Biochem. J. (1988) 256, 159–165 (Printed in Great Britain)

Effect of serum proteins on haem uptake and metabolism in primary cultures of liver cells

Peter R. SINCLAIR,*†¶ William J. BEMENT,* Nadia GORMAN,* Heng H. LIEM,‡ Allan W. WOLKOFF§ and Ursula MULLER-EBERHARD‡‖

*Veterans Administration Medical Centre, White River Junction, VT 05001, †Department of Biochemistry, Dartmouth Medical School, NH 03756, §Liver Research Center at Albert Einstein College of Medicine, Bronx, NY 10461, and Departments of ‡Pediatrics, ‖Pharmacology and ‖Biochemistry, Cornell University Medical College, New York, NY 10021, U.S.A.

A role of haemopexin in transporting haem to hepatocytes for degradation has been inferred from the high affinity of haemopexin for haem. We have examined this question in primary cultures of chick-embryo and adult rat liver cells. We present here the results of four sets of experiments which indicate that haemopexin retarded haem uptake by hepatocytes in culture. (1) Haem bound to bovine serum albumin is known to repress the activity of δ-aminolaevulinate synthase in chick cultures as indicated by decreased porphyrin accumulation. When haem–albumin was added in the presence of excess purified or freshly secreted chicken haemopexin, no haem-mediated repression of porphyrin production was observed. The haem-mediated repression of porphyrin accumulation was partially prevented when human, but not chicken, albumin was added to cultures. This finding reflected the higher affinity of human albumin for haem compared with that of chicken albumin. (2) Haemopexin inhibited the ability of haem to be incorporated into cytochrome *P*-450 induced in the chick cultures in the presence of the iron chelator desferrioxamine. (3) The rate of association of [^{55}Fe]haem with cultured rat hepatocytes when [^{55}Fe]haem–haemopexin was added was one-eighth of the rate observed when [^{55}Fe]haem–bovine serum albumin was used as the haem donor. (4) The presence of haemopexin also diminished the catabolism of haem by both rat and chick-embryo liver cell cultures. It is concluded that the uptake and subsequent metabolic effects of haem are inhibited in cultured hepatocytes by proteins such as haemopexin which have a high affinity for haem.

INTRODUCTION

Under physiological conditions the concentration of plasma haem is very low (Shinowara & Walters, 1963), but large amounts of circulating haem are found in acute and chronic states of haemolysis or during haem therapy for porphyria. It is generally assumed that plasma haem is transported to hepatocytes, where it is catabolized to bilirubin. However, not all haem that enters the hepatocyte is immediately catabolized. Some haem is incorporated into microsomal cytochromes *P*-450 (Bornheim *et al.*, 1987) and other intracellular proteins (for review, see Muller-Eberhard & Vincent, 1985). Exogenous haem also has several metabolic effects. Haem causes accumulation of cytosolic δ-aminolaevulinate synthase (ALA-S) (for review, see Ades, 1986) and decreases the mRNA for ALA-S (Ades *et al.*, 1987). Thus haem taken up by hepatocytes can enter at least three cellular compartments, i.e. those affecting feedback inhibition on ALA-S, incorporation into cytochromes *P*-450 and haem degradation.

Several mechanisms of haem uptake by cells have been proposed, some of which appear to be carrier-protein-mediated (Smith & Morgan, 1981; Smith, 1985), and others of which are not dependent on a specific circulating carrier protein (Galbraith *et al.*, 1985; Majuri & Graesbeck, 1987).

Experiments *in vivo* with rats (Muller-Eberhard *et al.*, 1970; Smith & Morgan, 1978, 1979) and *in vitro* with freshly isolated rat liver cells (Smith & Morgan, 1981) indicated that haemopexin, a serum protein which binds haem at an equimolar ratio and which possesses an exceptionally high affinity for haem (Hrkal *et al.*, 1974), has a role in hepatic haem uptake. Results of experiments performed *in vivo* in other species and in patients with elevated plasma haem concentrations owing to haemolysis or haem therapy for porphyrias were considered to be consistent with this hypothesis (see Muller-Eberhard & Liem, 1974; Muller-Eberhard & Morgan, 1975).

Whether a major portion of haem is delivered to liver cells by a haemopexin-mediated process under physiological conditions, however, remains unclear. Most previous studies with isolated liver cells have investigated the kinetics of haem association (Smith & Morgan, 1981) and the release of haem into the medium (Bissell *et al.*, 1979). That haem has entered cells and is not merely bound to their surface can be ascertained by determination of specific biological effects. Other studies, by Davies *et al.* (1979) and Smith (1985), reported that ^{55}Fe injected as haem–haemopexin was found in ferritin in the liver and distributed in various cell sub-fractions.

We have now investigated in cultured liver cells the effects of haemopexin not only on cell association of haem but also on subsequent intracellular events in cultured liver cells. Most of the experiments were carried out in primary cultures of chick-embryo liver cells. These

Abbreviations used: ALA-S, δ-aminolaevulinate synthase; BSA, bovine serum albumin; PIA, 2-propyl-2-isopropylacetamide.

