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INTERACTION OF CREMOPHOR EL WITH HUMAN PLASMA

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Abstract—1. Interaction of cremophor EL (CRM) with human plasma lipoproteins and nonlipoproteins has been investigated by ultracentrifugation.

2. VLDL has only a low or negligible capacity to bind CRM, i.e. there is little or no change in the optical absorption at 280 nm of VLDL when CRM is added.

3. A low density subfraction of low density lipoproteins seems to associate substantially with CRM at relatively low CRM concentrations (1–3 mg/ml), but such association is not evident for CRM concentrations in the region 12–116 mg/ml.

4. Low density lipoproteins (LDL) may act as a carrier for CRM-emulsions, yet there seems to be no concomittant change in the 280 nm optical absorption of the proteins of LDL.

5. The position in the gradient (i.e. in the centrifugation tube after centrifugation) of high density lipoproteins (HDL) is shifted towards lower density in the presence of 1–4 mg CRM/ml. For higher concentrations of CRM, a destruction of HDL can be observed: the HDL distribution is converted into a bimodal distribution of respectively lighter and heavier "HDL"-particles than the normal ones; the densities at the peaks of these distributions are ~1.07 g/ml (light), 1.20 g/ml (heavy) and 1.11 g/ml (normal HDL). The optical extinction coefficient is apparently the same for the proteins of normal—and modified HDL.

6. Even high CRM concentrations ($\leqslant$116 mg/ml) have no perceptible effect on the gradient positions and profile of human serum albumin (HSA) and/or other heavy proteins.

7. The possible biological significance of these findings is briefly touched upon.

INTRODUCTION

Cremophor EL (CRM) is a polyoxyethylated derivative of hydrogenated castor oil. It dissolves various hydrophobic drugs, and moreover acts as a solvent-emulsifier agent. As a drug emulsion-vehicle CRM has been employed, e.g. in conjunction with cyclosporine, an effective immunsuppressive agent for organ transplantation (Cohen *et al.*, 1984), and various antineoplastics, including the antimicrotubule agent taxol [see, e.g. Rowinsky *et al.* (1989)]. Intravenous administration of such drug preparations can elicit severe anaphylactoid and/or neurotoxic side effects which have been attributed to the CRM vehicle [see, e.g. Kahan *et al.* (1984); Chapuis *et al.* (1985); Lassus *et al.* (1985); Rowinsky *et al.* (1989)]. Substantial reduction in the incidence of acute taxol-CRM induced reactions was achieved by antiallergic premedication and longer duration of infusion (Donehower *et al.*, 1987; Wiernick *et al.*, 1987a,b).

In particular, CRM has been applied as a vehicle for purpurins in photosensitizing experiments on cells *in vitro* (Morgan *et al.*, 1987) and animal tumours *in vivo* (Morgan *et al.*, 1987a,b, 1988, 1990). Purpurins are modified chlorines which, among other tetrapyrroles, may become important for clinical photochemotherapy (PCT) of cancer [*cf* e.g. Roeder (1990)]. To our knowledge, no systemic toxicity subsequent to administration of purpurin-CRM vehicles has been reported [*cf* p. 1257, Selman *et al.* (1987); p. 197, Morgan *et al.* (1988)].

Knowledge about the effect of CRM-emulsions on such plasma constituents is thus of some interest in the light of the biological applications of CRM referred to above. In particular, interactions with and/or transport of CRM by plasma components may be related to some of the acute effects of CRM which have been observed subsequent to its i.v. administration. As far as we know, no study of the interaction of CRM with plasma lipoproteins and nonlipoproteins has appeared previously.

MATERIALS AND METHODS

CRM was obtained from Sigma Chemical Co., St Louis, Mo. Aqueous emulsions of CRM were obtained by adding 0.1 M Tris–HCl buffer pH 7.35, containing 0.15 M NaCl, to weighed amounts of CRM (a highly viscous liquid), followed by vigorous shaking and/or sonication.

