred Wistar rats (120–140g; specified pathogen-free; Hilltop Lab. Animals, Chatsworth, CA, U.S.A.) were allowed food (Purina Rat Chow) and water *ad libitum*.

Experimental design

The uptake by liver *in vivo* of apo-haemopexin and haem–haemopexin was studied in anaesthetized rats (pentobarbital; 0.15 grain/rat; intraperitoneal injection); body temperature was maintained by warming with an i.r. lamp. The protein in phosphate-buffered saline was injected into a lateral tail vein, and blood samples (100–200μl) were taken into calibrated heparinized capillary tubes after making a small cut across the tail artery. Serum was separated by centrifuging for 2min in a Beckman 'microfuge' (model 152) and rinsing a measured portion into phosphate-buffered saline (500μl) for direct counting of ^{125}I and/or ^{59}Fe. Serum samples for electrophoretic and immunological analysis of haemopexin were frozen with liquid N_2 and stored at −20°C.

Rats were killed by cervical dislocation; after decapitation the livers were immediately perfused (within 10s) through the portal vein with 50ml of ice-cold 0.15M-NaCl (at 15–20ml/min). Weighed samples of liver (two or three of about 1g each) were taken for direct counting of radioactivity; preliminary experiments established that there were no differences in uptake among the various lobes. Within an individual experiment two rats were used for each point, and each experiment was repeated. Radioactivity in unperfused organs, including kidney, lungs, bladder and spleen, was measured by counting the whole organ in a γ-counter.

Experiments with fetuin, asialofetuin and human serum albumin

We tested the experimental system used in this work with ^{125}I-labelled asialofetuin, ^{125}I-labelled fetuin and ^{125}I-labelled human serum albumin (specific radioactivities, 3×10^{16}–7×10^{16}c.p.m./mol). Uptake by liver and metabolism of these circulating

iodinated proteins were examined in anaesthetized rats by direct counting of liver-associated ^{125}I in the perfused organ and trichloroacetic acid-precipitable radioactivity in the blood and/or serum. The radioactivity (c.p.m./ml) in blood or serum samples taken 2min after injection, to allow for circulation time, was used to calculate an equilibrium circulation value (assuming no uptake and no intra-/extravascular equilibration). That is, the injected dose divided by serum and/or blood volume [4.2ml of serum/100g body wt. (Cunningham, 1975) or 7ml of blood/100g body wt. (Van Putten, 1958)] was expressed as a percentage of the observed acid-precipitable c.p.m./ml. The values for all proteins except asialofetuin routinely ranged from 90 to 110% of the theoretical value for the blood and were slightly lower for serum.

In agreement with LaBadie *et al.* (1975), rapid uptake by liver and metabolism of asialofetuin was shown; i.e., all detectable radioactivity from a tracer dose of 150pmol of asialofetuin was located in the liver by 5min ($t_{\frac{1}{2}}$ 140s). In contrast, when 360pmol of fetuin was injected, only 11% was in the liver by 15min. In addition, 4% ($n = 5$) of albumin was found in the liver by 15min, with the rest remaining in the circulation as trichloroacetic acid-precipitable material.

Results

Time course of uptake by liver of rat apo-haemopexin and [^{59}Fe]haem–^{125}I-labelled haemopexin

Distinct differences in the pattern of liver-associated ^{125}I were seen, depending on whether haemopexin was complexed with haem as shown in Fig. 1(*a*). Uptake by liver after injection of a tracer dose (700pmol) of apo-haemopexin gradually increased to 4pmol/g of liver by 10–15min and then declined to about 2pmol/g of liver. This was similar to the concentrations found for albumin, another serum protein metabolized by the liver. In contrast, after injection of the same amount of doubly labelled haem–haemopexin complexes, the liver ^{125}I increased rapidly to a maximum of 20±4.9pmol/g of liver at 10min, which is several times the concentration of apoprotein. By 1h the hepatic ^{125}I concentrations were less than 8pmol/g of liver and continued to decrease. Since normal circulating concentrations of haemopexin are 20–30μM, the 700pmol dose, giving a serum concentration of 0.1–0.2μM, represents less than 1% of the endogenous protein.

[^{59}Fe]Haem quickly associated with the liver and continued to accumulate, reaching 70pmol/g of liver at 1h (Fig. 1*a*). This represents 60% of the injected dose; the remaining ^{59}Fe was in the circulation. Thus the ratio of $^{59}Fe/^{125}I$ in the liver was 1:1 at 1min after injection, and increased to 2:1 by 10min and to 10:1 by 60min. The high affinity of haemopexin for haem ($K_d < 1$ pM) ensured that haem did not spontaneously dissociate from the protein during the experimental period.