¶ To whom correspondence should be addressed, at: VA Medical Centre (151), White River Junction, VT 05001, U.S.A.

cells are highly responsive to porphyrinogenic agents which induce the activities of ALA-S and cytochrome *P*-450. Previously, Sinclair & Granick (1976) reported that human, but not chicken, albumin prevented the cellular uptake of haem, as indicated by prevention of the haem-mediated repression of induced ALA-S. Here we studied the effects of the major haem-binding serum proteins, haemopexin and albumin, on the extent of haem uptake into chick liver cells and the effect of haem as reflected by its effects on three different intracellular metabolic events: (1) the feedback inhibition by haem of ALA-S; (2) the cytochrome *P*-450 content; and (3) the formation of the bile pigment biliverdin from added haem. Experiments on haem binding and catabolism were also conducted in short-term cultures of adult rat hepatocytes. Previous studies have revealed that the pathways of receptor-mediated endocytosis of asialoglycoproteins and transport of organic anions (Wolkoff *et al.*, 1987), as well as the expression and Fe-dependent modulation of the transferrin receptor (Muller-Eberhard *et al.*, 1988), are intact in these cells. We found that, in cultures from either species, haemopexin prevented the uptake of haem by hepatocytes over a wide range of haem concentrations and periods of incubation, whereas haem was readily taken up from bovine or chicken serum albumins.

MATERIALS AND METHODS

Chick-embryo liver cell cultures

Primary cultures from 16-day White Leghorn chick embryos were prepared as previously described (Sinclair *et al.*, 1982; Shedlofsky *et al.*, 1987). For the initial 18 h in culture, cells were maintained in Williams E medium (GIBCO, Grand Island, NY, U.S.A.), to which was added insulin (1 μg/ml), dexamethasone (0.3 μg/ml) and 3,3',5-tri-iodothyronine (1 μg/ml). The medium was then changed to Williams E medium containing dexamethasone and tri-iodothyronine, but no insulin, as described by Sinclair *et al.* (1982). Generally, experiments were performed on day 3 of culture without further change of the medium. In some experiments pig glucagon (Sigma, St. Louis, MO, U.S.A.) was added at 1 μg/ml, to suppress endogenous haemopexin production (Grieninger *et al.*, 1986: G. Grieninger, personal communication). Glucagon has been found to stimulate induced porphyrin accumulation (Fischer *et al.*, 1978).

Cultures of adult hepatocytes

Rat hepatocytes were isolated from 200–250 g male Sprague–Dawley rats after perfusion of the liver with collagenase (type 1; Sigma). Cells were suspended in medium consisting of Waymouth's 752/1 (GIBCO), 25 mM-Hepes, pH 7.2, 5% (v/v) heat-inactivated fetal-bovine serum (GIBCO), 1.7 mM additional $CaCl_2$, 5 μg of bovine insulin/ml (Sigma), 100 units of penicillin/ml (GIBCO) and 0.1 mg of streptomycin/ml (GIBCO). Approx. 1.5×10^6 cells in 3 ml were placed in 6 cm-diam. culture dishes. Approx. 2 h later, the medium was changed and cells were cultured for an additional 16–18 h until used (Wolkoff *et al.*, 1987).

Porphyrin measurements

Porphyrins were determined spectrofluorimetrically as described previously (Sinclair *et al.*, 1984).

Biliverdin production

Cells plus medium were mixed (1:1, v/v) with acetone/conc. HCl (39:1, v/v) adjusted to pH 3.5 with 10 M-NaOH and injected into a 3.9 mm × 300 mm C_{18} Waters μ-Bondapak column. Separation was achieved essentially as described previously (Bonkovsky *et al.*, 1986) by using a slightly modified mobile phase, Solution A (44% methanol/56% 0.1 M-ammonium phosphate, pH 3.15). The linear gradient was started at 40% Solution B (100% methanol) increasing to 100% B over 16 min. We have found that recovery of endogenous biliverdin from cultured hepatocytes is 85–100%, based on degradation of exogenous haem.

Other assays

Cytochrome *P*-450 was assayed spectrophotometrically in detergent-solubilized 8700 *g* supernatants of cultured hepatocytes as described by Sinclair *et al.* (1979). Proteins were determined by the method of Lowry *et al.* (1951), with BSA as standard. Haemopexin concentrations were determined by rocket immunoelectrophoresis (Laurell, 1966), with purified chicken haemopexin (Goldfarb *et al.*, 1986) as standard.

Preparation of haem–BSA solution

Haem (10 mg) was dissolved in dimethyl sulphoxide (1 ml). A 36 μl portion of this solution was added to 6 ml of BSA (6 mg/ml) in saline, making a 60 μg/ml haem solution at a molar ratio of haem to BSA of 1:1. This solution was diluted with water for additions of different amounts of haem–BSA to the culture medium. The exact concentration of haem in the stock solution was determined by the pyridine haemochrome method as described by Falk (1964), by using a millimolar absorption coefficient of 20.7 for absorbance difference between the 556 nm α maximum and the minimum between α and β peaks in the reduced-minus-oxidized difference spectrum.