The ultracentrifugation, salt-gradient fractionation of the centrifuged samples and their analysis were similar to the procedures described previously (Kongshaug *et al.*, 1989, 1990a,b).

Human plasma, sampled in EDTA tubes, was obtained from a healthy person who had fasted overnight (Kongshaug *et al.*, 1989), the same individual who had provided plasma for the previous studies (Kongshaug *et al.*, 1989, 1990a,b). The use of plasma with constant lipo- and nonlipoprotein contents provides straightforward comparison of plasma-dye binding patterns obtained at different times,

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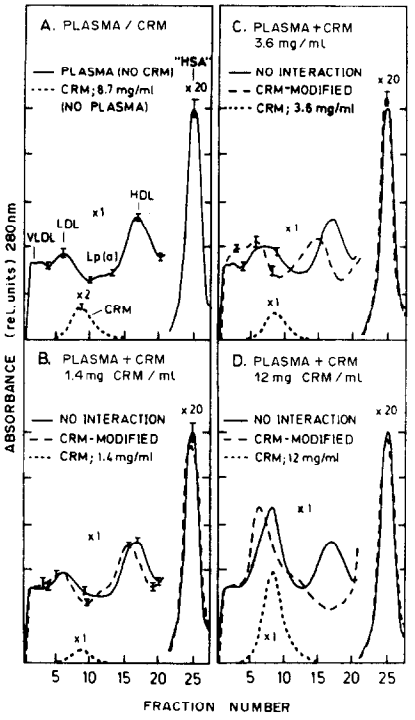

Fig. 1. Distribution of protein (0.2 ml plasma) and CRM in the gradient. (A) Whole line, plasma (no CRM); dotted line, CRM (8.7 mg/ml; no plasma). (B) Whole line, sum of plasma-profile (no CRM) and CRM-profile (no plasma) for concentration of CRM of 1.4 mg/ml; stippled line, plasma incubated with 1.4 mg CRM/ml (the curve is actually the mean of three individual samples, employing concentrations of CRM in the range 1.1–1.8 mg/ml, the mean concentration of CRM being 1.4 mg/ml); dotted line, CRM (1.4 mg/ml; estimated from the profile of 8.7 mg CRM/ml in panel A). (C) and (D) Corresponding curves for concentrations of CRM of 3.6 mg/ml (C) and 12 mg/ml (D).

whereas plasma from different persons may differ considerably in composition within the normal limits of concentrations. Since it is also desirable to be able to use the same plasma in future experiments, we have found it both necessary and convenient to store batches of the plasma in the frozen state. (The same person has donated many blood samples.) Thus, the present plasma was stored at −20°C until it was used. The lipo- and nonlipoprotein profiles of thawed plasma and fresh plasma are similar.

Ultracentrifugation was performed at 70,000 rpm, for 12 hr at 15°C, using a fixed angle 70.1 Ti rotor and a 4-step NaCl–CsCl gradient, as previously described (Kongshaug *et al.*, 1989). From top downwards, the steps are 3.5 ml of $d = 1.006$ g/ml (NaCl only), 3.5 ml of $d = 1.063$ g/ml (CsCl only), 4.5 ml of $d = 1.21$ g/ml (CsCl only) and 1.0 ml of $d = 1.35$ g/ml (CsCl only). These solutions are all made up with 0.1 M Tris–HCl buffer (pH = 7.35) containing 0.4 mg EDTA/ml. In present experiments, 0.2 ml of plasma was diluted with 0.2 ml of the Tris buffer or 0.2 ml of the Tris–CRM emulsion, incubated for ~1 hr at 37°C, and then layered at the top of the $d = 1.063$ g/ml layer, finally additing 3.1 ml of $d = 1.006$ g/ml on top of the sample layer. Other gradients in common use [see Chapman *et al.* (1981), Kelley and Kruski (1986)] result in similar resolution in the lipoprotein region as does our gradient, but only the latter yields a nonlipoprotein peak well separated from the bottom of the tube (Kongshaug *et al.*, 1990a).

