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Fluorimetric study of the binding of protoporphyrin to haemopexin and albumin

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Fluorescence spectra of protoporphyrin bound to its most affinitive site on human serum albumin, bound to human haemopexin and dissolved in human plasma reveal that, when present in plasma, at least 90% of this porphyrin is bound to albumin. Human serum albumin binds protoporphyrin with an affinity $K_A = 3 \times 10^9 M^{-1}$ in phosphate-buffered saline. The affinity of haemopexin for protoporphyrin is 4 times smaller. From these data it is concluded that less than 1% of plasma protoporphyrin is bound to haemopexin. Implications of the data for protoporphyrin transport and clearance are discussed.

Clearance of bilirubin and haem

The structurally related tetrapyrroles, bilirubin, haem and protoporphyrin, all relatively insoluble in water, are efficiently cleared from blood plasma by the liver. Bilirubin is transported in plasma bound to albumin, which does not enter the hepatocyte in its function as a transport protein (Schmid & McDonagh, 1978). This most abundant of the plasma proteins also binds haem (Liem & Muller-Eberhard, 1971) and protoporphyrin (Poh-Fitzpatrick & Lamola, 1977; Koskelo *et al.*, 1970; Marecek *et al.*, 1973). The clearance of haem involves haemopexin (Muller-Eberhard & Morgan, 1975; Muller-Eberhard, 1978), a plasma protein much less abundant than is albumin. Haemopexin has a high affinity for haem (Hrkal *et al.*, 1974) and actually carries the iron tetrapyrrole into hepatocytes (Muller-Eberhard, 1978). Haemopexin recirculates when delivering haem to the hepatocyte (Smith & Morgan, 1979), but is catabolized at an accelerated rate during this process (Sears, 1970; Wochner *et al.*, 1974) and, probably secondary to this, its production is enhanced. Haemopexin also binds porphyrins (Morgan & Muller-Eberhard, 1972), including protoporphyrin (Koskelo *et al.*, 1970; Marecek *et al.*, 1973; Seery & Muller-Eberhard, 1973) as well as bilirubin (Morgan *et al.*, 1978).

Protoporphyria

Protoporphyrin is not normally found in human plasma, as it does not accumulate either during haem synthesis or during haem catabolism. The presence of free base protoporphyrin in plasma is usually a consequence of aberrant haem biosynthesis. Although drug-induced protoporphyria is rarely observed in man, high doses of griseofulvin cause an hepatic protoporphyria in laboratory animals which results in elevated concentrations of plasma and erythrocyte free protoporphyrin (Poh-Fitzpatrick & Lamola, 1977) and increased levels of plasma haemopexin (Cripps *et al.*, 1977). In man, lead poisoning usually leads to the accumulation of zinc protoporphyrin in erythrocytes (Lamola & Yamane, 1974). Significantly abnormal concentrations of metal-free protoporphyrin are observed only in severe acute cases. However, in such cases only the metal-free protoporphyrin is ever found in plasma (Lamola & Yamane, 1974; Poh-Fitzpatrick & Lamola, 1976).

Congenital human protoporphyria is a well-recognized disease in which both the erythropoietic and hepatic tissues may be sites of excess porphyrin synthesis (Lamon *et al.*, 1980). Whenever liver function is unimpaired, plasma protoporphyrin is cleared rapidly and a flow of excess protoporphyrin from the erythrocytes to the plasma is maintained. Efficient hepatic clearance appears to be crucial for maintenance of a low concentration of porphyrin in the skin (Lamon *et al.*, 1980), thereby controlling concomitant photosensitivity. The fatal liver failure which occurs in protoporphyria may be the result of the continuous hepatic clearance of protoporphyrin (Lamon *et al.*, 1980). It is therefore important to

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define clearly the mechanisms of transport and excretion of protoporphyrin.

