Haematoporphyrin and *OO'*-diacetylhaematoporphyrin binding by serum and cellular proteins

Implications for the clearance of these photochemotherapeutic agents by cells

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Haematoporphyrin derivative (HpD), a mixture of porphyrins, is currently used as a photochemotherapeutic agent in the treatment of neoplasias. The interaction of purified components of HpD with serum and cellular proteins was investigated using absorption and fluorescence spectroscopy. The interactions of haematoporphyrin and *OO'*-diacetylhaematoporphyrin with human albumin and with haemopexin, the two major serum porphyrin-binding proteins, show stoichiometries of 1 mol of porphyrin bound per mol of protein. The apparent dissociation constants, K_d , are in the range of 1–2 μM for albumin and 3–4 μM for haemopexin. These two major components of HpD would, after intravenous injection, bind to albumin and circulate in serum as albumin complexes. Free porphyrin rather than porphyrin bound to albumin interacts with Morris hepatoma tissue culture cells. A rapid high-affinity saturable transport system operates at free porphyrin concentrations of less than 2 μM . In addition, fluorescence spectra show that components in rat liver cytosol can bind haematoporphyrin and *OO'*-diacetylhaematoporphyrin and distinguish these binders from those present in rat serum.

Successful treatment of different neoplasias with chemical derivatives of haematoporphyrin and visible light are now being regularly reported (Dougherty *et al.*, 1978; Cortese *et al.*, 1979; Kato *et al.*, 1980; Dougherty, 1981). Porphyrins localized in cancerous cells, when illuminated, produce cytotoxic radicals (Weishaupt *et al.*, 1976) that destroy these cells. The photocytotoxic effects of porphyrins have been investigated in detail both *in vivo* (Gregorie *et al.*, 1968; Dougherty *et al.*, 1975, 1978; Henderson *et al.*, 1980) and with cultured cells (Moan & Christensen, 1981; Kessel, 1981) and organelles (Sandberg, 1981; Sandberg & Romslo, 1981), but the uptake, cellular binding, metabolism and excretion processes involved in the clearance of porphyrins, by normal and cancerous cells, have not been addressed quantitatively. The mechanism of cell uptake and factors affecting this process *in vivo*, such as whether the porphyrin components of HpD circulate bound to serum proteins, have not been directly assessed.

There are two porphyrin-binding proteins in

Abbreviations used: Hp, haematoporphyrin; HpAc, *OO'*-diacetylhaematoporphyrin; HpD, haematoporphyrin derivative.

serum, albumin and haemopexin (Koskelo *et al.*, 1970; Muller-Eberhard & Morgan, 1975; Morgan *et al.*, 1980). Studies from this laboratory (Morgan *et al.*, 1980) and others (Lamola *et al.*, 1981) have shown that albumin is the carrier for the naturally occurring porphyrins in human serum. Hp and HpAc represent the major fluorescent components of the HpD used clinically. HpAc appears to be the most active component of HpD (Henderson *et al.*, 1980; Berenbaum *et al.*, 1982). However, the structure of the active component was not identified. The studies presented here were therefore undertaken to investigate the interaction of purified components of HpD with serum and cellular proteins to increase understanding of the mechanism of porphyrin clearance by cells. Here, we characterize the interaction of two, representative photocytotoxic porphyrins, Hp and HpAc, with human serum albumin and human haemopexin, using absorption and fluorescence spectroscopy. In addition, we examine the effect of porphyrin binding to albumin on the porphyrin–cell interaction using Morris hepatoma tissue culture (HTC 7288) cells, with well-defined mixtures of porphyrin and albumin.

