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UPTAKE AND LOCALISATION OF HAEMATOPORPHYRIN DERIVATIVE IN NORMAL RAT LIVER

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Abstract—Fluorescence microscopy and analytical subcellular fractionation were used to investigate the hepatic localisation of haematoporphyrin derivative (HPD) after intraperitoneal administration. HPD was found to rapidly accumulate in the liver and then to slowly decline over 48 hr. Fluorescence microscopy showed that although at early times porphyrins could not be localised to a particular cell type, at 24 hr porphyrins were preferentially localised to the Kupffer cells of the liver.

Subcellular fractionation studies indicated that the initial rapid uptake of HPD was to the cytosol. However, at 24 hr, porphyrins appeared to be localised to lysosomes. Lysosomal localisation was confirmed using the selective organelle perturbant, Triton WR 1339. No evidence was found either at the light microscope level or by subcellular fractionation to suggest association of HPD with other organelles.

HPLC analysis showed that the porphyrins present in the plasma and in the cytosol and lysosome fractions were mainly the (*RS, SR*) and (*RR, SS*) diastereoisomers of haematoporphyrin and the two position isomers 8-(1-hydroxyethyl)-3-vinyldeuteroporphyrin and 3-(1-hydroxyethyl)-8-vinyldeuteroporphyrin. There was no evidence for the involvement of dimers such as dihaematoporphyrin ether.

Haematoporphyrin derivative (HPD) photodynamic therapy is being used increasingly as a local treatment for neoplastic disease [1]. This relies on the preferential retention of HPD by tumour tissue in comparison with normal tissues from which it is rapidly cleared [2]. However, recent reports have shown that HPD causes damage to normal tissues as well as to malignant neoplasms [3], but few studies have been carried out to determine the cellular and subcellular localisation of internalised HPD components.

HPD is a complex mixture of porphyrins including diastereoisomers of haematoporphyrin (HP), the positional hydroxyethylvinyl deuteroporphyrin (HVD) isomers and protoporphyrin. A dimer of HP with an ether linkage, dihaematoporphyrin ether, has been suggested as the active tumour localising component in HPD [4], but HPLC and fast atom bombardment mass spectrometry (FABMS) [5] studied presented inconclusive evidence for such a compound. Previous reports have shown that HPD treatment can result in damage to mitochondria [6, 7], microsomes [8], DNA [9–12] and membranes [13–18]. It is implied that HPD is localised to these organelles but there is little direct evidence that nuclei or mitochondria are the sites of HPD accumulation in normal cells. Early studies with fluorescence microscopy implied the uptake of certain porphyrins by the lysosomes of isolated cells [19, 20].

This study utilised fluorescent microscopy and analytical subcellular fractionation to determine the cellular and subcellular localisation of haematoporphyrin derivative in rat liver. The nature of the internalised porphyrins were analysed by HPLC.

MATERIALS AND METHODS

Preparation and administration of haematoporphyrin derivative (HPD). HPD was prepared from the free base according to the method of Gomer and Dougherty [21], who showed that this preparation was a potent tumour sensitiser. Male Sprague–Dawley rats (200–250 g) were injected i.p. with 10 mg HPD in 0.15 M NaCl (1 ml). Control animals were injected with 1 ml 0.15 M NaCl. At various times after dosage, the animals were anaesthetised by methoxyfluorane inhalation and the livers perfused *in situ* with phosphate buffered–0.15 M NaCl via the portal vein. The liver was then immediately removed and 2 g of tissue from the periphery of the median lobe was homogenised in isotonic sucrose as in ref. 22.

Sucrose density gradient centrifugation. This was performed as in ref. 22 but with sucrose solutions containing 10 mM imidazole–HCl buffer, pH 7.2. Some gradient centrifugation was also performed in a vertical pocket rotor (Sorvall TV850, DuPont Instruments Ltd.). The sample (5 ml was layered onto a 28 ml linear gradient extending from density 1.05 to 1.32 g cm^{-3} and the rotor accelerated to 50,000 rpm for 30 min. The gradient was unloaded into 16 tared tubes by displacement with Maxidens (Nyegaard, Oslo, Norway). The fractions were thoroughly mixed, weighed and their density measured with an Abbé refractometer. Marker enzyme distributions obtained by this method were indistinguish-

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able from those obtained with the Beaufay zonal rotor. Marker enzyme recoveries were between 85 and 126%.