Preparation of [^{55}Fe]haem

The method of Adler *et al.* (1970) was followed for insertion of ^{55}Fe into protoporphyrin, and the haem was purified by the method of Healey *et al.* (1981). HCl washing of the haem in ether was continued until no further radioactivity of free ^{55}Fe could be extracted.

Uptake of ^{55}Fe-haem by cultured rat hepatocytes

Uptake was measured at 37 °C and 4 °C by the method described to determine uptake of sulphobromophthalein (Wolkoff *et al.*, 1987). After incubation with radioactive ligand, cells were washed twice with serum-free medium at 4 °C and incubated for 5 min in 5% BSA in phosphate-buffered saline (20 mM-sodium phosphate/0.9% NaCl), pH 7.4, at 4 °C to remove loosely bound haem. Longer incubations with BSA did not remove additional haem. The cultures were then washed three times with serum-free medium at 4 °C. Then the cells were scraped in 1 ml of phosphate-buffered saline, dissolved in 10 ml of Hydrofluor (National Diagnostics, Somerville, NJ, U.S.A) and radioactivity was determined in a liquid-scintillation counter.

Haem extraction

The method of Healey *et al.* (1981) was used to extract [^{55}Fe]haem from cells plus medium.

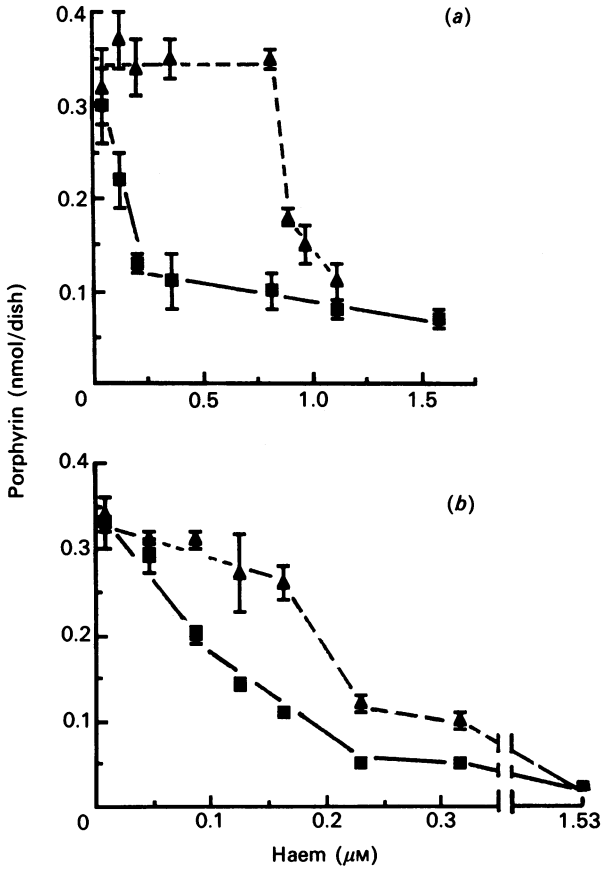

Fig. 1. Effect of haemopexin on the haem-mediated repression of porphyrin synthesis by chick-embryo hepatocytes

(*a*) Effect of exogenous haemopexin. Cells (0.5–0.7 mg of protein per 3.5 cm dish) were preincubated for 20 h in Williams E medium containing dexamethasone, triiodothyronine and glucagon. The medium was changed, and 0.14 mM-PIA and 0.15 mM-desferrioxamine were added to induce porphyrin synthesis maximally (Granick *et al.*, 1975); haem was added (at the same time as the drugs) as haem–BSA (1:1 molar ratio) at 0.01–1.60 μM. After a 6 h treatment period, porphyrins were extracted and measured spectrofluorimetrically as described in the Materials and methods section. Data are means ± S.D. for triplicate dishes. Key: ■, no added haemopexin; ▲, 0.77 μM chicken haemopexin added simultaneously with haem–BSA (1:1). (*b*) Effect of endogenous haemopexin. Cells (on 3.5 cm dishes) were preincubated for 20 h as described for Fig. 1(*a*), except that the medium did not contain glucagon. This conditioned medium was then removed, and portions of it were incubated with haem–BSA at the concentrations indicated for 1 h at 37 °C before being returned to the cells. A second set of dishes received fresh medium similarly preincubated with haem–BSA. The haemopexin content of the conditioned medium was 0.15 μM, determined immunoelectrophoretically as described in the Materials and methods section. After a 5 h incubation period with the inducers, total porphyrins were measured. Data represent means ± S.D. for triplicate dishes. Key: ▲, conditioned medium; ■, fresh medium.

Materials

2-Propyl-2-isopropylacetamide (PIA) was a gift from Hoffmann–La Roche, Nutley, NJ, U.S.A., and was dissolved in 50% (v/v) methanol. Desferrioxamine mesylate was purchased from Ciba Geigy, Camden, NJ, U.S.A., and was added in 0.9% NaCl. Haemin was given by Dr. S. Sassa, Rockefeller University, New York, U.S.A., or was purchased from Sigma. BSA and human and chicken albumin were from Sigma. Rat albumin was from ICN Biochemicals, Costa Mesa, CA, U.S.A. All four albumins were assayed for haemopexin. The contents of homologous haemopexin in each were: bovine, 0.1%; human, 1.3%; chicken, 5%; rat, < 0.1%. Haemopexin was purified from the plasma of chicken and rats by the method of Goldfarb *et al.* (1986).