After centrifugation the tubes were punctured as previously described (Kongshaug *et al.*, 1990b) and the gradients were analysed and fractionated from the top downwards, by means of a peristaltic pump and a Pharmacia double wavelength optical absorption meter (UV-2), allowing continuous recording of absorbances at 280 nm (proteins) and (when required) 405 nm (tetrapyrroles) (*cf* Kongshaug *et al.*, 1989). In the present work the distributions of both plasma proteins and CRM was determined by absorbance measurements at 280 nm.

Dialysed plasma and dialysed HDL were used in some centrifugations. The dialysis was performed with Sartorius bags (SM 13200) and associated equipment.

The present classification of lipo- and nonlipoproteins into various density classes is consistent with the accepted classification of plasma proteins (Patch and Patch, 1986). Although the present centrifugation time (12 hr) is shorter than that used previously (15–20 hr), resolution of proteins is nearly identical for centrifugation times in the region 12–20 hr. Hence, the density distribution in the centrifuged gradient may be considered (to a good approximation) to have the same form for 12–20 hr centrifugation times; the density distribution of our gardient, as it is after 15 hr centrifugation, has been shown previously [Fig. 1, Kongshaug *et al.* (1989)].

RESULTS AND DISCUSSION

Protein distribution (no CRM)

A good separation of the main protein classes is obtained [Fig. 1(A), whole line]. The present profile is similar to those previously obtained (Kongshaug *et al.*, 1989, 1990a,b). The gradient was divided into 27 equal volume fractions (i.e. ~0.46 ml/fraction). VLDL was found mainly in fraction 1–2, LDL peaked in fraction 6, there was a small (but significant) contribution from Lp(a) in the fraction range 10–13, HDL peaked in fraction 17 and HSA (and other heavy proteins) at fraction number 24.5. The positions of the various peaks were strictly reproducible within ±0.3 fractions over periods of months.

CRM distribution (no plasma)

The CRM-emulsion (8.3 mg/ml) centred at fraction number 8.3 (±0.3) [Fig. 1(A), stippled line]. It may be inferred from Fig. 1 in Kongshaug *et al.* (1989) that fraction number 8.3 corresponds to a gradient density of ~1.05 g/ml which is, as would be expected for (nonionic) CRM-emulsions, similar to that of undiluted CRM ($d \sim 1.05$ g/ml). The gradient peak position of CRM emulsions is consequently independent of the CRM concentration, as was observed (see below).

CRM–plasma interactions

When increasing concentrations of CRM (1–116 mg/ml) were added to plasma, there was a gradual change in the absorbance profile (Figs 1 and 2). We have compared, in some cases, the CRM-modified profiles (stippled lines) with the corresponding whole-line profiles obtained by the addition of the plasma profile (no CRM) and the CRM profile (no plasma); see Fig. 1(B)–(D). the latter profiles are therefore the profiles that would be observed if there were no significant interactions between CRM and plasma components; they will be referred to as non-interaction profiles.

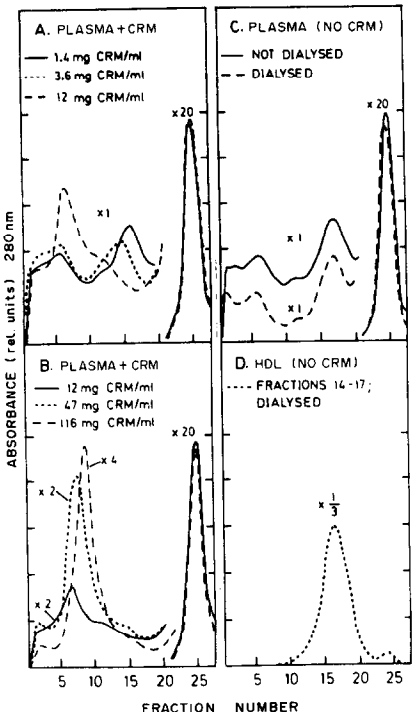

Fig. 2. (A) and (B) Effect of increasing concentrations of CRM (within region 1.4–116 mg/ml on plasma (0.2 ml). (C) Whole line, nondialysed plasma [see Fig. 1(A)]; stippled line, dialysed plasma (0.2 ml). (D) Distribution of HDL; fractions 14–17 of centrifuged dialysed plasma were pooled, dialysed, concentrated to 0.2 ml and centrifuged.