Preparation of subcellular fractions for HPLC. Livers from rats treated with HPD were homogenised in sucrose medium as above. This homogenate was centrifuged at 800 *g* for 10 min and the resulting supernatant centrifuged further at 15,000 *g* for 20 min to give a mitochondrial–lysosomal fraction. The supernatant was centrifuged at 100,000 *g* for 60 min to prepare a cytosolic fraction.

Enzyme and biochemical assays. Enzyme activities were measured as described in [22]. Porphyrin content was determined by mixing 500 μl of fraction or liver homogenate with 4 ml of ethyl acetate and acetic acid (4:1, v/v), mixing thoroughly and then centrifuging for 10 min at 2500 rpm. The fluorescence of the supernatant was measured at an excitation wavelength of 400 nm and emission wavelength of 618 nm in a Perkin Elmer LS-3 spectrofluorimeter. The distribution of enzymes and porphyrins in the gradients is expressed as relative concentration versus density curves, calculated as in ref. 23.

Fluorescence microscopy. Fluorescence microscopy was performed with a Carl Zeiss Fluorescence microscope (Carl Zeiss Ltd., Welwyn Garden City, Hertfordshire), with a mercury vapour lamp as source. Fresh liver was frozen in liquid nitrogen, sectioned and mounted.

High performance liquid chromatography. HPLC was performed with a Waters 660 solvent programmer and two model 6000A pumps connected to a Perkin Elmer LS-3 fluorimeter at an excitation wavelength of 400 nm and emission wavelength of 618 nm. Aliquots (75 μl) of rat plasma, cytosol and resuspended mitochondrial–lysosomal fraction were extracted with 200 μl DMSO/10% TCA (1:1, v/v) and, after centrifugation at 2000 *g* for 10 min 100 μl clear supernatant was injected onto the column. Injection of samples was by a Rheodyne 7125 injector fitted with a 200 μl loop. Reverse-phase separation was carried out on a 5 μm particle size, 25 cm by 5 mm MOS-Hypersil (C8) column (Shandon Southern) with a linear gradient elution system from 60% methanol, 40% 1 M ammonium acetate, pH 4.6 to 100% methanol over 30 min. The flow rate was 1 ml min^{-1}.

Extraction of HPD from liver homogenates and subcellular fractions. Porphyrins were extracted from the samples with either the well established ethyl acetate/acetic acid technique or the more recent DMSO/10% TCA procedure [24]. To assess recoveries, a known amount of HPD was added to liver homogenates and each of the subcellular fractions and then extracted with either ethyl acetate/acetic acid (4:1, v/v) or DMSO/10% TCA (1:1, v/v). The fluorescence intensities were compared to those of standard solutions in identical solvents. The recoveries of HPD were virtually the same for both extractants, being 95 ± 5.5% for the former and 98 ± 4.6% for the latter (mean ± SD, N = 15). The high recoveries also suggested that fluorescence quenching in the extracts was not a problem. Ethyl acetate/acetic acid extracts were unsuitable for direct HPLC analysis as they cause peak shifting and broadening, DMSO/10% TCA extracts are compatible with the present system and were therefore used for the HPLC analysis.

Materials. Haematoporphyrin (free base), bovine serum albumin, and NADH were purchased from Sigma Chemical Co. (Poole, Dorset). 4-Methylumbelliferone substrates were purchased from Koch Light Laboratories Ltd (Colnbrook, Berkshire). Methanol was HPLC grade from Rathburn Chemicals Ltd. (Walkerburn, Scotland). [^{3}H]-Adenosine-5-monophosphate was purchased from Amersham International (Amersham, Bucks., U.K.). All other chemicals were of Analar grade and obtained from BDH Ltd. (Poole, Dorset).

RESULTS

Cellular localisation of haematoporphyrins

Figure 1 shows the amount of porphyrin in rat liver homogenates as fluorescence intensity at various times after administration of HPD. It can be seen that the total amount in the tissue is at a maximum at the earliest time point and decreases thereafter. In order to investigate whether the HPD was being preferentially accumulated by a particular cell type, frozen sections of liver from the experiments described above were examined by fluorescence microscopy. No concentration of fluorescence could be detected by eye or by long exposure photography if the rat has been dosed 1.5 hr before sacrifice. However, at 24 hr after dosage a pattern of discrete areas of red fluorescence is apparent (Fig. 2). Comparison with sections stained with haematoxylin and eosin, showed that the fluorescence was associated with Kupffer cells.