RESULTS

Haemopexin and haem repression of ALA synthase

Two different experimental conditions were used to monitor the effect which haemopexin had on the haem-mediated repression of ALA-S activity in cultured chick-embryo liver cells. The activity was assessed indirectly during a 6 h incubation period by measuring porphyrin production in the presence of desferrioxamine, a ferric iron chelator that prevents conversion of protoporphyrin into haem (Granick *et al.*, 1975), but does not remove iron from haem. Fig. 1(*a*) shows the results of experiments in which we compared the effects of haem taken up from haem–BSA in the presence and absence of chicken haemopexin. Haemopexin prevented the haem-mediated decrease in porphyrin production caused by haem added as haem–BSA complex. Haem in excess of the equimolar haem-binding capacity of haemopexin exerted its expected effect, i.e. a decrease in porphyrin production.

In the second set of experiments the effect of endogenously produced haemopexin on porphyrin production was examined, by using the finding by Grieninger *et al.* (1983) that considerable amounts of protein M [more recently identified as haemopexin (Grieninger

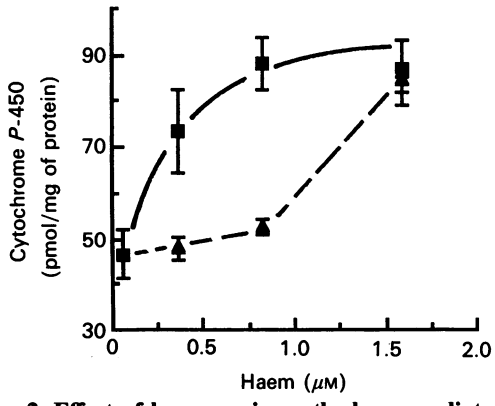

Fig. 2. Effect of haemopexin on the haem-mediated restoration of the cytochrome *P*-450 content in chick-embryo hepatocytes

Cells (~ 1 mg of protein per 6.0 cm dish) were pre-incubated for 20 h and treated with PIA and desferrioxamine for 6 h as described in the legend to Fig. 1. Addition of haem was as haem–BSA (1:1 molar ratio) in the absence (■) or the presence (▲) of 0.77 μM chicken haemopexin. The cytochrome *P*-450 content was measured spectrophotometrically in the detergent-solubilized 8700 *g* supernatant as described in the Materials and methods section.

et al., 1986)] are secreted into the medium of chick-embryo liver cell cultures. The data shown in Fig. 1(*b*) indicate that endogenous haemopexin significantly decreased the inhibitory effect of haem on porphyrin production. The concentration of haemopexin in the conditioned medium was 10 μg/ml, which binds 0.15 μM-haem. Fig. 1(*b*) shows that at haem concentrations above 0.15 μM porphyrin accumulation was suppressed. These data indicate that endogenously formed and secreted haemopexin, as well as exogenous haemopexin, prevented haem uptake into chick-embryo liver cells in culture.

Haemopexin and haem incorporation into cytochrome *P*-450

Fig. 2 shows that the presence of desferrioxamine prevented the increase of cytochrome *P*-450 induced by PIA. The cytochrome *P*-450 content of the cells treated with PIA and desferrioxamine was no greater than that of the untreated cells. When haem was added, there was a dose-dependent increase in cytochrome *P*-450 content, suggesting that exogenous haem was incorporated into apocytochrome *P*-450 as reported previously (Lincoln *et al.*, 1988). The presence of exogenous haemopexin prevented this increase in cytochrome *P*-450 until the haem-binding capacity of haemopexin was exceeded (at > 1 μM-haem). This result is consistent with the conclusion drawn from the data presented in Fig. 1, that haemopexin prevents the uptake of haem by the cells.

Haemopexin and haem degradation

Cultured chick hepatocytes degrade haem to biliverdin (biliverdin is the final product of haem degradation, since avian liver lacks biliverdin reductase). Table 1 summarizes the effect of haemopexin on the conversion of haem into biliverdin during a 6 h incubation period. Although the biliverdin values varied, addition of haemopexin to the culture medium decreased biliverdin production by 90%.

Our next experiment examined whether the inhibition by haemopexin of haem entry into the degradative pathway was a peculiarity of the chick-embryo liver cell system. Under nearly identical conditions we measured the stability of [^{55}Fe]haem added to cultures of liver cells prepared from both chicken embryos and adult rats. The data in Table 2 demonstrate that approx. 25% of the [^{55}Fe]haem was metabolized during the 3 h period when haemopexin was not present in the medium, whereas in the presence of haemopexin essentially no haem was metabolized. Identical observations were made for both systems. We interpret the data to mean that incubation with haemopexin inhibited the cellular uptake and subsequent metabolism of haem. It should be noted that the method of extraction, which includes washing the haem in ether with HCl, would have separated the extracted haem from free ^{55}Fe.