Methods

Haemopexin was prepared (Vretblad & Hjorth, 1977) from defibrinated non-haemolysed human and rat plasma. The purity of preparations was assessed immunologically and with polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Laemmli & Favre, 1973). Concentrations of freeze-dried human albumin (Kabi AB; lot no. 0175953) and rat albumin (Sigma; fraction V, lot no. 30F-9330) solutions were determined by using an $A_{280}^{1\%}$ of 5.5 (Beaven *et al.*, 1974) and a mol.wt. of 68000 for human and $A_{280}^{1\%}$ of 6.9 and mol.wt. of 65000 for rat (Peters, 1975). Immunological analysis using a monospecific antibody to rat haemopexin raised by us in goats, detected a contamination (approx. 10%) of the commercial rat albumin with haemopexin. The rat haemopexin was removed by passing the albumin, dissolved in 0.05 M-sodium phosphate/0.2 M-NaCl, pH 7.0, over a wheatgerm lectin-Sepharose affinity column (Vretblad & Hjorth, 1977). The albumin fractions were pooled, dialysed against 5 mM-sodium phosphate buffer, pH 7.4, and freeze-dried. Cytosol (100000g supernatant of a 30% tissue homogenate) was prepared from rat liver as described previously (Davies *et al.*, 1979) after the liver was perfused with ice-cold 0.9% NaCl to remove serum (Smith & Morgan, 1979).

Preparation of porphyrins

HpD was formed by treating haematoporphyrin dihydrochloride (Porphyrin Products, Logan, UT, U.S.A.) with 5% H_2SO_4 in acetic acid for 1 h at 20°C (Bonnett *et al.*, 1981). This HpD preparation, dissolved in dimethyl sulphoxide, was applied to thin-layer plates and chromatography was carried out by the method of Henderson *et al.* (1980), using silica gel G with benzene/methanol/98–100% formic acid (140:25:2, by vol.) as solvent system. HpAc (R_F 0.5) was eluted from the gel with methanol and portions were dried under N_2 without delay to minimize hydrolysis of the diacetate and the formation of methoxy derivatives (Bonnett *et al.*, 1981). Subsequent chromatography of this eluted material showed it to be greater than 90% HpAc with two minor components (R_F 0.1 and 0.3) that co-migrate with authentic haematoporphyrin and the monoacetate respectively. Stock solutions (approx. 1 mM) of porphyrins were made up fresh in dimethyl sulphoxide, and protected from light. Concentrations were determined using an ϵ_{402}^{mm} of $320 M^{-1} \cdot cm^{-1}$ for Hp in 1 M-HCl (Tipping *et al.*, 1978) and an ϵ_{396}^{mm} of $188 M^{-1} \cdot cm^{-1}$ for HpAc in methanol (Bonnett *et al.*, 1981).

Porphyrin-albumin complexes were formed by pre-incubating the porphyrin in the dark with various concentrations of human serum albumin for 30 min at pH 7.2 and 22°C to ensure equilibrium

binding. By increasing the albumin/porphyrin molar ratio in these mixtures the cells can be exposed to controlled amounts of free and albumin-bound porphyrin. This closely mimics the situation in the plasma *in vivo*. The concentration of free porphyrins in such mixtures can be calculated from the experimentally determined values for the dissociation constant, K_d (see Table 2), for the porphyrin-albumin complexes. Only a small amount of hydrolysis of HpAc is expected to occur at this pH and was confirmed by the lack of apparent change in the absorption spectra.

Spectral analysis: absorption and fluorescence experiments

Absorption spectra were recorded using a Cary 210 spectrophotometer and fluorescence experiments were carried out using a Perkin-Elmer 650-40 spectrofluorimeter. Titrations of protein fluorescence were performed by adding portions of concentrated porphyrin solutions to proteins and analysed as described previously (Morgan *et al.*, 1980) using the double-logarithmic method of Chipman *et al.* (1967) to obtain apparent K_d values and binding stoichiometries.