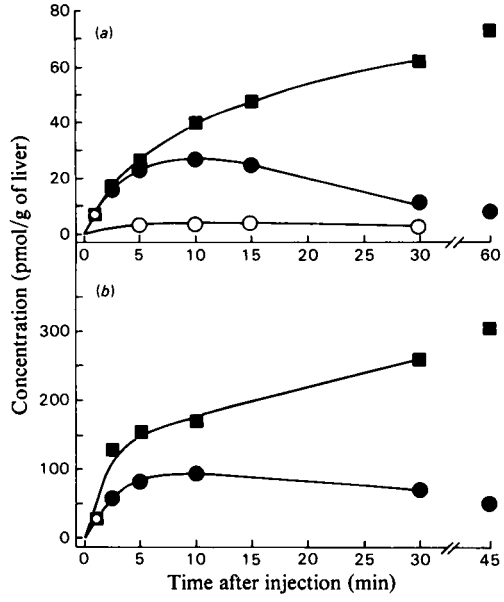

Fig. 1. *Time course of ^{59}Fe and ^{125}I uptake in rat liver after injection of ^{125}I-labelled apo-haemopexin or equimolar [^{59}Fe]haem–^{125}I-labelled haemopexin complexes*

(*a*) Rats were injected with 700pmol of either rat ^{125}I-labelled apo-haemopexin or [^{59}Fe]haem–^{125}I-labelled haemopexin and killed at the indicated times. The results are from a representative experiment with male rats and each point represents an average of two animals. Further details are given in the Materials and Methods section. (*b*) Rats were injected with 4nmol of [^{59}Fe]haem–^{125}I-labelled haemopexin and killed at the indicated times. Further details are given in the Materials and Methods section. ○, Apo-haemopexin, ^{125}I; ●, haem-haemopexin, ^{125}I; ■, haem-haemopexin, ^{59}Fe.

The transport of haem by haemopexin appeared to be specific for the liver. Within the first 15min, less than 1–2% of the dose of ^{125}I-labelled apo-haemopexin or [^{59}Fe]haem–^{125}I-labelled haemopexin was located in unperfused lungs, spleen or kidney or had been excreted in the urine. Radioactivity that was not detected in these organs was accounted for in the circulation. The average total recovery in liver and serum of the injected dose was 86±10% for ^{125}I and 86±8% for ^{59}Fe for each animal. Serum ^{125}I was both trichloroacetic acid- (98%) and immuno- (92%) precipitable for up to 1h (discussed in detail below).

Liver ^{59}Fe (about 400pmol total) was maintained

for at least 1–2h. During this time the ^{59}Fe content of spleen increased from 8pmol at 10min to 14pmol, whereas the ^{125}I decreased from 6 to 2pmol. In bone marrow [estimated by multiplying the radioactivity in the femur by 14.4 (Garby & Obara, 1960)], the initial $^{59}Fe/^{125}I$ ratio was approx. 1:1 (15pmol), and remained constant for 2h, whereas in the kidneys the ratio, although constant, was about 3:1 (10pmol:3pmol). Only a trace of ^{59}Fe was found in the urine, and the total ^{125}I excreted was about 18pmol at 2h. The state of the ^{125}I in the urine, i.e. whether attached to protein or catabolic products such as iodotyrosine, was not determined. Serum concentrations of injected ^{125}I-labelled haemopexin decreased to 50pmol of protein/ml of serum in 2h. This was probably due mainly to equilibration with the extravascular compartment rather than to catabolism, considering the low ^{125}I content of the urine. The relatively low specific radioactivity of ^{59}Fe available to label the haem precluded a definitive analysis of whether any haemopexin-mediated haem uptake by extrahepatic tissues occurred; if it does occur it is small compared with uptake by the liver.

Influence of increasing the amount of injected apo-haemopexin and haem–haemopexin

The uptake by liver of apo-haemopexin was a constant proportion of the injected dose (range 200–11000pmol) averaging 3% at 5min (Fig. 2*a*) and 5% at 10min (Fig. 2*b*) after injection. However, increasing the dose of haem–haemopexin increased the amount of protein associated with the liver 10min after injection up to a plateau of about 200pmol/g of liver (Fig. 2*b*). The [^{59}Fe]haem uptake was greater than that of the protein, reaching 350pmol/g of liver at 10min. Saturation of both labels was reached at a dose of 9nmol, producing initial serum concentrations of approx. 1–1.5μM. This difference in ^{125}I and ^{59}Fe plateau values is related to the phenomenon of recycling of the haemopexin, as discussed in more detail below. The pattern of ^{125}I and ^{59}Fe in the liver (Fig. 2*a*) at 5min after injection suggests that saturation of uptake is not reached as readily before the maximum of ^{125}I at 10min (Fig. 1*a*).