Table 1. Effect of haemopexin on haem catabolism by cultured chick-embryo liver cells

Cells on 3.5 cm-diam. dishes were treated with 3 nmol of haem–BSA and 7.5 nmol of chicken haemopexin as indicated. After incubation for 6 h, haem and biliverdin were extracted from cells plus medium and separated by h.p.l.c. as described in the Materials and methods section. Data are means, with individual values in parentheses.

Time of incubation (h)	Haemopexin	Content (pmol/dish)	
		Biliverdin	Haem
0	−	98 (95, 101)	1768 (1680, 1856)
6	−	576 (473, 678)	632 (464, 800)
0	+	60 (17, 102)	1883 (1616, 2150)
6	+	113 (34, 192)	1600 (1536, 1664)

Association of [^{55}Fe]haem with cultured rat hepatocytes

The rate and extent of association to rat hepatocytes of [^{55}Fe]haem added as haem–rat haemopexin was compared with that added as haem–rat albumin. The results

Table 2. Effect of haemopexin on ^{55}[Fe]haem metabolism by cultured chick-embryo and rat liver cells in culture

Chick-embryo cells on 6 cm-diam. dishes were rinsed twice with fresh medium and incubated as indicated with [^{55}Fe]haem–BSA (0.38 nmol of haem) or with [^{55}Fe]haem–chicken haemopexin in 1 ml of medium at a molar ratio of haem:protein of 0.9. At harvesting, dishes were placed on ice, and cells plus medium were collected by scraping. Haem was extracted as described in the Materials and methods section. Data are means ± S.D. for triplicate dishes. For rat liver cultures, cells on 6 cm-diam. dishes were rinsed twice with phosphate-buffered saline 24 h after culture and were then incubated for 30 min at 37 °C to release preformed proteins. Dishes were then washed twice with phosphate-buffered saline and then incubated in 1 ml of medium containing 0.38 nmol of [^{55}Fe]haem–BSA or 0.38 nmol of [^{55}Fe]haem–rat haemopexin at a molar ratio of haem:protein of 0.9. Incubation times were identical with those used for the chick cultures, as were harvested and haem extractions. Zero-time haem radioactivity (d.p.m.) was 55854, 70894, 54513 and 45752 for chick cultures with and without haemopexin, and rat cultures with and without haemopexin, respectively.

Time of incubation (h)	Additional treatments...	Residual haem radioactivity (%)			
		Chick		Rat	
		None	Haemopexin	None	Haemopexin
0		100.0±3.5	100.0±2.8	100.0±7.6	100.0±14.7
1		83.3±5.0*	89.8±5.8	80.2±1.4*	100.5±4.4
3		76.0±11.8*	95.1±3.2	72.8±6.3*	101.5±21.5

* Significantly different from zero time ($P < 0.05$) by Student's *t* test.

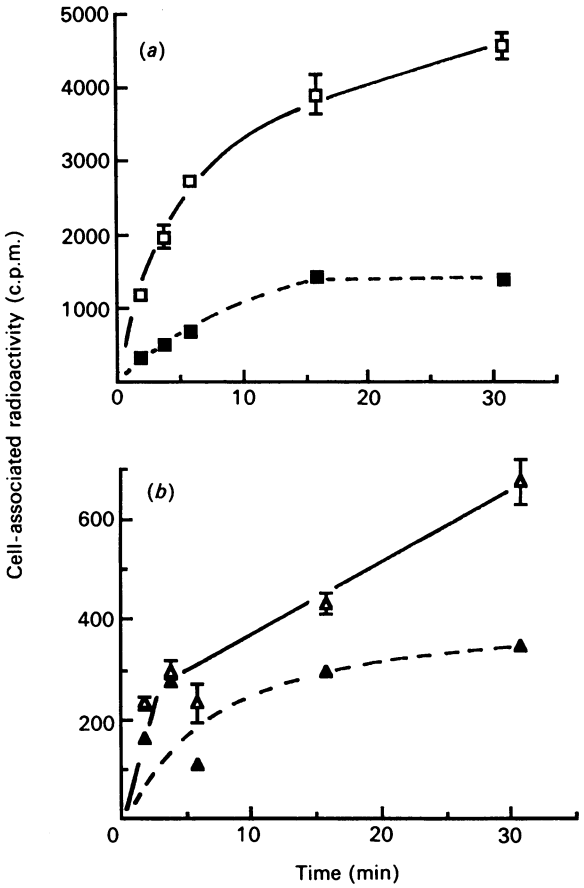

Fig. 3. Dependence of cellular association of [^{55}Fe]haem on the haem–protein complex added to rat hepatocytes in primary culture

Rat liver cells (1–2 mg of protein per 6.0 cm dish) were pre-incubated for 24 h in Waymouth medium as specified in the Materials and methods section. After thorough washing of the cells with phosphate-buffered saline, pH 7.4, to remove pre-existing protein in the medium; 50000 c.p.m. of [^{55}Fe]haem (7.3×10^8 c.p.m./μmol) haem–protein complexes (1:1 molar ratio) were incubated with the cells for the indicated times at 4 °C and 37 °C. The cells were then washed thoroughly with and without 0.5% BSA, and the radioactivity was determined as described in the Materials and methods section. The scales of the ordinates in (*a*) (haem–albumin) and (*b*) (haem–haemopexin) are quite different. The data are from duplicate dishes of a single representative experiment. Key: (*a*) rat albumin: ■, 4 °C; □, 37 °C; (*b*) rat haemopexin: ▲, 4 °C; △, 37 °C.

indicate a much greater extent of association of added haem with the cells when it was supplied as haem–albumin (Fig. 3*a*) rather than as haem–haemopexin (Fig. 3*b*). The rate of haem association from BSA was about 8-fold that from rat haemopexin at 37 °C.

