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# Effect of Density-gradients on the Binding of Photosensitizing Agents to Plasma Proteins

KATHRYN WOODBURN,* DAVID KESSEL†

*Departments of Pharmacology and Medicine, Wayne State University School of Medicine, Detroit MI 48201, U.S.A.*

**The binding of photosensitizing agents to low-density lipoprotein is considered an important factor in tumor localization. We examined the affinity of a group of photosensitizers, varying in charge and hydrophobicity, for LDL, under conditions designed to determine whether the high salt concentration involved in conventional KBr gradients affected the results. Density-gradients containing KBr vs $D_2O$ were evaluated; the latter can delineate VLDL and LDL from other plasma components, while the KBr gradient readily resolved VLDL, LDL, HDL and albumin. Distribution of the photosensitizers to plasma fractions was assessed, along with the effect of Cremophor EL, an emulsifier used for formulation of water-insoluble drugs. Both the $D_2O$ and KBr gradients provided similar results with regard to the affinity of anionic, neutral or cationic photosensitizers for LDL. The use of Cremophor EL for drug formulation was associated with an altered electrophoretic lipoprotein profile. In some cases, affinity of CRM-solubilized sensitizers for plasma components varied with the density-gradient employed. The high salt concentration used in density-gradient fractionation had little effect on the affinity of photosensitizing agents to low-density lipoprotein but may introduce artifacts when emulsifiers are used in drug formulation.**

**Keywords: Photosensitizers Density-gradient Lipoproteins Drug-delivery vehicles**

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## INTRODUCTION

The efficacy of photosensitizing agents in the photodynamic therapy (PDT) of cancer depends in part on the selectivity of drug biodistribution. The relative affinity of photosensitizers for different plasma components has been proposed as one determinant of tissue localization. Tumor cells are known to exhibit an elevated number of LDL receptors on the cytoplasmic membrane (Maziere *et al.*, 1990). This could play a role in the localization of sensitizers which show affinity for LDL (Jori and Reddi, 1990; Kessel, 1986). Albumin may also play a role in sensitizer biodistribution within the tumor stroma (Milanesi *et al.*, 1988; Kessel *et al.*, 1987).

Ultracentrifugation is the most reliable experimental method for studying the distribution of photosensitizers among plasma proteins and lipoproteins (Kongshaug, 1992). Affinity relationships can vary with drug hydrophobicity, structural symmetry and charge. In this study, we examined the *in vitro* partitioning of a diverse series of photosensitizing agents among the protein and lipoprotein components of human plasma. Sensitizers were dissolved in dimethylformamide (DMF) or formulated with Cremophor EL (CRM), a non-ionic emulsifier which has been used as a parenteral vehicle for many hydrophobic drugs (Morgan *et al.*, 1988; Woodburn and Kessel, 1994). Our aim was the delineation of the association between photo

*Current address: Pharmacyclics, 995 East Arques Avenue, Sunnyvale, CA 94086, U.S.A.

†To whom all correspondence should be addressed.

*Abbreviations*: CRM, Cremophor EL; DCC, dicationic ketochlorin; DMF, N,N-dimethylformamide; $D_2O$, deuterium oxide; HDL, high density lipoprotein; KBC, ketochlorin photosensitizer; KBr, potassium bromide; LDL, low density lipoprotein; MCP, monocationic porphyrin; MP, mesoporphyrin; NPe6, mono-L-aspartyl chlorin e6; PDT, photodynamic therapy; SnET2, tin etiopurpurin; $TPPS_4$, tetraphenyl porphine tetrasulphonate; VLDL, very low density lipoprotein.

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sensitizers and plasma macromolecules. We examined two density-gradient systems utilizing 1KBr or $D_2O$ to provide the density variation. The latter system can be useful for examining drug: LDL binding under conditions where high salt concentrations are avoided (Hallberg *et al.*, 1994).