Interaction with VLDL

VLDL presumably concentrates mainly in fractions 1 and 2. As the absorbance in this region was not significantly changed by CRM, Figs 1 and 2, it seems that VLDL is not appreciably affected by CRM.

Interaction with LDL

Relative to the area of the noninteraction profile (3.6 mg CRM/ml), the areas of the corresponding modified profile was lower by an amount Δ in the fraction region 5–11 and larger by an amount $\sim\Delta$ in the fraction range 1.5–5; the difference area Δ is moreover some 60–70% of the area of the CRM component [Fig. 1(C)]. Apparently substantial amounts of CRM have been shifted from the fraction region 5–11 (wherein CRM is concentrated in the absence of plasma) to the fraction region 1.5–5, without affecting the absorbance at 280 nm of the lipoproteins in the region. A similar effect was seemingly perceptible, even at 1.4 mg CRM/ml [Fig. 1(B)]. There is thus some kind of interaction between the lighter subfraction of LDL, on one hand, and the CRM-emulsion, on the other. It seems as if such lipoproteins destroy the CRM-emulsion and associate with CRM. For larger CRM concentrations (12–116 mg/ml) there is no evidence from our results for an increasing shift of CRM into the 1.5–5 fraction range (Figs 1 and 2). In fact, there was less CRM in that region for CRM concentration of 12 mg/ml than for CRM concentration of 3.6 mg/ml [Fig. 1(C) and (D)]. These findings are not easy to understand. It should be noted, though, that the nature/size of CRM-emulsion droplets may conceivably vary with the CRM concentration and, moreover, the average distance between the droplets will be affected (i.e. decreased) by the centrifugation.

For CRM concentration of 12–47 mg/ml there was, none the less, a shift of substantial amounts of CRM into the 4–8 (i.e. LDL−) fraction region [Figs 1(D), 2(A) and (B)]. The fraction of CRM being shifted into the fraction region 4–8 decreased with increasing concentration of CRM. Thus, at CRM concentration of 116 mg/ml the maximum in the modified absorption profile occurred at the same position as does CRM when no plasma is present [Fig. 2(B)]. Not surprisingly, a given amount of LDL has thus a limited capacity to associate with CRM.

Interaction with HDL

Even a relatively small CRM concentration (1.4 mg CRM/ml) significantly shifted the HDL distribution towards lower densities [Fig. 1(B)], this shift being still more pronounced at CRM concentration of 3.6 mg/ml [Fig. 1(C)]. What is more, at this higher concentration the absorbance of modified HDL is substantially reduced [Fig. 1(C), stippled line]. For CRM concentration of 12 mg/ml, the peak of the normal HDL-absorption was not observable [Fig. 1(D)]. Further increase in CRM concentration