Clearance of protoporphyrin

In patients with protoporphyria, the rate of protoporphyrin excretion into the bile has been reported to be as high as 50mg/day (Lamon *et al.*, 1980). It appears that protoporphyrin is, for the most part, excreted unchanged, and that very little is catabolized (Ibrahim *et al.*, 1966; Ibrahim & Watson, 1968). This is unlike haem, which is in great part rapidly catabolized in the liver, and unlike bilirubin, which is esterified in the liver and excreted in conjugated form (Schmid & McDonagh, 1978).

The concentration of protoporphyrin in plasma of patients with uncomplicated protoporphyria is of the order of 1μM. This concentration is smaller than those of either haemopexin (about 5–10μM) or albumin (about 500μM). Concentrations of haemopexin and turnover time for this plasma protein in these patients do not appear to differ significantly from the normal range of values. Gel-filtration chromatography and electrophoretic fractionation of normal plasma to which protoporphyrin is added reveals that most of the porphyrin is associated with fractions containing albumin and haemopexin (Koskelo *et al.*, 1970). The fluorescence emission and excitation spectra of plasma specimens from patients with protoporphyria are identical with those of protoporphyrin bound to albumin. Although these observations and the binding studies of Seery & Muller-Eberhard (1973) suggest that protoporphyrin in the plasma of patients with protoporphyria is chiefly bound to albumin, binding of a significant fraction of the protoporphyrin to haemopexin and a role for haemopexin in the clearance of protoporphyrin cannot be excluded. Metabolic studies of haemopexin (Wochner *et al.*, 1974; Muller-Eberhard *et al.*, 1974) in patients with protoporphyria left uncertain the involvement of this protein in the disposal of protoporphyrin. We therefore undertook to assess further *in vitro* the relative roles of human albumin and haemopexin in protoporphyrin binding. We characterized the haemopexin–protoporphyrin complex spectroscopically and determined the affinities of haemopexin and albumin for protoporphyrin.

Experimental

Preparation of materials

Protoporphyrin (>90%) (Porphyrin Products, Logan, UT, U.S.A.) was used as received. A few crystals were dissolved in a small volume (5μl) of concentrated ammonia. The resulting solution was diluted with 20ml of buffered saline (0.15M-NaCl/0.01M-sodium phosphate, pH 7.4), left at 4°C for 1h, and filtered through paper (Whatman no. 1). The concentration of protoporphyrin was determined by measuring the A_{406} of a sample made 2.4M in HCl by using $\varepsilon_{mM} = 242$. The solution (usually about 10μM) was then diluted with buffered saline to make stock solutions of concentrations 0.1–1μM. Protoporhyrin solutions were stored at 4°C shielded from light and used within a few hours of preparation.

Human serum albumin was prepared from the plasma of adult male volunteers by a variation (Lamola *et al.*, 1979) of the method of Flodin & Killander (1962) and stored at pH3 and 4°C. The protein was used within 5 days of its preparation. Its purity was assayed by the Bromocresol Green colorimetric method (Doumas *et al.*, 1971), by A_{280} measurement by using $\varepsilon = 36000$, and fluorimetrically by using bilirubin binding (Lamola *et al.*, 1979). Only preparations assayed to be over 90% pure by all three methods were used.

Haemopexin was prepared from plasma by the method described by Hrkal & Muller-Eberhard (1971). Freeze-dried specimens, stored at −20°C, were dissolved in buffered saline and used immediately. Haemopexin was assayed by measuring the A_{280} by using $A^{1\%}_{1cm} = 20$ (Seery *et al.*, 1972).

Fluorescence spectroscopy

Fluorimetric measurements were made with a Perkin–Elmer MPF4 spectrofluorimeter. A cut-off filter (Hoya 0–56) was used to decrease scattered excitation light for specimens with low fluorescence signals.

Results

Fluorescence of protoporphyrin in different microenvironments

The fluorescence emission and excitation maxima of protoporphyrin as well as its fluorescence quantum yield vary with its microenvironment. Representative emission spectra of protoporphyrin in the presence and absence of protein are shown in Fig. 1. Lists of the wavelengths of the maxima of the most intense (0–0) band of the fluorescence spectra, the maxima of the Soret bands of the fluorescence excitation spectra and some relative fluorescence yields are given in Table 1. The data refer to specimens in buffered saline (pH 7.4) containing concentrations of proteins sufficient to ensure that virtually all the protoporphyrin (0.1μM) was bound to the strongest protein-binding site.