## MATERIALS AND METHODS

### *Materials*

Triton X-100 and CRM were obtained from Sigma Chemical Co. St Louis, Mo. Mesoporphyrin (MP) and tetraphenyl porphine tetrasulphonate ($TPPS_4$) were purchased from Porphyrin Products, Logan, Utah; tin etiopurpurin (SnET2) from PDT Pharmaceuticals Inc., Santa Barbara, Calif. KBC, DCC and MCP were provided by Dr C. K. Chang, Michigan State University, East Lansing, Mich. Synthesis, purification and characterization of the latter three will be described elsewhere. Structures of these sensitizers are shown in Fig. 1. All compounds were dissolved (1 mg/ml) in DMF or 20% CRM (Woodburn and Kessel, 1994).

### *Plasma binding studies*

Binding of the different sensitizers to protein and lipoprotein components of human plasma was assessed using two density-gradient systems. For these studies, plasma was collected from donors using EDTA as the anticoagulant to minimize lipoprotein oxidation. Erythrocytes were removed by centrifugation for 10 min at 4°C. Sensitizers were incubated with plasma for 30 min at 37°C (8 $\mu$g/ml).

The KBr gradient system is a modification of a previously published procedure (Sykes *et al.*, 1992). Plasma (250 $\mu$l) was mixed with 500 $\mu$l of 0.15 M NaCl and brought to a density of 1.21 g/ml with solid KBr. A 750 $\mu$l portion of the resulting mixture was layered over 750 $\mu$l of KBr solution (density of 1.27 g/ml) in 13 × 38 mm polyallomer centrifuge tubes (Beckman Instruments Inc., Palo Alto, Calif.). The tubes were then filled with isotonic saline (total Vol. = 3.9 ml).

The deuterium oxide ultracentrifugation method was based upon a procedure described by Hallberg *et al.*, 1994. Plasma samples (250 $\mu$l) were mixed with 1.325 ml $D_2O$ containing 140 mM NaCl, and placed at the bottom of polyallomer centrifuge tubes which were then filled with 0.15 M NaCl (made up in $H_2O$).

All ultracentrifugation procedures were carried out with a TLN rotor (9° from vertical orientation) in a Beckman TL-100 tabletop ultracentrifuge for 60 min at 100,000 rpm ($r_{av} = 254{,}000\,g$) at 20°C. A setting of 6 was used for both the acceleration and deceleration rates. The tubes were fractionated from the top using an ISCO Model 184 tube piercer and fraction collector. Samples were displaced by introducing a dense solution (1.85 g/ml) of Fluorinert FC-40 (3M Co.) into the tube bottoms at a rate of 0.5 ml/min, using a Harvard Apparatus Model 11 syringe drive. A total of 25 fractions were collected, approx. Vol. = 150 $\mu$l each. The fractions were diluted with 3 ml of 10 mM Triton X-100, and the concentration of sensitizer was assessed by fluorescence, using optimal wavelengths of excitation and emission for each sensitizer.

For studies involving gel electrophoresis, selected samples were pooled and concentrated using Lyphogel (Gelman Instrument Co., Ann Arbor, Mich.): solid polyacrylamide particles designed for macromolecule concentration from aqueous solutions.

### *Gel electrophoresis*

Lipoproteins were characterized by gel electrophoresis, using the Paragon lipoprotein agarose gels (Beckman Instrument Co., Fullerton, Calif.). After electrophoresis in 50 mM barbital buffer pH 8.6, the gels were fixed in MeOH/acetic acid mixture and stained with Sudan Black. Chylomicrons (if present) remain at the origin; the order of migration from the origin is LDL < very low-density lipoproteins (VLDL) < high-density lipoproteins (HDL).

## RESULTS

The photosensitizers used in this study varied in terms of structure, charge and hydrophobicity (Fig. 1). We used a modified procedure for the KBr gradients, outlined above, which provides a better separation of HDL from albumin than does the original method (Sykes *et al.*, 1992). The $D_2O$ procedure was adapted for the table-top ultracentrifuge from the method described by Hallberg *et al.* (1994).