Haem uptake from haemopexin compared with other serum proteins

In additional experiments the effect of haem-binding serum proteins of varying affinities for haem (added as haem–BSA) on induced porphyrin production was assessed in cultured chick hepatocytes. The data in Fig. 4 demonstrate that both chicken haemopexin or a mixture

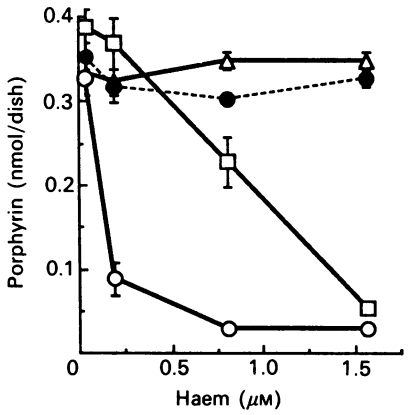

Fig. 4. Effect of various serum haem-binding proteins on the haem-mediated repression of porphyrin synthesis in chick-embryo hepatocytes

Cells on 3.5 cm dishes were pre-incubated for 20 h and treated for 6 h as described in the legend for Fig. 1. Haem was added as haem–BSA (1:1 molar ratio) in the concentrations indicated. In addition to haem–BSA, duplicate sets of dishes contained chicken albumin (200 μg/ml) (○), human albumin (200 μg/ml) (□), chicken haemopexin (100 μg/ml) (△) or chicken haemopexin (100 μg/ml) plus chicken serum albumin (200 μg/ml) (●). The data for haem alone and for haem plus chicken haemopexin were essentially superimposable. The data are means and ranges of duplicate dishes. Unless otherwise shown, the ranges fell within the symbols.

of chicken haemopexin and chicken albumin prevented haem uptake. Addition of chicken albumin had no inhibitory effect on porphyrin synthesis, probably because its affinity for haem is equal to or even lower than that of BSA. Human albumin ($K_D = 10^{-8}$ M; Beaven *et al.*, 1974) decreased the effect of haem on porphyrin production, but was less effective than haemopexin (K_D = approx. 10^{-12} M, determined for human haemopexin; Hrkal *et al.*, 1974).

DISCUSSION

Previous studies on the delivery of haem to hepatocytes *in vitro* have been restricted to investigations of the binding of haem from either haemopexin or serum albumin as donor (Smith & Morgan, 1981, 1984). There have been few studies showing that the haem is actually taken up into the cells, For example, Lodola (1985) reported that haem taken up by isolated rat hepatocytes from BSA was preferentially directed to the endoplasmic reticulum, in conformity with results obtained *in vivo* (Liem *et al.*, 1975). Grandchamp *et al.* (1981) presented evidence that 0.01 μM exogenous haem added to cultured rat hepatocytes was taken up into cellular haem pools, where it equilibrated with the precursor pool for cytochrome *P*-450.

The major finding of the present study is that apparently insufficient haem entered the cells, when haem was presented as haem–haemopexin, to affect the three haem-dependent metabolic events that we studied. This apparent lack of uptake was found when the molar ratio of haem to haemopexin was less than 1:1. Once this ratio was exceeded, the haem had the same effect on the cells as did haem delivered from BSA (Figs. 1 and 2). The

decreased haem uptake in the presence of equimolar haemopexin was not due to alterations of haemopexin incurred during purification, since fresh, endogenously synthesized and secreted haemopexin also retarded haem uptake (Fig. 1*b*).

These results appear to conflict with the currently held view (Wintrobe *et al.*, 1981; Robinson, 1983; Jandl, 1987) that the role of haemopexin is to mediate hepatic haem uptake, especially once the haemoglobin-binding capacity of haptoglobin is exhausted and free haem is released from circulating methaemoglobin. Both in the present studies with monolayer cultures and in those with isolated rat hepatocytes (Smith & Morgan, 1981), haem from haemopexin associated with the cells to a much lesser extent than did haem from BSA (Fig. 3). Evidence has been obtained from studies *in vivo* (Smith & Morgan, 1978, 1979) and with isolated hepatocytes (Smith & Morgan, 1981) for receptor-mediated haem–haemopexin binding and release of haem. Our studies do not directly address this point. Although a small amount of haem is bound when added to cultures as haem–haemopexin (Fig. 3*a*), we saw no effects of this haem on intracellular events (Figs. 1 and 2, and Table 1). This suggests (i) that haem binding is not equivalent to delivery of haem to the cell's interior, (ii) that the small amount of haem entering the cells in our experiments was insufficient to trigger the cellular events, or (iii) that the methodologies used to monitor the effects of haem were too insensitive. The most sensitive of the three assays used was haem repression of ALA-S, which was measured fluorimetrically by porphyrin production. We can estimate that only one-eighth of the haem from haemopexin (derived from data of Fig. 3), relative to that from BSA, would bind to and enter the cells. At low haem concentrations (0.1 μM), in the presence of BSA, porphyrin accumulation was detectably decreased (Fig. 1*b*). Even with larger amounts of haem (0.8 μM), as long as it was bound to haemopexin, there was no effect on porphyrin production (Fig. 1*a*).