Characteristic fluorescence excitation and emission spectra of the porphyrin fluorophore in buffer or associated with proteins were obtained with spectral slits of ≤ 5 nm using the conditions described by Morgan *et al.* (1980). Care was taken to minimize exposure of samples to exciting light to prevent photo-oxidation or bleaching of protein and porphyrin. In experiments with serum, normal human serum or haemopexin-free serum (Conway *et al.*, 1975) was diluted 1:100 with buffer and porphyrin was added to a final concentration of $0.1 \mu M$. These diluted serum samples showed no fluorescence characteristic of porphyrins in the 500–700 nm region before the addition of porphyrin.

Incubation of hepatoma tissue culture cells with porphyrins

Hepatoma tissue culture (HTC) cells, originally from Morris hepatoma 7288C, were maintained in Swim's S-77 medium fortified with 5% calf serum and 5% foetal-calf serum ('5/5 medium'). The HTC 7288 cells were carried by splitting (1:10) confluent cells from one 75 cm² tissue culture flask. The cells were released from the flask with a sterile iso-osmotic trypsin/EDTA solution (Gibco) and then plated (2×10^6 cells) on a 75 cm² flask with 15 ml of 5/5 medium. The cells were incubated at 37°C with media changes at 24 h and 72 h. The doubling time of HTC 7288 cells in 5/5 medium under these conditions was 22–24 h (Mapes & Aultman, 1982). To maintain a low passage number, the cells were plated every 6 months from ampoules of frozen cells

that had been stored in liquid N₂ in Swim's medium plus 20% serum and 7% dimethyl sulphoxide. These seed cultures were generously provided by Dr. J. P. Mapes of this Department.

To avoid photoactivation of the porphyrins and consequent cellular damage, cell-associated porphyrin was determined in non-illuminated cells and all porphyrin-containing solutions were protected from exposure to light to minimize photo-oxidation. These experiments were carried out on cells in their exponential phase of growth. After harvesting by brief trypsin exposure the cells were washed free of medium and stored in incubation buffer (25 mM-Hepes/0.15 M-NaCl/2 mM-CaCl₂, pH 7.2) on ice until use. Control experiments determined that addition of soya-bean trypsin inhibitor to the cells immediately after harvesting and to the wash media did not increase porphyrin uptake in these cells and, therefore, was not routinely used. After 10 min pre-incubation at 37°C with shaking at 100 cycles per min [(0.5–1.0) × 10⁶ cells per ml] porphyrin or well-defined porphyrin-albumin complexes were added to the cells and the incubation was continued.

The porphyrin associated with the cells was determined in duplicate portions (1.0 ml) from paired vials taken at 0.5, 5, 10, 30 and 60 min. The variation (standard deviation) in cell-associated porphyrin was less than 10% of the mean in these samples. Cells, collected by centrifugation (approx. 50 g for 1 min), were then washed by resuspension in 5 ml of ice-cold 10 mM-sodium phosphate buffer, pH 7.4–7.2, containing 0.15 M-NaCl ('PBS') and centrifuged. The cells (volume approx. 200 µl) were resuspended once more in 5 ml of fresh, ice-cold PBS, representing more than a 1000-fold dilution, and re-centrifuged. Throughout, polypropylene tubes and vials, to which porphyrins do not adhere, were used. Since porphyrins have limited solubility in aqueous solutions, forming dimers and aggregates, control experiments to measure background levels of porphyrin precipitation were carried out. Hp or HpAc were added at 0.2–10 µM to buffer and subjected to identical conditions and treatment as the cell samples. These experiments established that at free porphyrin concentrations up to 3.5 µM, background porphyrin levels were equivalent to less than 10% of the cell-associated porphyrin at 30 s and less than 5% and 1.5% of cell-associated Hp and HpAc respectively, at 30 min over this range of free porphyrin. All time-course, temperature and inhibitor experiments were carried out at free porphyrin concentrations of 0.2 µM (10 µM porphyrin/100 µM-human serum albumin), so that the experimentally-determined background levels would be equivalent to <1 pmol/10⁶ cells, a negligible amount compared with cell-associated porphyrin (generally greater than 20–50 pmol per 10⁶ cells).