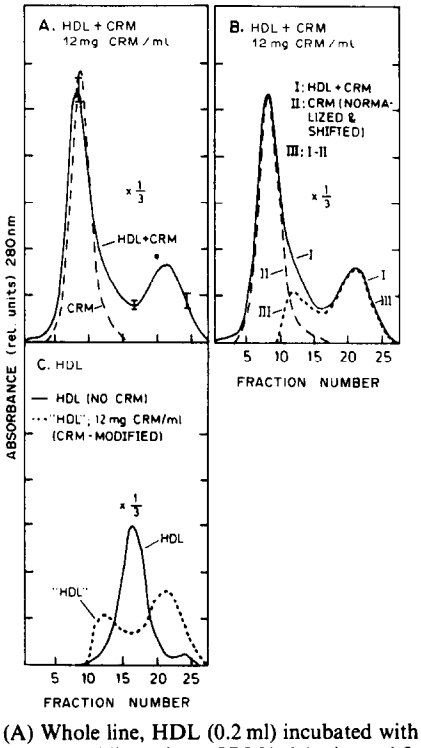

Fig. 3. (A) Whole line, HDL (0.2 ml) incubated with 12 mg CRM/ml; stippled line, 12 mg CRM/ml (estimated from the CRM-curve in Fig. 1A); (B) Whole line (I), HDL incubated with 12 mg CRM/ml (same as in panel A); stippled line (II) 12 mg CRM/ml, normalized and slightly shifted (i.e. by $\sim$0.3 fractions) to the left; dotted line (III), difference between the curves I and II. (C) Whole line, HDL (no CRM; see Fig. 2, panel D); dotted curve, CRM-modified HDL (12 mg CRM/ml; see curve III in Fig. 3, panel B).

induced no further change in the absorption profile in the normal HDL region [Fig. 2(A) and (B)].

As the effect of CRM on HDL is partly concealed by the presence of the other plasma components, HDL fractions 14–19 were pooled, dialysed against $d = 1.006$ g/ml Tris–NaCl solution and concentrated to a volume of 0.2 ml (to obtain a similar concentration of HDL as in 0.2 ml plasma). Dialysis not only reduced the concentration of salt to physiological level but also eliminated more or less free peptides and amino acids. The absorption profiles of dialysed and undialysed plasma are compared in Fig. 2(C), *cf* Kongshaug *et al.* (1990a).

The consequence of the interaction between HDL and CRM (12 mg/ml) is shown in Fig. 3. The centrifugation of HDL (no CRM) resulted in a major distribution at the expected HDL position; in addition there was a small contribution in the HSA region [Figs 2(D) and 3(C)]. The latter contribution might be due to the presence of some small unseparated amounts of heavy proteins in the fraction range 14–19 when whole plasma is centrifuged and/or some slight destruction of the plasma/HDL may have occurred during centrifugation/dialysis. In the presence of CRM the original (normal) HDL peak is absent, but a modified HDL peak arises at higher fractions [Fig. 3(A)]. Also, as the modified profile is wider than that of CRM alone in the fraction region 1–15 [Fig. 3(A) and (B)], the destruction of HDL also yields lighter products than HDL. In fact, when the contribution from CRM is subtracted from the modified profile [Fig. 3(B)] a bimodal distribution of modified HDL results [Fig. 3(B) and (C)]. Further, CRM–HDL interactions may yield a minor contribution of products in the 1–5 fraction region [not shown, but compare curves I and II in Fig. 3(B), in the region 1–5]. The densities at the peaks of the various distributions are ~ 1.07 g/ml for modified light HDL, 1.11 g/ml for normal HDL and 1.20 g/ml for modified heavy HDL; these densities may be estimated from a previously published gradient-density profile [see Fig. 1 in Kongshaug *et al.* (1989)]. Of further interest is the fact that the sum of the absorbance areas of HDL (no CRM) and CRM (no HDL) is equal to the absorbance area of the mixture of HDL and CRM (*cf* Fig. 3). The modification of HDL thus seemed to leave the HDL-proteins unaffected.

CRM–nonlipoprotein interactions

Although conceivably CRM-emulsions may interact with HSA (and/or other heavy proteins) there was no indication in our results that such possible interactions lead to significant modification of nonlipoproteins or to substantial transport of CRM by such proteins. Thus, the position and form of the heavy protein profile was unaffected by the presence of CRM for all concentrations of CRM studied (Figs 1 and 2). In particular, the error in the peak nonprotein absorbance is within 5% of that absorbance, implying that CRM at most contributes some 5% to the same absorbance. (If a 5% contribution of CRM was present for CRM 116 mg/ml, this would correspond to *ca* 20% of the total CRM.)

The nonlipoproteins are relatively polar as compared to the apolar CRM-emulsion. Hence, the affinity/binding capacity of nonlipoproteins for CRM emulsions may well be small or negligible, consistent with the present findings.