The affinity of the different photosensitizers for human plasma components, as assessed on KBr and $D_2O$ gradients, is summarized in Table 1. With the KBr gradient (Woodburn and Kessel, 1994) VLDL is found mainly in fraction 1, LDL in fractions 3–7, HDL in fractions 13–18, and albumin in fractions 20–25. Gel electrophoresis was used to provide information

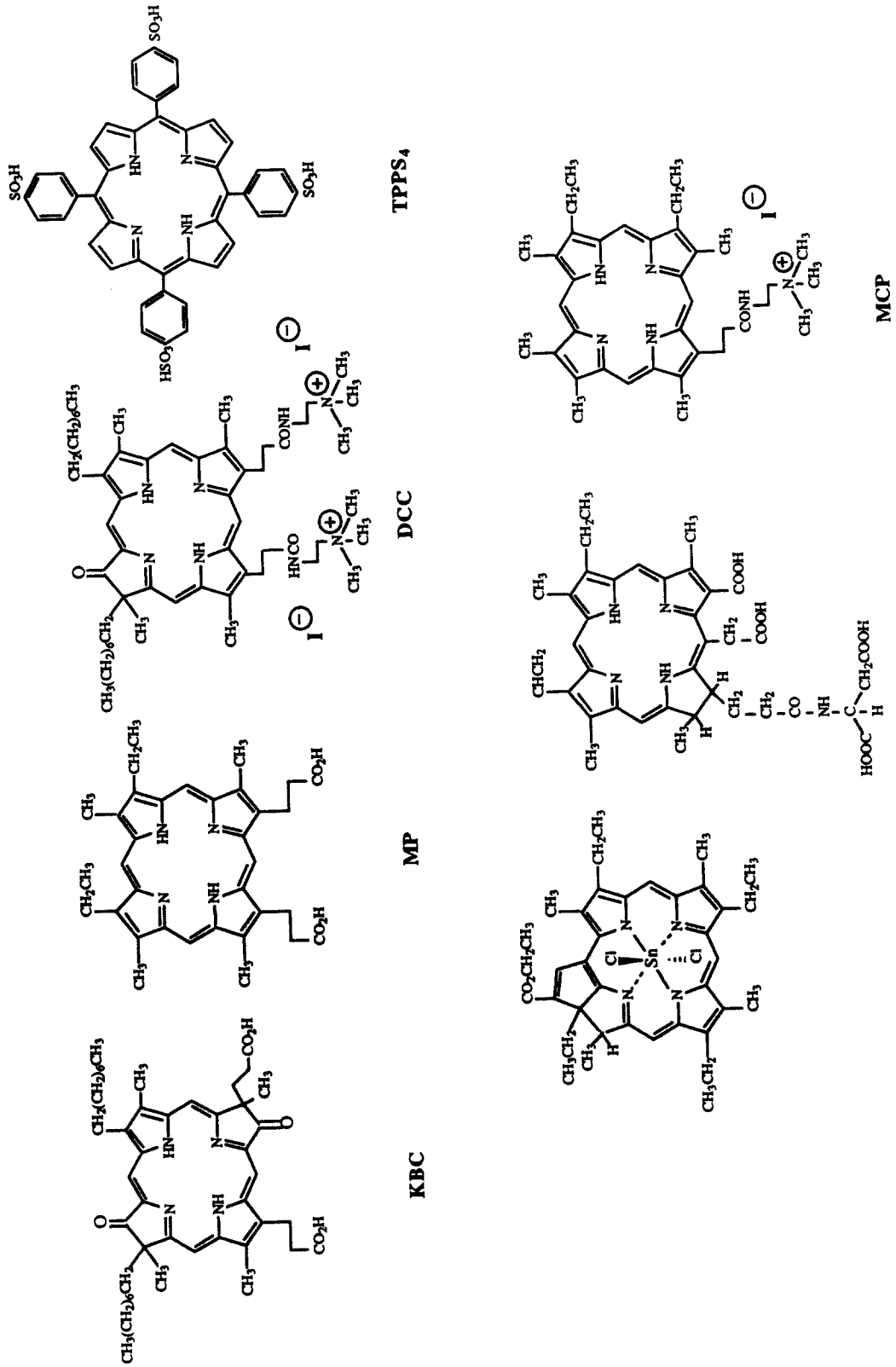


Fig. 1. Structures of photosensitizers used in this study.

Table 1. Distribution of sensitizers to plasma fractions expressed as percent of the total

| Drug | | KBr gradient | | | | $D_2O$ gradient | | |
|---|---|---|---|---|---|---|---|---|
| Name | Charge | VLDL | LDL | HDL | Albumin | VLDL | LDL | HDL/albumin |
| DCC | +2 | 2.5 | 30 | 67 | 0 | 0 | 35 | 65 |
| MCP | +1 | 5 | 50 | 30 | 15 | 5 | 55 | 40 |
| SnET2 | 0 | 2 | 9 | 55 | 34 | 0 | 10 | 90 |
| KBC | −2 | 0 | 20 | 80 | 0 | 0 | 20 | 80 |
| MP | −2 | 0 | 20 | 30 | 50 | 0 | 20 | 80 |
| NPe6 | −4 | 0 | 1 | 13 | 86 | 0 | 0 | 100 |
| $TPPS_4$ | −4 | 0 | 0 | 20 | 80 | 0 | 0 | 100 |

These data represent results from typical experiments. The range of variation in replicate determinations indicates that these values are accurate to ±10% of the numbers shown.

on the separation with the $D_2O$ gradient (Fig. 2). VLDL was found in fractions 1–3 (these fractions were only faintly stained with Sudan black), LDL in fractions 7–14 and HDL in fractions 19–25. The latter fractions also contain albumin and other 'heavy' plasma proteins which do not stain with Sudan black.

When DMF was used as the formulation vehicle, we observed a slightly reduced affinity of the cationic sensitizers DCC and MCP for LDL with the KBr gradient. This may be within the expected experimental error of the procedure, since replicate determinations usually yield a reproducibility within ±10% of numbers shown in Table 1. Otherwise, we could detect no variation in affinity of the sensitizers examined for plasma components when the two gradients were compared.

An example of the improved separation of VLDL from LDL afforded by the $D_2O$ gradient is provided by comparison of plasma-binding studies involving the monocationic porphyrin MCP (Fig. 3). In the KBr gradients, VLDL is found in the first fraction, LDL is centered in fraction 5, HDL in fraction 17 and albumin in fraction 22. With the $D_2O$ gradient, VLDL (fractions 1 and 2) is cleanly separated from LDL (centered at fraction 12).

The ability of the $D_2O$ gradient to resolve components of plasma treated with 0.16% (v/v)