Background levels of 10 µM free porphyrin in buffer, a porphyrin level used only in the experiment whose results are shown in Fig. 4, were equivalent to 12 pmol/10⁶ cells, whereas the 'signal' was >150 pmol/10⁶ cells.

The term cell-associated porphyrin has been used throughout and is defined as porphyrin residing within or tightly bound to the cell surface. The cell-associated porphyrins assessed by this procedure are tightly bound or within the cell since addition of human serum albumin (100 µM) to the wash buffer did not decrease the porphyrin concentration in the final cell pellet. Human serum albumin ($K_D < 2 \mu\text{M}$) would decrease the amount of cell-associated porphyrin if it was adhering to the surface loosely and thus likely non-specifically. Total porphyrin associated with the cells was measured by dissolving the cell pellet to a final volume of 2.0 ml with 2% sodium dodecyl sulphate in PBS, which simultaneously extracted and solubilized the porphyrin. Porphyrin concentration was determined by comparison of the extracted fluorescence with a standard curve of porphyrin in the same solvent. Porphyrin fluorescence is linear with concentration up to 0.04 µM-porphyrin.

Results and discussion

As shown in Fig. 1, characteristic red-shifts in the absorption maximum and enhanced absorbance of the porphyrin occur when Hp or HpAc bind to albumin or haemopexin. Similarly, porphyrin-albumin and porphyrin-haemopexin complexes have characteristic fluorescence excitation and emission properties (Table 1). The absorption spectrum for Hp in serum (Table 1) is similar to those previously reported (Moan & Christensen, 1981; Reddi *et al.*, 1981). In order to determine whether albumin or haemopexin is the predominant binding agent in human serum for these porphyrins the stoichiometry and affinity of albumin and haemopexin for Hp and HpAc were investigated using quenching of intrinsic protein fluorescence. The interaction of albumin or haemopexin with Hp and HpAc quenches the fluorescence of the protein in proportion to the amount of ligand added (Fig. 2). Titrations of albumin and haemopexin with both Hp and HpAc were similar, with stoichiometries of 1 mol of porphyrin bound per mol of protein and apparent K_d values in the range 1–2 µM for albumin and 3–4 µM for haemopexin (Table 2). In view of the 25–50-fold molar excess of albumin to haemopexin circulating in serum (Morgan *et al.*, 1980), we can conclude that Hp and HpAc will be preferentially bound to albumin. Although endogenous substances associated with albumin in serum, e.g. fatty acids, may alter the environment where the porphyrin binds, the spectra of the porphyrins in haemopexin-free serum closely resemble that of the

Fig. 1. Absorption spectra of free and protein-bound HpAc

Absorption of HpAc ($1\mu\text{M}$) in 25 mM-Hepes/0.15 M-NaCl, pH 7.2 (—), with human serum albumin ($100\mu\text{M}$; ---), in human serum (diluted 1:10 with buffer; — · —) and in haemopexin-free serum (diluted 1:10 with buffer; ----) are shown.

porphyrin-albumin complexes. The more intense signal and the longer wavelength maxima of the porphyrin-albumin complexes suggest that the albumin porphyrin-binding site has an apolar or hydrophobic character as previously reported (Morgan *et al.*, 1980).

The apparent preferential accumulation of porphyrins by cancerous cells must be the result of differences in the clearance of porphyrins by these cells compared with normal tissue. Clearance involves many processes, including binding by serum proteins, cell uptake, which may involve interaction with a membrane component, intracellular binding and elimination. The experiments described below

Table 1. Summary of the absorption and fluorescence properties of porphyrin-albumin, -haemopexin and -human serum mixtures

Abbreviation used: n.d., not determined. For the absorption measurements the concentration of porphyrin was $1\mu\text{M}$ (an average molecular weight of 640 was assumed for porphyrins present in the HpD mixture), whereas for the fluorescence measurements it was $0.1\mu\text{M}$. Porphyrins were added to 25 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/0.15 M-NaCl buffer, pH 7.2, or to the buffered protein solutions $100\mu\text{M}$ -human albumin, $40\mu\text{M}$ -human haemopexin or -serum (i.e. diluted 1:100). The λ_{max} values were determined using 5 nm slits and have a 1 nm resolution limit. This serum displayed no fluorescence in the 590–700 nm region when excited at 400 nm, before the addition of porphyrin. Fluorescence spectra were recorded in a temperature-controlled cuvette at 25°C .