Affinity of protoporphyrin for serum albumin and haemopexin

The spectra of Fig. 1 show that the binding of protoporphyrin to albumin can be well monitored by the increase in intensity at 635nm during titration of protoporphyrin in buffered saline with albumin.

Such fluorimetric titrations were carried out at protoporphyrin concentrations (1–10nM) at which binding at the strongest albumin site(s) could be observed. Typical data are shown in Fig. 2; 17 independent titrations were performed, with three different preparations of albumin. In all the experiments an excellent fit (see Fig. 2) of the data was obtained to the simple first-order (one-site) binding equation with a binding constant of 3.2 (± 0.8) $\times 10^9 M^{-1}$ (25°C).

Weaker secondary sites for binding of protoporphyrin and albumin were observed fluorimetrically at much higher porphyrin and protein concentrations than those necessary to observe primary binding. Since plasma protoporphyrin concentrations observed even in the most severe cases of protoporphyria do not approach the albumin concentration, these secondary sites were not further investigated.

The affinity of haemopexin for protoporphyrin was determined relative to that of albumin. Both the quantum yield and the fluorescence maximum of the haemopexin complex differ from those of the albumin–protoporphyrin complex (Fig. 1). Thus, at constant protoporphyrin concentration and combined concentrations of the proteins that ensure

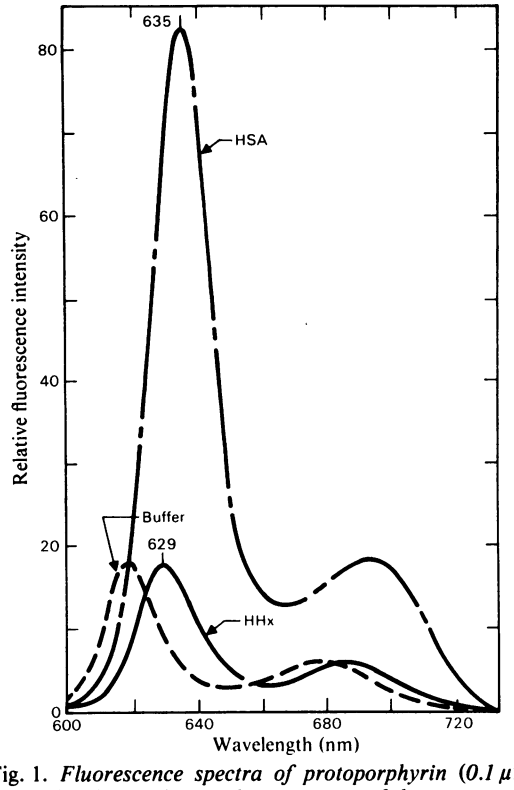

Fig. 1. *Fluorescence spectra of protoporphyrin (0.1 μM) in buffer (pH 7.4), in the presence of human serum albumin (HSA; 0.2 μM) and in the presence of human haemopexin (HHx; 1 μM)*
The excitation wavelength was 408nm.

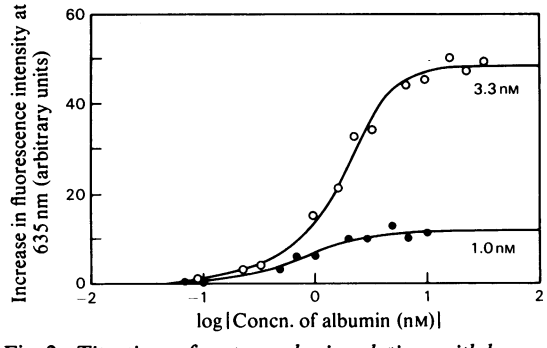

Fig. 2. *Titrations of protoporphyrin solutions with human serum albumin followed fluorimetrically*
The data points represent the increase in fluorescence intensity at 635nm obtained when albumin was added. The curves through the data points are the binding curves expected for a single site with an affinity constant of $3 \times 10^9 M^{-1}$. Concentrations of protoporphyrin are given beside the curves.