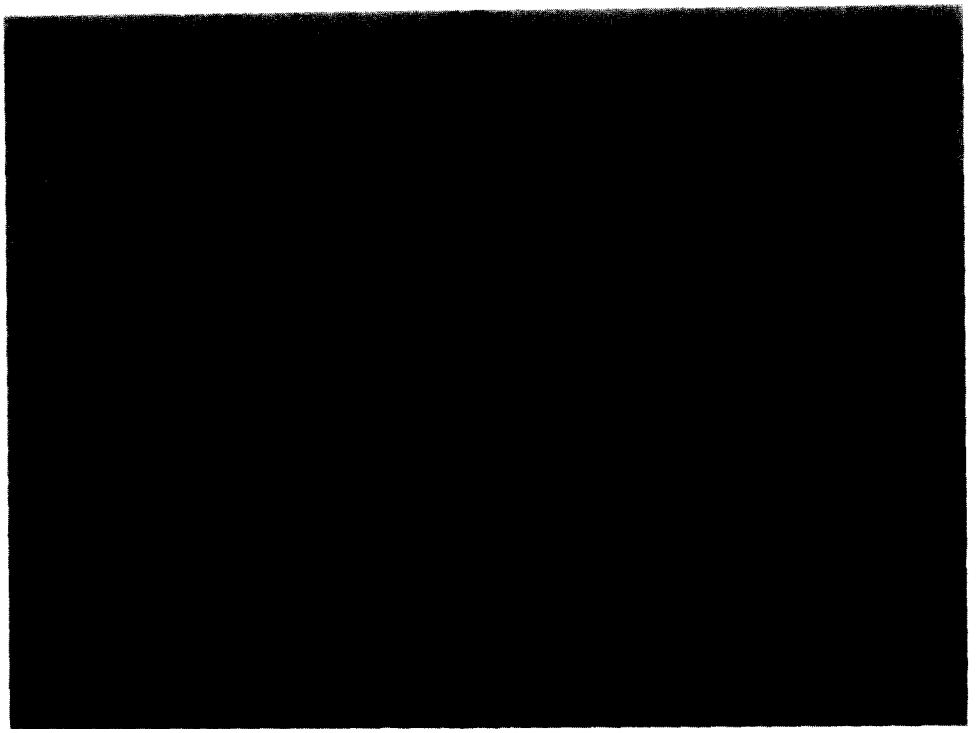

Fig. 2. Gel electrophoresis analysis of concentrated plasma fractions obtained from ultracentrifugation with the $D_2O$ gradient. Lane 1 = fractions 1–3 (VLDL); lane 2, fractions 4–6; lane 3, fractions 7–9 (LDL); lane 4, fractions 10–12 (LDL); lane 5, fractions 13–15 (LDL); lane 6, fractions 16–18; lane 7, fractions 19–21 (HDL); lane 8, fractions 22–25 (HDL).

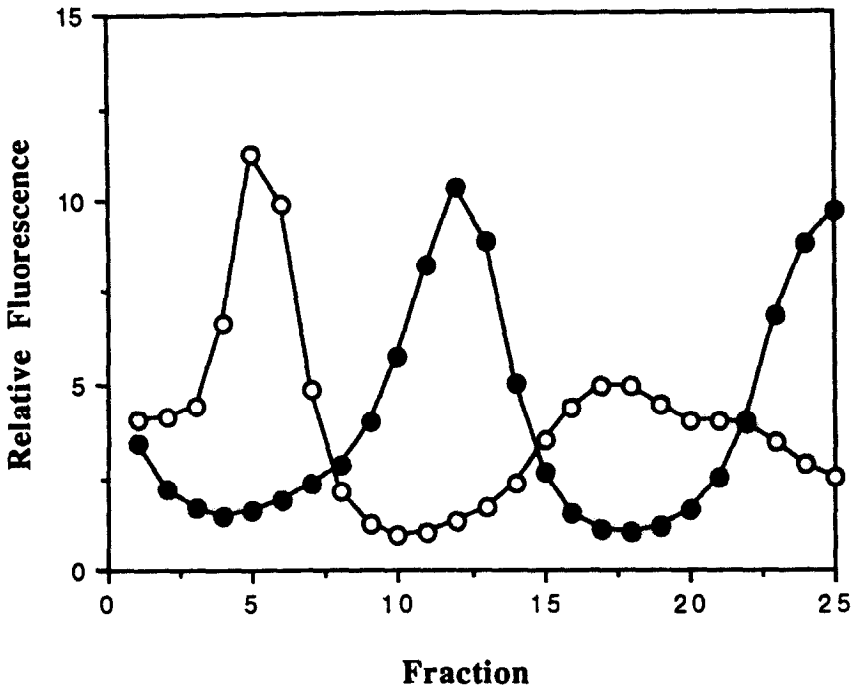


Fig. 3. Density-gradient analysis of plasma containing MCP (vehicle = DMF) analyzed with a KBr (-○-) or a $D_2O$ (-●-) gradient.