Protein	Porphyrin	Fluorescence		
		Absorption λ_{max} (nm)	Excitation λ_{max} (nm)	Emission λ_{max} (nm)
None	Hp	393	394	612
	HpAc	393	395	615
	HpD	394	n.d.	n.d.
Human albumin	Hp	400	398	621
	HpAc	402	399	622
	HpD	401	n.d.	n.d.
Human serum	Hp	401	398	622
	HpAc	402	400	622
	HpD	402	n.d.	n.d.
Human haemopexin	Hp	398	400	620
	HpAc	400	400	622
	HpD	n.d.	n.d.	n.d.
Haemopexin-free serum	Hp	400	400	620
	HpAc	401	401	623
	HpD	400	n.d.	n.d.

show that, although Hp and HpAc are bound by albumin in serum, non-protein-bound monomeric porphyrin is the kinetically-active species in this transport process. HpAc associates to a greater extent than Hp with both HTC cells and S49.1 lymphoma cells. Both porphyrin structure and cell type are shown to be important determinants of the rate and extent to which porphyrin associates with cells.

The concentration of free porphyrin presented to the cells was carefully controlled by adjusting the molar proportions of albumin and porphyrin. The amount of free porphyrin in these mixtures was calculated using the experimentally determined K_d values (Table 2). A range of free porphyrin concentrations was studied, reflecting the estimated intravascular free porphyrin in patients ($0\text{--}2\mu\text{M}$). HpD is normally given to patients at doses ranging

from 5 to 7mg/kg body wt. (Dougherty *et al.*, 1978), and 8 μ M-Hp in serum has been recorded 1 h after HpD infusion in patients (Zalar *et al.*, 1977).

Fig. 2. Quenching of intrinsic protein fluorescence, by Hp and HpAc

The quenching of the protein fluorescence, as a function of the porphyrin/protein molar ratio, of human albumin by Hp (●) and HpAc (■) and of human haemopexin by Hp (○) and HpAc (□) is shown. Protein solutions (whose absorption values at the exciting wavelength were <0.08) in 15mM-sodium phosphate buffer, pH 7.5, were illuminated at 290nm (albumin) or 295nm (haemopexin) and the emission at 335nm was recorded after adding the indicated amounts of porphyrin to portions of protein and incubating for 30 min in the dark.

Table 2. Summary of the interactions of human serum albumin and human haemopexin with Hp and HpAc determined by intrinsic protein fluorescence quenching

Fluorescence data were obtained from duplicate titrations of the quenching of the intrinsic fluorescence of 5 μ M-human albumin or -human haemopexin by added porphyrins. These solutions (A_{290} or A_{295} <0.08) in 25mM-Hepes/0.15M-NaCl buffer, pH 7.2, were illuminated at 290 or 295 nm and the emission at 335 nm was recorded after additions of porphyrin. The data were treated by the double-logarithmic method of Chipman *et al.* (1967) as reported previously (Morgan *et al.*, 1980). Uncertainties based on our observed experimental variations are approx. $\pm 50\%$ for K_d values and ± 0.1 for molar ratios.