Biological significance

The biological significance of the present results cannot be adequately assessed at present. We have found, however, that CRM-emulsions are far from inert in their interactions with human plasma. Firstly, they can be associated to a substantial degree with LDL. Secondly, there is a marked destructive effect of CRM on HDL, more or less observable at all concentrations which we have employed, i.e. 1–116 mg/ml, the effect being especially dramatic for concentrations $\geqslant 3.6$ mg CRM/ml. Of particular interest therefore is the fact that a number of applications in biology have involved similar concentrations of the CRM vehicle as used in this study. Thus, culture media in photosensitizing cellular experiments contained 1 mg CRM/ml [*cf* Kessel (1989)] and 10 mg CRM/ml [*cf* Morgan *et al.* (1987)]; i.v. administration of photosensitizers to tumour bearing animals was made with solutions containing 33 mg CRM/ml [*cf* Morgan *et al.* (1987, 1990)]; and 100 mg CRM/ml (Morgan *et al.*, 1988); chemotherapeutical treatment of cancer has involved i.v. taxol formulated with 2.5–50 mg CRM/ml [see, e.g. Wiernik *et al.* (1987a); Green *et al.* (1987); Donehower *et al.* (1987); Rowinsky *et al.* (1989)].

Even acute reactions (characterized by bronchospasm, hypotension, stridor, tachy- and bradyarrhythmias, and even death) have been attribued to taxol's CRM vehicle (Rowinsky *et al.*, 1989), since similar cardiovascular effects were observed with other drugs formulated with it and when the vehicle alone was administered to animals [*cf* Chapuis *et al.* (1985); Lassus *et al.* (1985); Green *et al.* (1987); Donehower *et al.* (1987); Rowinsky *et al.* (1989)]. It is not known whether the mechanism behind these adverse effects of CRM is true anaphylaxis or whether it is nonimmunologic in character (Donehower *et al.*, 1987). Nor is anything known about the biological effects of CRM-associated and/or CRM-modified lipoproteins as studied herein. It would be of interest to investigate whether CRM-modified light—or heavy HDL have acute toxic and/or allergic effects. The HDL fractions may easily be produced and separated by the present technique (so as to contain relatively little of CRM; see Fig. 3; the contribution of CRM to light HDL presumably could be reduced by further centrifugation).

Since conceivably CRM associated- and/or modified lipoprotein may behave more or less abnormally, it would be of further interest to find out whether the observed increase in total plasma lipids, cholesterol and triglycerides in dogs treated with taxol-CRM [*cf* Grem *et al.* (1987)] is due mainly to taxol or to CRM (or both). Presumably, a possible abnormal behaviour of CRM-associated or CRM-modified lipoproteins would be counteracted by increased production of lipoproteins (and hence of plasma lipids, cholesterol and triglycerides).

The association of CRM with plasma lipoproteins may affect the tissue distribution of CRM and its elimination. It is noteworthy that small plasma concentrations of CRM may nevertheless result in high

local tissue concentrations. The destructive effect of CRM on HDL presents a warning that it may also severely affect other important entities in the circulation and tissues of the body; playing an important role in reverse cholesterol transport from peripheral cells back to the liver (Brown and Goldstein, 1983; Eisenberg, 1984; Miller *et al.*, 1985), HDL thus binds to specific cellular receptors on fibroblasts, arterial smooth-muscle cells, hepatocytes, kidney cells (Oram *et al.*, 1983; Eisenberg, 1984), macrophages (Brown and Goldstein, 1983; Schmitz *et al.*, 1985a,b,c) and leukocytes (Schmitz *et al.*, 1987). In spite of the limitations of the present work it offers some of the possible clues as to why it is necessary or advisable when administering CRM to use as low doses and low rates of infusion as possible.

Finally, the tissue distribution and pharmacology of hydrophobic drugs may be influenced by the type of vehicle employed [*cf* Chowdhary *et al.* (1990)]. In particular, suitable vehicles may deaggregate dyes and increase the efficiency of PCT [*cf* Chowdhary *et al.* (1989)].

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