Subcellular localization of haematoporphyrins

Figure 3 shows the result of analytical subcellular fractionation of the whole liver homogenates. After 1.5 hr, accumulation is into the cytosol fraction. Over 48 hr this cytosolic peak declines and a new peak can be seen at a density of 1.21 g cm^{-3}. This reaches

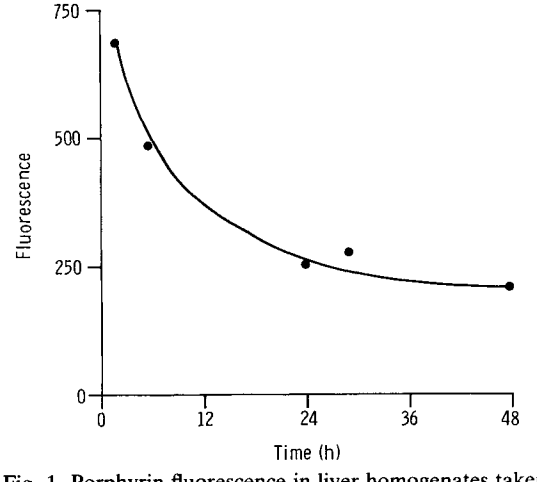

Fig. 1. Porphyrin fluorescence in liver homogenates taken at different time points after intraperitoneal dosing with 10 mg of HPD. Measurements were made at excitation and emission wavelengths of 400 nm and 618 nm, respectively, after extraction of 500 μl of homogenate with 4 ml of ethyl acetate/acetic acid (4:1, v/v).

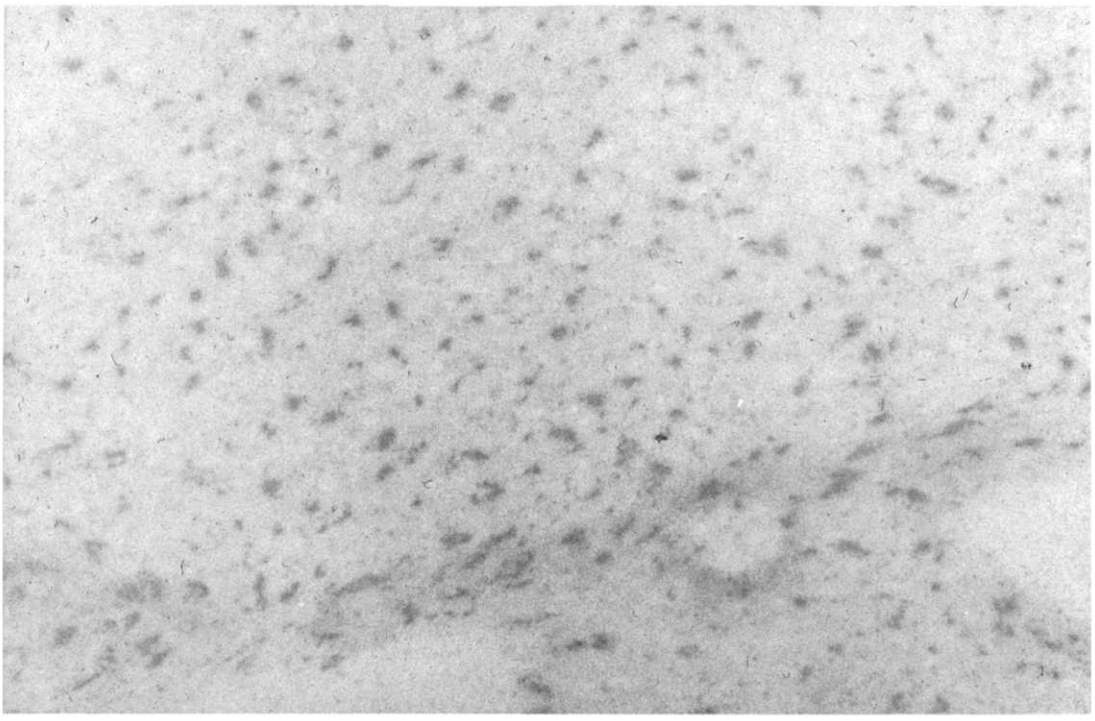

Fig. 2. Fluorescence micrograph of a frozen, unstained section of rat liver 24 hr after intraperitoneal injection of HPD (×160).