We were concerned whether the activity of the protein had been affected by iodination. The uptake of ^{125}I-labelled apo-haemopexin is a rigorous control because the haem–haemopexin complexes are made from the same preparation of apoprotein as used for the control. Since the liver is the site of synthesis and catabolism of most plasma proteins, including haemopexin (Thorbecke *et al.*, 1973), it is likely that the apoprotein can associate with the liver by a route different from the haem complex. In addition, injecting 1mg (17nmol) of unlabelled rabbit haem–haemopexin 2.5 or 5min before a

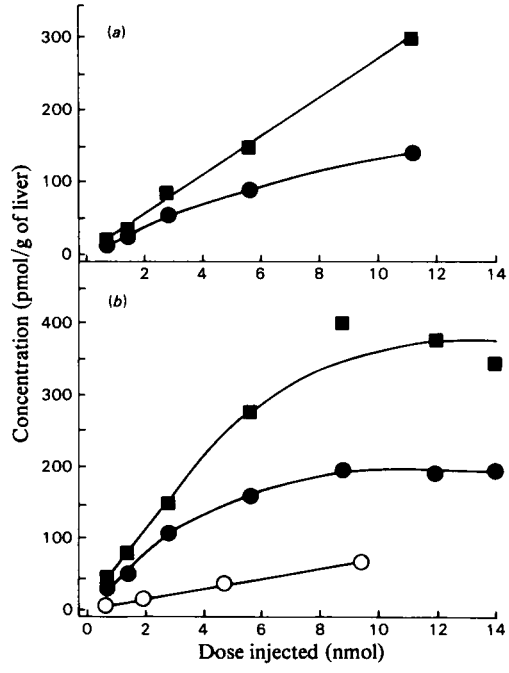

Fig. 2. *Effect of increasing the dose of rat apo-haemopexin or haem–haemopexin on liver uptake*

Rats were injected with the indicated amounts of ^{125}I-labelled apo-haemopexin or [^{59}Fe]haem–^{125}I-labelled haemopexin and killed 5 (*a*) or 10 (*b*)min later. Further details are given in the Materials and Methods section. ○, Apo-haemopexin, ^{125}I; ●, haem–haemopexin, ^{125}I; ■, haem–haemopexin, ^{59}Fe.

tracer dose of rat haem–^{125}I-labelled haemopexin almost completely blocked the uptake of the latter. This indicates that the iodinated protein was binding at the same site as unlabelled material.

Time course of liver uptake of a high dose of haem–haemopexin

We investigated the time course and rate of uptake of a larger amount of complex. As shown in Fig. 1, injection of 4nmol, which is approx. 6 times the dose usually employed, produced the same pattern of uptake over 1h as the smaller dose. The maximum association of protein with the liver again occurred at 10min, but was increased approx. 5-fold for both ^{125}I and ^{59}Fe. Assuming linear uptake for the first 5min, we calculated a rate of association of 11.6pmol of haemopexin/min per g of liver compared with 2.3 for the tracer dose, also a 5-fold increase. Thus the initial rate of hepatic association of protein is proportional to the circulating haem–haemopexin concentration.

Table 1. *Effect of haemopexin species and haem analogues on the haem–haemopexin–liver interaction: comparison with apo-haemopexin and albumin*

Rats were injected with 700pmol of the sample described, and killed at the times indicated. For experiments with more than two animals, the results are expressed as means±S.D. The numbers of animals are in parentheses. Further details are in the Materials and Methods section. Abbreviation: NA, not applicable.