Protein	Porphyrin	K_D apparent (μ M)	Porphyrin/protein stoichiometry (mol of porphyrin/mol of protein)
Human albumin	Hp	1.9	1.1
	HpAc	1.5	0.9
Human haemopexin	Hp	2.8	1.2
	HpAc	4.0	1.05

Depending on the rate of infusion, porphyrin levels as high as 50 μ M could be reached [assuming a mol.wt. of approx. 640 for the porphyrins and a plasma volume of 42ml/kg body wt. (Dagher *et al.*, 1965)].

Hp and HpAc rapidly associate with HTC cells and the amount of porphyrin that accumulates was time- (Fig. 3) and dose-dependent (Fig. 4). The time course of association of porphyrin with the cells (Fig. 3) shows a rapid porphyrin accumulation for the first 15 min followed by a lower rate over the next 30 min. HpAc is accumulated at a clearly higher rate (>2pmol of HpAc/10⁶ cells per min) than is Hp (Fig. 3). At least three classes of association are observed. First, a dose-dependent and saturable association of porphyrin occurs within 30s at 37°C in these extensively washed cells under conditions of low levels of free porphyrin (less than 2 μ M). It seems likely that porphyrin would be predominantly membrane-associated at this early stage. Eadie-Hofstee analyses were consistent with a high-affinity saturable process with a K_m of approx. 10nM for both Hp and HpAc. The maximum porphyrin association was 20pmol/10⁶ cells. After this rapid initial association a slower but also saturable process and of higher capacity is evident (Fig. 4). Thus the difference in cell-associated porphyrin at 30 min and 30s is plotted in Fig. 4 to reflect the major saturable process observed. This process is half-saturated at 1.0 μ M free porphyrin and reaches a maximum of 60pmol of HpAc/10⁶ cells. At free porphyrin levels >2 μ M a non-saturable association of porphyrin occurs. This suggests that previous investigations on the mechanism of cell-porphyrin interactions (Kessel, 1981; Moan & Christensen, 1981; Berns

Fig. 3. Accumulation of HpAc and Hp by HTC cells Cells (1 $\times 10^6$ cells per ml) were incubated at 37°C with well-defined preformed porphyrin-albumin complexes (10 μ M-porphyrin/100 μ M-protein); either Hp (●) or HpAc (■) was used. Quantification of the cell-associated porphyrin was carried out as described in the Methods section.

et al., 1982) conducted under conditions likely to produce free porphyrin concentration greater than $2\mu\text{M}$ must be interpreted with caution. The data in Fig. 4 show that there is a direct, but complex, relationship between free porphyrin concentration in the media and the amount of porphyrin associated with the cells. Thus, the same data plotted against albumin concentration (inset in Fig. 4) show a decrease in cell-associated porphyrin with increasing albumin concentration. This supports the argument that the free porphyrin, not the porphyrin-albumin complex, interacts with the cells. Similar results were obtained with Hp (results not shown); over the same range of free porphyrin concentrations the amount of cell-associated porphyrin was about 5-fold lower with Hp compared with HpAc, consistent with the results shown in Fig. 3. Although most of the porphyrin in serum will circulate bound to albumin, which is present at $500\mu\text{M}$ in serum, binding is a dynamic process and ligand constantly dissociates and becomes available to interact with cells. These experiments directly show that porphyrin from well-defined porphyrin-albumin complexes readily associates with cells at concentrations likely to be present in serum during photochemotherapy.

In addition, porphyrin associated with S49.1 lymphoma cells was investigated using the same protocol described for the HTC cells. In side-by-side

incubations the lymphoma cells accumulated both Hp and HpAc but at a significantly lower rate than the HTC cells, even after taking into account the smaller size of the lymphoma cells. Again, more HpAc than Hp was taken up (0.5 pmol of HpAc/ 10^6 lymphoma cells per min compared with $1.5\text{ pmol}/10^6$ HTC cells per min), indicating the importance both of porphyrin structure and cell type in porphyrin-cell interactions. This is consistent with the observation of Henderson *et al.* (1980) that more HpAc than Hp accumulated in cancerous mouse squamous tissue after intravenous injection.