a maximum at 24 hr and then remains relatively constant. To identify the subcellular localization of the accumulated porphyrins, the distribution profile of the 24 hr treated animal was compared with the distribution profiles of various marker enzymes (Fig. 4). The peak at density 1.21 g cm^{-3} is in a similar region of the gradient to N-acetyl-β-glucosaminidase, succinate dehydrogenase and catalase. There is no association with neutral α-glucosidase, a marker for the endoplasmic reticulum. Rats were treated as described in [25] with Triton WR1339, a selective lysosomal perturbant, prior to administration of HPD. Figure 5 shows that the distribution for the lysosomal marker, N-acetyl-β-glucosaminidase is shifted by the Triton treatment to a lower equilibrium density. The catalase and succinate dehydrogenase activities were unaffected, but the porphyrin distribution is shifted to lower densities for the lysosomal enzymes.

HPLC

Figure 6 shows the HPLC analysis of porphyrins

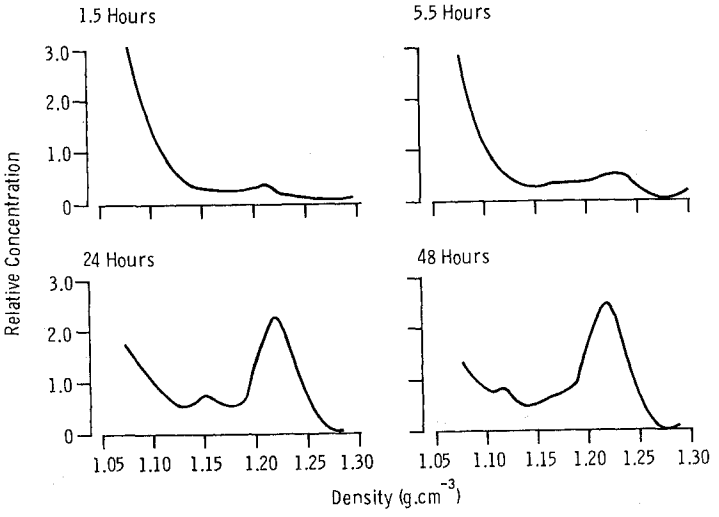

Fig. 3. Variation with time in the subcellular distribution of porphyrins in rat liver. Rats were dosed with 10 mg HPD and sacrificed at the indicated times. The results shown are the mean of two separate experiments at each time point.