CRM is shown in Fig. 4. Control plasma samples were placed in lanes 1 and 8, plasma + CRM in lane 2. The decreased electrophoretic mobility of HDL and LDL induced by CRM is shown, as is the appearance of a new stained band which migrates toward the cathode. In studies to be reported elsewhere, we found that this material represents components of CRM which are sufficiently hydrophobic to be stained with Sudan black. VLDL was detected in fractions 1–5, LDL and CRM in 6–15; HDL slightly contaminated with LDL in 16–20; only HDL in 21–25. Since the extent of concentration of the pooled fractions by Lyphogel is variable, the results shown in Figs 2 and 4 provide qualitative, rather than quantitative information on lipoprotein separation.

KBC and SnET2 are sufficiently hydrophobic to need formulation with an emulsifier, e.g. CRM. When CRM formulations of these agents were examined (CRM concentration = 0.16% v/v), we found no significant differences in sensitizer binding to plasma components with the bouyant-density of LDL (fractions 6–15) when either $D_2O$ or KBr gradients were employed (Table 2). This was also true for NPe6, a water-soluble sensitizer. Both gradient systems detected enhanced binding of sensitizers to the 'LDL fraction'. This fraction also contains some CRM components which stain with Sudan black (Fig. 4). The presence of CRM did not affect distribution of MP among plasma components using the KBr gradient, but eliminated sensitizer binding to the 'LDL fraction' of the $D_2O$ gradient.

## DISCUSSION

Lipoproteins are defined on the basis of their hydrated density characteristics (Chung *et al.*, 1986). Ultracentrifugation is therefore the preferred method for the examination of the delineation of binding of photosensitizers to different lipoprotein fractions. Using a NaCl–CsCl gradient Kongshaug (1992) found that protoporphyrin and hematoporphyrin binding was similar regardless of salt concentration and the relative positioning of the plasma within the gradient. In an earlier publication (Kongshaug *et al.*, 1989), it was proposed that a high salt concentration promoted binding of hematoporphyrin to the 'heavy protein fraction', and might lead to salting out of porphyrins.

In the reporting of relative binding of sensitizers to plasma components (Tables 1 and 2), we have defined the fractions which represent each separable species. Some overlap is unavoidable with the small tubes (13 × 38 mm), but the tabletop ultracentrifuge does substantially shorten the time of exposure of plasma to