The cell-associated porphyrin represents an accumulation of porphyrin over the initial concentration of free porphyrin in the incubation medium. For example, taking a value of cell-associated HpAc of $45\text{ pmol}/10^6$ cells (see Fig. 4) and using the HTC cell volume of 1.9×10^{-12} litre/cell (Mapes & Aultman, 1982) the concentration of porphyrin in the cell pellet is $24\mu\text{M}$, compared with an initial free porphyrin level of $1\mu\text{M}$. Moreover, pre-incubation of the HTC cells with KCN (10 mM for 2 h at 37°C) reduced the amount of cell-associated porphyrin at 30 min by 50%. Other metabolic inhibitors including sodium fluoride, ouabain and oligomycin also decreased the amount of cell-associated porphyrin uptake but were less effective (20–35% inhibition). These results suggest that at least part of the process involved in the interaction of porphyrin with HTC cells involves active transport. The association of porphyrin with the HTC cells was also temperature-dependent. The

Fig. 4. The effect of free porphyrin concentration on cell-associated porphyrin

The data represent the difference between porphyrin associated with the cells at 30 min and 30 s in HTC cells after incubation with preformed complexes of HpAc and albumin. HpAc ($10\mu\text{M}$) was added to albumin (0 – $100\mu\text{M}$) to produce a range of free porphyrin concentrations calculated from the experimentally-determined K_d values. In addition, the cell-associated porphyrin is shown versus the albumin concentration (see the inset).

Table 3. Summary of fluorescence properties of Hp and HpAc with rat serum proteins and liver cytosol

The fluorescence maxima were determined as described in the legend to Table 2. Rat liver cytosol prepared as described in the Methods section was diluted 1:10 with 15 mM-sodium phosphate buffer, pH 7.4, for these spectral measurements. Neither the rat serum nor cytosol displayed any fluorescence in the 590–700 nm region when excited at 400 nm before the addition of porphyrin.

Protein	Porphyrin	Fluorescence excitation $\lambda_{\text{max.}}$ (nm)	Fluorescence emission $\lambda_{\text{max.}}$ (nm)
None	Hp	394	612
	HpAc	395	616
Rat liver cytosol	Hp	394	617
	HpAc	398	623
Rat serum	Hp	400	619
	HpAc	401	622
Rat albumin	Hp	403	621
	HpAc	403	623
Rat haemopexin	Hp	399	618
	HpAc	400	620

rate observed at 4°C (results not shown) paralleled the slower phase of accumulation at 37°C. By 1 h at 4°C cell-associated porphyrin was only 50% of that observed at 37°C.

The affinity that normal and cancerous cells have for porphyrins can be ascribed to binding sites on the plasma membrane (Kessel, 1981) or to intracellular porphyrin-binding proteins. When Hp and HpAc are mixed with rat cytosol or with rat serum proteins (albumin and haemopexin), distinct fluorescence spectra are generated (see Table 3). These fluorescence spectra provide direct evidence for the presence of components in rat liver cytosol that can bind Hp and HpAc, and further distinguish these cytosolic binders from rat albumin and haemopexin. The fluorescence maxima of the porphyrin-rat-albumin and porphyrin-rat-haemopexin complexes are too similar to allow identification of the complex in serum. Interestingly, the spectrum (emission maximum 624 nm) generated by HpAc (0.1 nM) in cytosol from HTC cells resembles that of normal cytosol. The relationship of this protein(s) to the cytosolic proteins in normal rat liver that interact with naturally occurring porphyrins (Koskelo *et al.*, 1976), and their role in porphyrin clearance by cells, require investigation.

In summary we clearly show that albumin and haemopexin both have appreciable affinities for certain photochemotherapeutic porphyrins, but that albumin is the principal binding protein in serum. In addition, the free porphyrin, not porphyrin-albumin, interacts with cells and, further, evidence is presented for intracellular porphyrin-binding molecules.

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