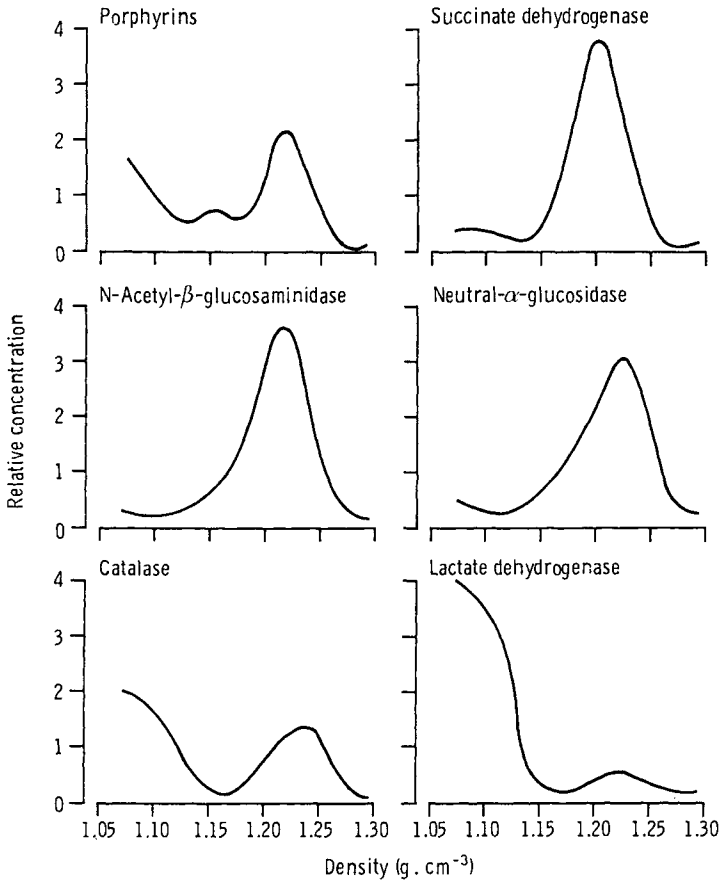

Fig. 4. Comparison of the subcellular distribution of porphyrin with marker enzymes, 24 hr after administration of HPD. Marker enzymes are shown for the lysosomes (*N*-acetyl-β-glucosaminidase), peroxisomes (catalase), mitochondria (succinate dehydrogenase), endoplasmic reticulum (neutral α-glucosidase) and the cytosol (lactate dehydrogenase).

in the plasma and gradient fractions. They show that the amounts in the plasma and cytosol are considerably reduced at 24 hr. The main peaks are the two diastereoisomers of haematoporphyrin and the two positional isomers of hydroxyethylvinyl-deuteroporphyrin. There is an extra unidentified peak eluting at about 3–4 min in both particulate and cytosol samples. This peak is also found in the bile of rats not given HPD.

DISCUSSION

In this study the time course of haematoporphyrin uptake has been followed at three different levels: whole liver by fluorometry, cell type by flourescence microscopy and the subcellular localisation with analytical subcellular fractionation. The time course for the whole liver showed that uptake was rapid being at a maximum at the first time point (90 min) examined and thereafter declining. Fluorescence microscopy did not indicate that this initial uptake was to any particular cell type, fluorescence being at a low level and evenly distributed throughout the entire section. The subcellular fractionation studies showed that this initial uptake was to the cytosolic compartment. It is not clear from these experiments whether or not this represents uptake by the hepatocytes but in separate experiments (not shown) where HPD was administered intravenously, the porphyrins rapidly (6 min) appeared in the bile, suggesting that under some circumstances there is rapid transit through the hepatocytes.

The whole liver uptake experiments indicated that porphyrin levels declined over 48 hr following the initial uptake. Subcellular fractionation showed that although there was a decline in the amount of fluorescent material associated with the cytosolic compartment over this period, there was a concomitant increase in the fluorescence associated with lysosomes. Fluorescence microscopy at 24 hr post administration of HPD showed that this lysosomal compartment was associated with the Kupffer cells. These results are in agreement with the report by Bugelski *et al.* [26] who, using autoradiography concluded that the distribution after 24 hr HPD treatment is mainly to stromal cells. It also confirms the suggestions of Allison and Young [20] who favoured a lysosomal localisation for HPD. The experiments reported here do not allow us to determine whether or not the porphyrins that are accumulated in the