Sample injected	Time (min)	Liver uptake: ^{125}I-labelled haemopexin (pmol/g of liver)	Liver uptake: [^{59}Fe]Haem (pmol/g of liver)	
Rat [^{59}Fe]haem–^{125}I-labelled haemopexin	5	18.5±5.9	23.6±4.3	(4)
	15	21.4±3.2	49.8±5.5	(4)
Rabbit [^{59}Fe]haem–^{125}I-labelled haemopexin	5	28.4±3.3	60.9±8.6	(4)
	15	21.4±3.8	79.2±5.9	(4)
Human [^{59}Fe]haem–^{125}I-labelled haemopexin	5	41.2±4.4	59.9±9.0	(3)
	15	51.0	83.0	(2)
Rat [^{59}Fe]haem–^{125}I-labelled haemopexin	10	20.0±4.9	37.5±2.9	(8)
Rat deuterohaem–^{125}I-labelled haemopexin	10	22.0	NA	(2)
Rat mesohaem–^{125}I-labelled haemopexin	10	22.0	NA	(2)
Human ^{125}I-labelled albumin	5	4.0	NA	(2)
	15	4.0	NA	(2)
Rat ^{125}I-labelled apo-haemopexin	5	3.0	NA	(2)
	10	4.2±1.0	NA	(4)
	15	3.8	NA	(2)

Investigations of the state of haemopexin during haem transport: is the protein altered by its interaction with the liver cell membrane?

Previous experiments (Smith & Morgan, 1978) using rabbit haem–^{125}I-labelled haemopexin complexes showed a reciprocal relationship between the peak of liver-associated ^{125}I and circulating serum concentrations of acid-precipitable ^{125}I. The time course of serum radioactivity indicated that after transporting haem to the liver the protein returned to the circulation.

We used sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and immunoprecipitation with monospecific antibody to haemopexin to test serum samples for alterations in circulating ^{125}I-labelled haemopexin, such as radioactive fragments produced by proteolysis. Serum samples were taken from rats killed at 1, 5, 10, 30 and 60min after injection of rat [^{59}Fe]haem–^{125}I-labelled haemopexin. There was no significant difference between the pattern of ^{125}I on the gels of ^{125}I-labelled haemopexin added to control rat serum *in vitro* and the serum samples. An average of 93% of the ^{125}I applied to the gel was recovered. Moreover, the ^{125}I in all serum samples was precipitable, 92±1.3% (mean±S.D.), with monospecific antibody to rat haemopexin. Any extensive alterations of haemopexin should have been readily detected, since by 30min 50–60% of the dose of [^{59}Fe]haem was located in the liver, so that at least half of the injected haemopexin, of which >80% remained in the serum, had interacted with the liver. This is evident in the decrease in ^{59}Fe/^{125}I in serum from 1:1 at 2min to 0.3:1 by 30min and to 0.14:1 by 2h. More detailed studies (A. Smith & W. T. Morgan, unpublished work) have shown that there were no large changes in the haemopexin molecule during haem transport.

Investigations on the interaction of different species of haemopexin with rat liver

Our earlier studies (Smith & Morgan, 1978) showed that a peak of liver-associated ^{125}I occurred 5min after injection into rats of rabbit haem–^{125}I-labelled haemopexin and was already declining by 10min. We therefore measured the uptake by liver of tracer doses of rat, rabbit and human haem–haemopexin complexes at 5 and 15min after injection. The results are summarized in Table 1.

The amount of rabbit haemopexin associated with the liver was higher than the rat protein 5min after injection (28±3.3pmol/g of liver compared with 19±5.9) and they were the same at 15min. In addition, twice as much human haemopexin was associated with the liver at both times, i.e. 40 and 50pmol/g of liver respectively. Consistent with the higher protein values for rabbit and human haemopexin, the amount of [^{59}Fe]haem in the liver was increased, averaging about 60 and 80pmol/g of liver at 5 and 15min for both rabbit and human haemopexin compared with 24 and 50pmol/g for the rat protein at these times.

Data for the apoprotein control and human serum albumin are also presented in Table 1. Uptake by liver of rat apo-haemopexin at 5min was 4pmol/g of liver, which is the same as for albumin but lower than for the haem–haemopexin complexes. More

radioactivity, averaging 8 pmol/g of liver, was found in the liver using rabbit and human apo-haemopexin (results not shown).

Role of haem in the interaction of haem–haemopexin with the liver

Although it is likely that recognition of the haem–haemopexin complex by a receptor involves the protein moiety primarily, there is a possibility that specific groups on the haem molecule might be involved, at least partially, in the recognition process. We prepared complexes of rat haemopexin with haem analogues differing in their 2,4-substituents: haem (vinyl), mesohaem (ethyl) and deuterohaem (hydrogen). There was no significant difference in the amount of rat haemopexin associated with the liver 10 min after injection of haem–, deuterohaem– or mesohaem–haemopexin complexes (see Table 1). These experiments suggest that small differences in the haem molecule do not affect the interaction of haem–haemopexin with the liver.