Smith & Morgan (1981) recognized that BSA may deliver potentially toxic haem non-specifically to hepatocytes. They suggested that haemopexin may specifically direct haem to the site of catabolism. However, our results for biliverdin production (Table 1) as well as those on the stability of labelled haem (Table 2) failed to detect enhanced delivery of haem for degradation. Rather, as for the other cellular haem-dependent processes studied here, haemopexin decreased degradation of added haem (Tables 1 and 2). More sensitive radioactive techniques would be necessary to detect whether, in the presence of haemopexin, very small amounts of haem were entering the cells and were being degraded.

The highly effective uptake of haem from BSA suggests that there may be a receptor on hepatocytes for haem itself. Evidence for such a receptor in other cell types has been presented (Galbraith *et al.*, 1985; Majuri & Graesbeck, 1987). Our data suggest that uptake of haem by cultured hepatocytes may be required by the relative affinity of the extracellular proteins for haem (Fig. 4). This is analogous to the role of the affinity for haem of intracellular proteins suggested by Granick *et al.* (1975) and more recently discussed by Bissell (1986).

Under physiological conditions the haemopexin capacity for binding haem is unlikely to be exceeded. Muller-Eberhard *et al.* (1968) showed that haemopexin is severely depleted in chronic haemolytic diseases and also acutely during haem therapy (Lamon *et al.*, 1978). A mechanism for haem uptake from serum albumin may co-exist with that mediated by haemopexin. In a qualitative study, using tracer amounts of haem, the uptake of haem from haemopexin and albumin did not differ within 15 min after injection (Muller-Eberhard *et al.*, 1970). However, at present no quantitative data are available concerning the relative hepatic uptake of haem *in vivo* from haem–haemopexin and haem–albumin.

The ability of haemopexin to bind haem tightly (Hrkal *et al.*, 1974) may have an additional, entirely different, role. Haemopexin protects completely against haem-catalysed production of malondialdehyde from microsomal lipids, whereas other haem-binding proteins promote malondialdehyde formation (Vincent & Muller-Eberhard, 1988). Further work will be required to ascertain whether this is a physiologically relevant process.

In conclusion, our studies with cultured liver cells show that haemopexin retards haem uptake into cultured hepatocytes.

We thank Dr. D. M. Bissell, Dr. A. Mason, Dr. J. Sinclair and Dr. L. Smith for their critical comments, and Ms. Y. Remache for performing haemopexin assays. The work was supported by grants from the National Institutes of Health (CA 25012, HL 09011, DK-30203, DK-23026, DK-17702, DK-30664) and by research funds from the Veterans Administration.

REFERENCES

Ades, I. Z. (1986) IRCS Med. Sci. **14**, 1170–1173
Ades, I. Z., Stevens, T. M. & Drew, P. (1987) Arch. Biochem. Biophys. **253**, 297–304
Adler, A. D., Longo, F. R., Kampass, F. & Kim, J. (1970) J. Inorg. Nucl. Chem. **32**, 2443–2445
Beaven, G. H., Chen, S. H., D'Albis, A. & Gratzer, W. B. (1974) Eur. J. Biochem. **41**, 539–546
Bissell, D. M. (1986) in Bile Pigments and Jaundice (Ostrow, J. D., ed.), p. 141, Marcel Dekker, New York
Bissell, D. M., Liem, H. H. & Muller-Eberhard, U. (1979) Biochem. J. **184**, 689–694
Bonkovsky, H. L., Wood, S. G., Howell, S. K., Sinclair, P. R., Lincoln, B., Healey, J. F. & Sinclair, J. F. (1986) Anal. Biochem. **155**, 56–64
Bornheim, L. M., Underwood, M. C., Calders, P., Rettie, A. E., Trager, W. F., Wrighton, S. A. & Correia, M. A. (1987) Mol. Pharmacol. **32**, 299–308
Davies, D. M., Smith, A., Muller-Eberhard, U. & Morgan, W. (1979) Biochem. Biophys. Res. Commun. **91**, 1504–1511
Falk, J. E. (1964) Porphyrins and Metalloporphyrins, pp. 181–183, Elsevier Publishing Co., Amsterdam
Fischer, P. W. F., Stephens, J. K. & Marks, G. S. (1978) Mol. Pharmacol. **14**, 717–721
Galbraith, R. A., Sassa, S. & Kappas, A. (1985) J. Biol. Chem. **260**, 12198–12202
Goldfarb, V., Trimble, R. B., De Falco, M., Liem, H. H., Metcalfe, S., Wellner, D. & Muller-Eberhard, U. (1986) Biochemistry. **25**, 6555–6562
Grandchamp, B., Bissell, D. M., Licko, V. & Schmid, R. (1981) J. Biol. Chem. **256**, 11677–11683
Granick, S., Sinclair, P. R., Sassa, S. & Grieninger, G. (1975) J. Biol. Chem. **250**, 9215–9225
Grieninger, G., Plant, P., Liang, T., Kalb, R., Amrani, D., Mosesson, M., Hertzberg, K. & Pindyck, J. (1983) Ann. N.Y. Acad. Sci. **408**, 469–489