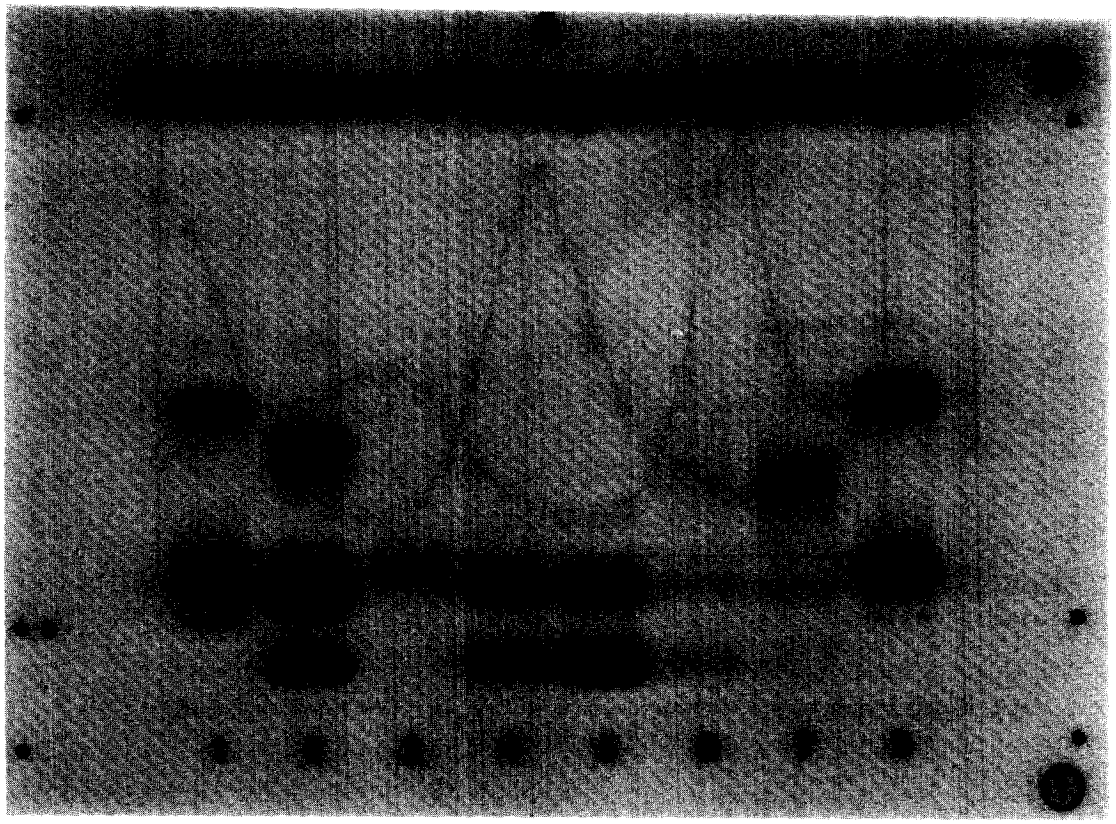

Fig. 4. Gel electrophoresis of plasma and concentrated plasma fractions showing effects of CRM. Lanes 1, 8 = control plasma; lane 2 = plasma + 0.16% (v/v) CRM; lanes 3–7, density-gradient fractions ($D_2O$ gradient) from plasma + CRM: lane 3 = 1–5, lane 4 = 6–9, lane 5 = 10–15, lane 6 = 16–20, lane 7 = 21–25.

high salt concentrations. The use of a rotor which holds tubes at 9° angle minimizes adherence of VLDL and heavy proteins to the inboard and outboard sites of the tube (Sykes *et al.*, 1992). While the gradient used in the latter publication was modified here to enhance separation of HDL from the heavier proteins, we still observed incomplete separation of these species.

The $D_2O$ gradient permits a better separation of VLDL and LDL from other plasma components, permits the maintenance of physiological ionic conditions (Hallberg *et al.*, 1994) and eliminates the need for subsequent dialysis before fractions can be examined, e.g. by gel electrophoresis. The major disadvantage is that the albumin and HDL fractions are not resolved; both are important carriers of photosensitizing agents in plasma (Jori *et al.*, 1990; Kongshaug *et al.*, 1989; Maziere *et al.*, 1990). Hallberg *et al.* (1994) reported that the use of a density gradient containing $D_2O$ indicated reduced affinity of the free-radical scavenger probucol for LDL was reduced, as compared with a KBr gradient. Human LDL isolated from a $D_2O$ gradient was found more resistant to $Cu^{2+}$-catalyzed oxidation than was LDL isolated from a KBr gradient. It is not clear whether these phenomena derive from effects of

Table 2. Distribution of sensitizers (percentage) formulated with CRM to plasma fractions which correspond to the bouyant density of lipoproteins shown in the last row

| | KBr gradient fractions | | | | $D_2O$ gradient fractions | | |
|---|---|---|---|---|---|---|---|
| Drug | 1–3 | 3–7 | 13–18 | 20–25 | 1–3 | 7–14 | 19–26 |
| SnET2 | 0 | 25↑ | 45↓ | 30 | 0 | 25 | 75 |
| KBC | 0 | 25 | 75 | 30 | 0 | 27↑ | 73↓ |
| MP | 0 | 20 | 35 | 45 | 0 | 0↓ | 100↑ |
| NPe6 | 0 | 8↑ | 10 | 82 | 0 | 10↑ | 90↓ |
| | VLDL | LDL | HDL | Albumin | VLDL | LDL | HDL/Albumin |