Table 1. *Fluorescence parameters for protoporphyrin–protein complexes*

Complex (1 : 1) with	Excitation maximum (nm) (Soret band)	Emission maximum (nm) (0–0 band)	Relative yield*
Human serum albumin	408	635	[1]
Rabbit serum albumin	408	633	0.40
Bovine serum albumin	408	626	0.25
Human haemopexin	408	629	0.20
Rabbit haemopexin	403	623	0.33
In buffered saline (pH 7.4)	~400	619	0·23

* Fluorescence quantum yields relative to that of the human albumin complex.

binding of all the porphyrin, the ratio of protoporphyrin bound to albumin to that bound to haemopexin can be obtained directly by comparing fluorescence spectra from mixtures of the two proteins with spectra in the presence of each alone. Spectra from such a competition experiment are shown in Fig. 3. The reference spectra for this experiment are shown in Fig. 1. The results of several such experiments indicated that equal quantities of protoporphyrin are bound to albumin and to haemopexin when the molar ratio of the proteins is approx. 1:4 respectively. Thus the strongest site on albumin binds protoporphyrin with an affinity 4 times that of haemopexin, assuming a single strongest binding site for the latter. Therefore the effective association constant for the haemopexin–protoporphyrin complex (single site) is approx. 8×10^8 M^{-1} (25°C).

Protoporphyin in plasma

The fluorescence spectrum of human plasma to which was added 0.3 μM-protoporphyrin was identical with that of plasma from patients with protoporphyria (Fig. 4). The intensities of the fluorescences were approximately in proportion to the protoporphyrin concentrations in the specimens as determined by extraction methods (Poh-Fitzpatrick *et al.*, 1973). Spectra from the native and synthetic protoporphyrin–plasma complexes were identical, within experimental certainty, with that of the albumin–protoporphyrin complex.

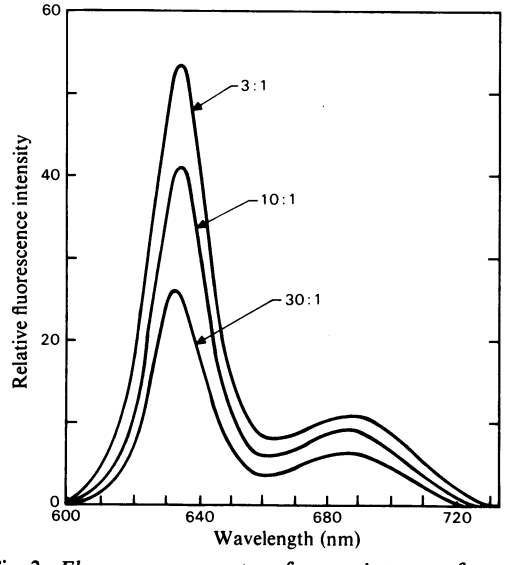

Fig. 3. *Fluorescence spectra from mixtures of protoporphyrin (0.1 μM), human serum albumin and human haemopexin*

The albumin concentration was 0.2 μM. Reference spectra with either albumin or haemopexin present alone are shown in Fig. 1. Numbers beside curves refer to haemopexin/albumin ratios.

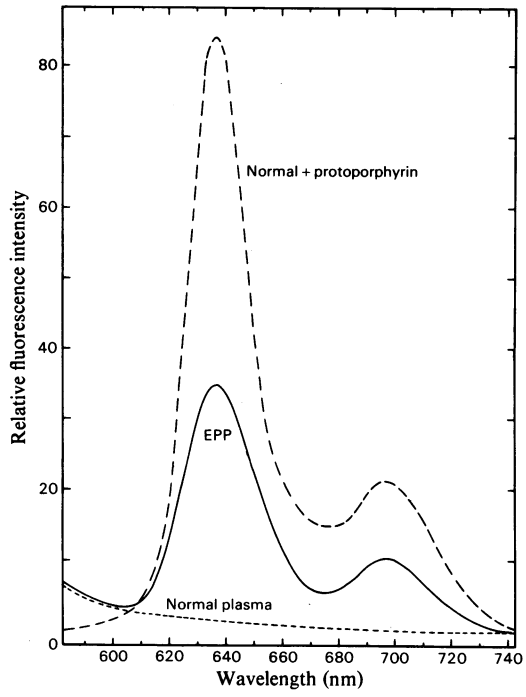

Fig. 4. *Fluorescence spectra of plasma specimens diluted 1:4 with buffered saline*