Discussion

The physiological function of haemopexin, reviewed by Hershko (1975), is to act together with haptoglobin and albumin to salvage iron from intravascularly released haemoglobin. The possibility that haemopexin might act in the transport of haem from other sources, e.g. myoglobin, remains to be fully examined. The role for haemopexin in haem catabolism was originally proposed from two types of observations: first, that injections of haematin increase the fractional catabolic rate of haemopexin and the plasma clearance of the protein (Lane *et al.*, 1972, 1973; Wochner *et al.*, 1974; Liem *et al.*, 1975*b*); second, that, in certain human haemolytic states, haemopexin concentrations are decreased (Muller-Eberhard, 1970; Engler & Jayle, 1976) and the catabolism of haemopexin is increased (Wochner *et al.*, 1974). However, after injection of haematin, the reported plasma half-clearance times of haemopexin are unusually long, 0.8 day in humans (Wochner *et al.*, 1974), 7–8 h in rabbits (Liem *et al.*, 1975*b*) and 5–6 h in rats (Liem, 1976), compared with other receptor-mediated uptake processes, for example, 10–30 min for haemoglobin–haptoglobin (Garby & Noyes, 1959; Freeman, 1964) and 90–100 s for asialofetuin (LaBadie *et al.*, 1975). On the other hand, the localization of radioactive haem and haemopexin by radioautography only in liver parenchymal cells (Muller-Eberhard *et al.*, 1970) is consistent with the known function of these cells in receptor-mediated uptake (Tolleshaug *et al.*, 1977).

Detailed studies *in vitro* (Bunn & Jandl, 1968; Hrkal *et al.*, 1974; Morgan *et al.*, 1976) have established the relative affinities of globin, apo-haemopexin and albumin for haem. Haem is bound by haemopexin with high affinity, $K_d < 1$ pM (Hrkal *et al.*, 1974); at equilibrium, 50–70% of haemoglobin haem is bound to haemopexin in experiments with equimolar apo-haemopexin and methaemoglobin. Even at physiological ratios of albumin and haemopexin (70 albumin:1 haem:1 haemopexin), haem is transferred over several hours from albumin to haemopexin (Morgan *et al.*, 1976). These studies *in vitro* emphasize the problems inherent in attempting to quantify haem–haemopexin uptake by liver after haematin injection *in vivo*. Intravenous injections of haematin (typically 1–4 mg/kg body wt.; Dhar *et al.*, 1975) produce, in serum, a complicated mixture of haem–protein complexes as well as haem aggregates, which would be removed from the circulation by cells of the reticuloendothelial system, e.g. Kupffer cells.

Although albumin binds haem (K_d about 10 nM; Beaven *et al.*, 1974), it appears only to store haem before transport to the liver as haem–haemopexin. For example, after injection of doubly labelled haemoglobin–haptoglobin or methaemalbumin, a comparison of accumulation of radioactive isotope by the liver and microsomal haem oxygenase activity indicated that haem dissociates from the albumin before its hepatic uptake and catabolism (Bissell *et al.*, 1972).

Further work is necessary to elucidate the nature of the interaction of haem–haemopexin with liver. In this study of the role of haemopexin in haem transport, we initially have asked the following questions. What is the time course of the interaction of haem–haemopexin with the liver? How specific is it? Is the process receptor-mediated? Does the whole ligand–protein complex enter the liver cell, or does haem alone pass through the cell membrane?

We have shown, using small doses of well-defined equimolar rat haem–haemopexin complexes in untreated animals, that haemopexin transports haem to the liver in a manner that is rapid and saturable. Occurring within minutes, the association is on the same time scale as the receptor-mediated uptake of asialoglycoproteins (LaBadie *et al.*, 1975) and of iron–transferrin complexes (Gardiner & Morgan, 1974). Furthermore the association of haemopexin with the liver during haem uptake has been shown to be a saturable process. Saturation is indicative of an interaction with a rate-limiting step and a finite number of binding sites and is characteristic of receptor-mediated uptake.

The hypothesis (Morgan, 1976) that the conformational change (Morgan & Muller-Eberhard, 1972) that haemopexin undergoes on binding haem is necessary for its recognition by a specific receptor molecule on the liver cell surface is supported by the greater association of the haem–haemopexin complex with the liver, compared with the apoprotein. In addition, our results indicate that during the process

of hepatic haem uptake the protein returns to the circulation intact. Firstly, the ratio of $^{59}Fe/^{125}I$ radioactivity in the liver was already 2:1 by 10min; secondly, there was a reciprocal relationship between the liver and serum ^{125}I concentrations (Smith & Morgan, 1978); thirdly, no difference was found in the circulating trichloroacetic acid- and immunoprecipitable haemopexin sampled at various times when most of the haem had been delivered to the liver.