In replicate determinations, we found that these values can differ by as much as ±10% of the numbers shown. Arrows indicate statistically significant effects of CRM on binding of sensitizers to plasma fractions.

high salt concentrations on LDL, or from differences in hydrogen bonding or other physical properties between $H_2O$ and $D_2O$.

We detected no statistically-significant differences in binding of a series of photosensitizers (DMF formulation) to the VLDL/LDL fraction when KBr and $D_2O$ gradients were compared (Table 1). Since the latter gradient cannot resolve HDL from high-density plasma proteins, we cannot determine whether the high salt concentrations inherent in the KBr gradients alter the relative affinity of the sensitizers for HDL vs albumin and othr heavy plasma proteins. Since many solubilizing agents, e.g. DMF and Triton X-100 are too toxic for injection, and other vehicles have been used to solubilize hydrophobic photosensitizers. A drug-delivery vehicle with current popularity is CRM (Morgan *et al.*, 1988; Woodburn *et al.*, 1994). Kongshaug *et al.* (1991) had reported a lipoprotein:CRM interaction which resulted in altered behavior of HDL and LDL in a NaCl–CsCl density gradient. This was an important pioneering study, but did not include an electrophoretic examination of CRM-induced effects on lipoproteins, and relied upon measurements of optical density at 280 nm to quantitate the appearance of CRM in density-gradients. This value does not correspond to the 230 nm absorbance maximum observed with our CRM preparation and, in preliminary studies designed to assess the activity of CRM fractions, we have found that optical density was not a reliable method of assessing the concentration of the biologically-active components.

Lorenz *et al.* (1982) reported that CRM contains both hydrophobic and hydrophilic products; the latter are stained with Sudan black (Fig. 4). CRM, at concentrations $\geqslant 0.4$ mg/ml, decreased the electrophoretic mobility of both LDL and HDL (Woodburn and Kessel, 1994; Woodburn *et al.*, 1994), but the effects of these changes in sensitizer-binding capacity of the different lipoproteins are dependent on the sensitizer being examined. We found no significant effect of CRM on the relative affinity of KBC for plasma components, using either gradient system. Enhanced affinity of SnET2 and NPe6 for fractions with the bouyant density of LDL were observed with either gradient. This likely reflects formation of a lipoprotein:CRM adduct which binds these sensitizers, since hydrophobic components of CRM are found in these fractions (Fig. 4). The distribution of SnET2 or NPe6 was not affected by the choice of gradient. MP exhibited an anomalous behavior when CRM was present. The KBr gradient did not detect enhanced binding of MP to the 'LDL fraction', but with the $D_2O$ gradient, only binding of MP to the HDL/albumin fraction was observed.

These results suggest that MP shows a greatly enhanced affinity for HDL or the albumin/protein fraction when CRM is present and the ionic strength is low. Since CRM modifies HDL with regard to both density (Kongshaug *et al.*, 1991) and the electrophoretic mobility (Woodburn and Kessel, 1994; Woodburn *et al.*, 1994), it is tempting to consider that it is the HDL which is responsible for this effect. Additional studies, utilizing purified lipoproteins, may shed additional light on this question.

It is possible that the differences in the physical chemistry of $H_2O$ vs $D_2O$ mask phenomena which might otherwise be revealed in the absence of high salt concentrations. Leaving this problem aside for the moment, the data of Table 1 indicate that high ionic strength does not appear to influence affinity of the sensitizers examined here for LDL. Based on results described here, we conclude that the affinity of photosensitizers for LDL can be reliably determined using density-gradients containing KBr or other salts.

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