Greininger, G., Liang, T. J., Beuving, G., Goldfarb, V., Metcalfe, S. A. & Muller-Eberhard, U. (1986) J. Biol. Chem. **261**, 15719–15724
Healey, J. F., Bonkovsky, H. L., Sinclair, P. R. & Sinclair, J. F. (1981) Biochem. J. **198**, 595–604
Hrkal, Z., Vodrazka, Z. & Kalousek, I. (1974) Eur. J. Biochem. **43**, 73–78
Jandl, J. H. (1987) Blood, pp. 88–89, Little, Brown and Co, Boston
Lamon, J. M., Tschudy, D. & Muller-Eberhard, U. (1978) in Diagnosis and Therapy of Porphyrias and Lead Intoxication (Doss, M., ed.), pp. 285–292, Springer-Verlag, Berlin
Laurell, C.-B. (1966) Anal. Biochem. **15**, 45–52
Liem, H. H., Tavassoli, M. & Muller-Eberhard, U. (1975) Acta Haematol. **53**, 219–225
Lincoln, B. C., Healey, J. F. & Bonkovsky, H. L. (1988) Biochem. J. **250**, 189–196
Lodola, A. (1985) Biosci. Rep. **5**, 609–614
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
Majuri, R. & Graesbeck, R. (1987) Eur. J. Haematol. **38**, 21–25
Muller-Eberhard, U. & Liem, H. H. (1974) in Structure and Function of Plasma Proteins (Allison, A. C., ed.), vol. 1, pp. 35–53, Plenum, London
Muller-Eberhard, U. & Morgan, W. T. (1975) Ann. N.Y. Acad. Sci. **244**, 624–650
Muller-Eberhard, U. & Vincent, S. H. (1985) Biochem. Pharmacol. **34**, 719–725
Muller-Eberhard, U., Bosman, C. & Liem, H. H. (1970) J. Lab. Clin. Med. **76**, 426–431
Muller-Eberhard, U., Javid, J., Liem, H. H., Hanstein, A. & Hanna, M. (1968) Blood **32**, 811–815
Muller-Eberhard, U., Liem, H., Grasso, J., Giffhorn-Katz, S., DeFalco, M. & Katz, N. (1988) J. Biol. Chem., in the press
Robinson, S. (1983) in Hematology (Williams, W. J., Beutler, E., Erslev, A. J. & Lichtman, M. A., eds.), 3rd edn., pp. 388–394, McGraw-Hill, New York
Shedlofsky, S. I., Sinclair, P. R., Bonkovsky, H. L. & Healey, J. F. (1987) Biochem. J. **248**, 229–236
Shinowara, G. Y. & Walters, M. I. (1963). Am. J. Clin. Pathol. **40**, 113–122
Sinclair, J., Sinclair, P. R. & Bonkowsky, H. (1979) Biochem. Biophys. Res. Commun. **86**, 710–717
Sinclair, J. F., Sinclair, P. R., Healey, J. F., Smith, E. L. & Bonkovsky, H. L. (1982) Biochem. J. **204**, 103–109
Sinclair, P. R. & Granick, S. (1976) Ann. Clin. Res. **8**, suppl. **17**, 250–258
Sinclair, P., Bement, W., Bonkovsky, H. & Sinclair, J. (1984) Biochem. J. **222**, 737–748
Smith, A. (1985) Biochem. J. **231**, 663–669
Smith, A. & Morgan, W. T. (1978) Biochem. Biophys. Res. Commun. **84**, 151–157
Smith, A. & Morgan, W. T. (1979) Biochem. J. **182**, 47–54
Smith, A. & Morgan, W. T. (1981) J. Biol. Chem. **256**, 10902–10909
Smith, A. & Morgan, W. T. (1984) J. Biol. Chem. **259**, 12049–12053
Vincent, S. & Muller-Eberhard, U. (1988) Arch. Biochem. Biophys., in the press
Wintrobe, M. M., Lee, G. R., Boggs, D. R., Bithell, T. C., Foerster, J., Athens, J. W. & Lukens, J. N. (1981) Hematology, 8th edn., p. 180, Lea and Febiger, Philadelphia
Wolkoff, A., Samuelson, A. C., Johansen, K. L., Nakata, R., Withers, D. M. & Sosiak, A. (1987) J. Clin. Invest. **79**, 1259–1268

Received 12 February 1988/15 June 1988; accepted 5 July 1988