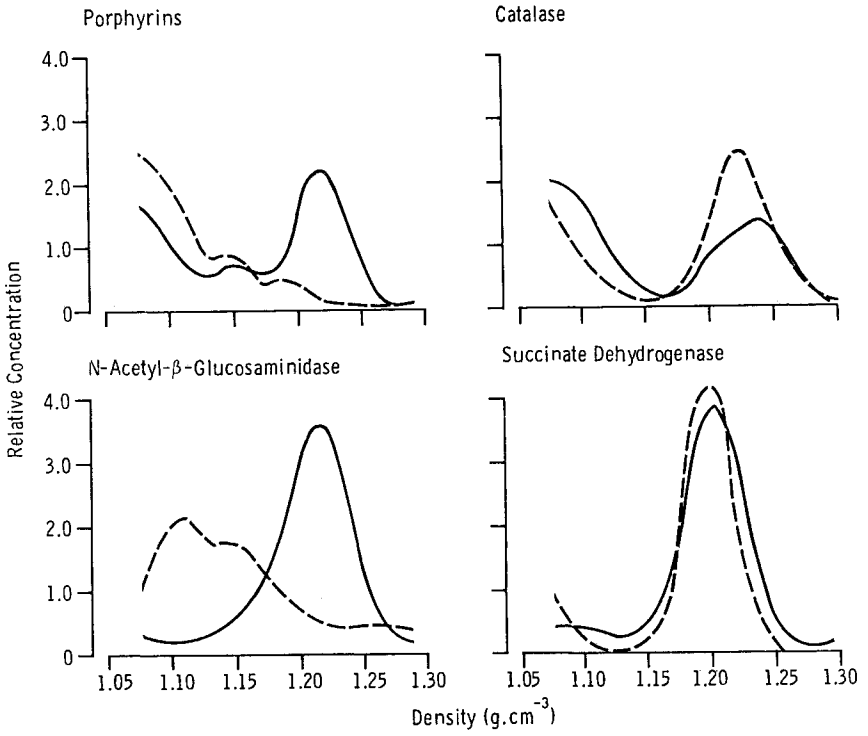

Fig. 5. The effect of Triton WR-1339 on the subcellular distribution of porphyrins. Rats were treated at 3.5 days before sacrifice with 85 mg/100 g body weight of Triton WR-1339 (i.p.) and at 24 hr prior to sacrifice with HPD (10 mg i.p.). Results obtained after treatment with Triton WR-1339 (interrupted line) are compared with those obtained on treatment with HPD alone (solid line). The results are the mean of three separate experiments.

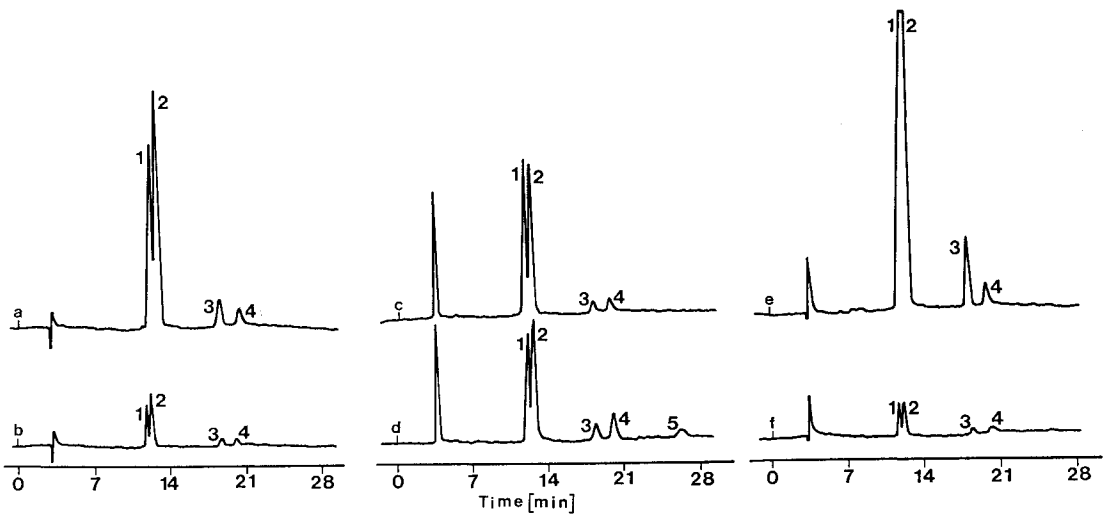

Fig. 6. HPLC of porphyrins in rat plasma and liver subcellular fractions. Samples were extracted with DMSO/10% TCA (1:1, v/v) and chromatographed as described in Methods. a, Plasma at 1.5 hr; b, plasma at 24 hr; c, mitochondrial–lysosomal pellet at 1.5 hr; d, mitochondrial–lysosomal pellet at 24 hr; e, cytosol at 1.5 hr and f, cytosol at 24 hr post treatment with HPD. Peaks: 1, (*RS* + *SR*)-haematoporphyrin; 2, (*RR* + *SS*)-haematoporphyrin; 3, 8-(1-hydroxyethyl)-3-vinyldeuteroporphyrin; 4, 3-(1-hydroxyethyl)-8-vinyldeuteroporphyrin; 5, protoporphyrin.

lysosomal compartment of the Kupffer cells are transferred from the hepatocytes or are taken up directly.

Although other reports have suggested that HPD was associated with nuclei [9–12], mitochondria [6, 7], microsomes [8] and unspecified membranes [13–18], no evidence was found to support any of these claims. However, these other studies used malignant cells, *in vitro*, whereas this study used normal cells *in vivo* and previous studies also used preparative centrifugation techniques rather than analytical fractionation procedures.

The HPLC studies show that haematoporphyrin and the hydroxyethylvinyldeuteroporphyrin isomers are the principle porphyrins in the mitochondrial–lysosomal fraction, cytosol and plasma. There is little evidence for the involvement of ether dimer formation as previously suggested [4], nor any evidence for the selective accumulation of any of the isomers comprising HPD.

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