Plasma obtained from a normal adult male volunteer with 0.3 μM-protoporphyrin added was compared with that from a patient with protoporphyria (EPP). The excitation wavelength was 405 nm.

Discussion

Affinities of serum albumin and haemopexin for protoporphyrin

The observations that the fluorescence spectra of protoporphyrin added to normal plasma, of plasma from patients with protoporphyria and of the albumin–protoporphyrin complex are the same within experimental certainty are verified. The fluorescence emission spectrum of haemopexin–protoporphyrin was found to be shifted from that of the albumin–protoporphyrin complex by 6 nm. This shift is sufficient to support the conclusion that only a small fraction, 10% at most, of the protoporphyrin in plasma could be bound to haemopexin. Further quantification of this conclusion can be obtained from the relative association constants. Both binding strength and concentration favour binding of protoporphyrin by albumin over that by haemopexin. The ratios for these are 4 and 50–100 respectively,

indicating that the ratio of protoporphyrin bound to albumin to that bound to haemopexin in plasma is 200–400, i.e. less than 0.5% of the protoporphyrin in plasma is bound to haemopexin.

Our results settle a disagreement which has existed in the literature between results of Koskelo *et al.* (1970), who suggested that protoporphyrin is mostly bound to albumin in plasma, and Marecek *et al.* (1973), who suggested that protoporphyrin is mostly bound to haemopexin. Neither groups employed methods that could assess the relative affinity constants for the first protoporphyrin bound to each of the proteins. Because of the necessity to use very low concentrations of protoporphyrin and albumin, the fluorimetric titration method that we employed appears to be the best approach presently available for accurate determination of the binding constants. Furthermore, the fluorescence method has allowed direct competition experiments without the necessity to separate the proteins.

Implications for protoporphyrin clearance

The very high value for the association constant of the albumin–protoporphyrin complex, $3 \times 10^9 M^{-1}$, predicts that, for the concentrations of protoporphyrin and albumin found in the typical patient with protoporphyria, the concentration of unbound plasma protoporphyrin is of the order of 0.001 nM. It is interesting to compare this exceedingly low concentration of unbound protoporphyrin and the apparent 50 μmol of protoporphyrin/day cleared through the liver of a patient with protoporphyria with the situation for bilirubin clearance. Unconjugated bilirubin, which is taken up by the liver at a rate of the order of 500 μmol/day, normally presents an unbound concentration in the plasma of the order of 1 nM (Schmid & McDonagh, 1978), i.e. the unbound bilirubin concentration is 1000 times higher than the unbound protoporphyrin concentration, whereas the uptake rates differ perhaps by only a factor of 10. If hepatocytes take up both tetrapyrroles only in unbound form, the specific rate of uptake of protoporphyrin would have to be very much higher than that for bilirubin. Alternatively, the relatively rapid uptake of plasma protoporphyrin by the liver in the face of an extremely low unbound protoporphyrin concentration may involve a carrier protein. The concentration of plasma protoporphyrin bound to haemopexin, although low relative to that bound to albumin, is expected to be about 1000 times the unbound protoporphyrin concentration. Thus haemopexin, known to carry haem into hepatocytes, cannot be excluded from a role in protoporphyrin excretion.

Results of analyses by gel filtration of plasma specimens from patients with protoporphyria indicated that up to 5% of the protoporphyin is bound to proteins other than albumin and haemopexin (A. A. Lamola & M. B. Poh-Fitzpatrick, unpublished work). Whether or not these other proteins are important for protoporphyrin clearance awaits further investigation.

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