The clearance of haemopexin from plasma after large doses of haematin (Lane *et al.*, 1973; Wochner *et al.*, 1974; Liem *et al.*, 1975*b*) and the lowered concentrations of haemopexin observed in cases of severe haemolysis (Sears, 1970; Muller-Eberhard, 1970) must be reinterpreted in terms of the recycling uptake process demonstrated in this work. Certain aspects of the clearance of haemopexin after intravenous haem administration have been enigmatic until now. For example, although haem binding to haemopexin is equimolar, haemopexin amounts did not decline in molar proportion to the haem dose (Lane *et al.*, 1973; Wochner *et al.*, 1974). Furthermore, as pointed out above, the clearance of haemopexin after injection of haem is slower than that observed for other receptor-mediated uptake processes. Although this might relate in part to the transfer of haem from albumin to haemopexin (Morgan *et al.*, 1976), no effect on the catabolism of haemopexin is expected in view of this work showing that haemopexin acts in a recycling fashion. The reported changes in haemopexin fractional catabolic rate and circulating concentrations suggest that during delivery of haem to the liver, the recycling of the protein may not be 100%, at least under conditions of unusual haem load. However, indirect effects in liver and kidney resulting from circulation of excessive quantities of haem, whether exogenous or haemolytic, cannot be ruled out. A corollary to the recycling mechanism we propose here is that the extent of the function of haemopexin in haem transport is not directly related to its circulating serum concentrations.

The liver-associated radioactivity of the protein and the haem moiety was affected by the species of haemopexin used. The increased amount of rabbit and human protein associated with the liver was accompanied by enhanced haem uptake by liver. It is possible that rabbit and human haemopexin have a higher affinity for the rat liver receptor under the experimental conditions. Consistent with these differences between the three species of haemopexin investigated, the normal plasma clearance of rabbit and human haemopexin was much faster than for the homologous protein in rats (Liem, 1976). Nevertheless, it is likely that the three species of protein interact with a common receptor. The saturation concentrations of haemopexin with the liver during haem transport (about 200pmol/g of liver) were similar for both rat and rabbit haemopexin (Fig. 2*b*; Smith & Morgan, 1978), and rabbit haem–haemopexin complexes blocked rat haem–haemopexin uptake (see above).

The plateau concentration of ^{59}Fe in liver at 60–70% of the injected tracer dose (initial serum concentration of 0.1–0.2μM) is of interest. One explanation can be derived from the affinity of the receptor molecule for the haem–haemopexin complex. Applying Michaelis–Menten kinetics over the first 10min from the saturation experiments (Sando & Neufeld, 1977), we estimate an apparent affinity constant of 0.73μM and an apparent maximum velocity of 54pmol/min per g of liver for rat haem–haemopexin in rats. Thus as the concentration of the complex decreases, the rate of uptake declines. An alternative explanation involves reutilization of the haem iron transported via transferrin from liver, perhaps after storage on ferritin, to the spleen. Since, during 2h after injection, there was no detectable urinary excretion of ^{59}Fe radioactivity, this would be consistent with the concept of conservation of the haemoglobin iron (Hershko, 1975). Although the available evidence indicates that haemopexin transports haem specifically to the liver, the pattern of radioactivity found in the spleen is consistent with a small uptake in a manner similar to that found for the liver. Haem with a higher specific radioactivity will be needed to resolve whether there is low uptake of haem by extrahepatic tissues.

In summary, we conclude that haem–haemopexin interacts with the liver in a rapid saturable manner similar to that proposed for iron–transferrin (Gardiner & Morgan, 1974; Beamish *et al.*, 1975). In this receptor-mediated uptake process, the protein is recycled rather than being taken up as a complex and degraded in the lysosomes, as demonstrated for cobalamin–transcobalamin II (Youngdahl-Turner *et al.*, 1978) and proposed for haemoglobin–haptoglobin (Franklin *et al.*, 1960). Further information is needed to elucidate fully the haem–haemopexin–liver interaction and how haem reaches microsomal haem oxygenase, possibly via cytosolic proteins. We are also interested in factors that affect the number of receptors, and preliminary experiments with isolated hepatocytes and with liver-cell membranes (A. Smith & W. T. Morgan, unpublished work) support the mode of action of haemopexin proposed in this paper.

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