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Research Article

Thermosensitive Sterically Stabilized Liposomes: Formulation and *in Vitro* Studies on Mechanism of Doxorubicin Release by Bovine Serum and Human Plasma

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Purpose. To formulate thermosensitive sterically stabilized liposomes and to study the effects of plasma and serum components *in vitro*.

Methods. The rate of release of encapsulated doxorubicin (Dox) from liposomes of various compositions was followed by fluorometric assay at 37°, 42° and 45°C, in buffer and also in both calf serum and human plasma up to 50% by volume.

Results. The optimal composition for the maximal differential release of doxorubicin between 37°C and 42°C in human plasma was a mixture of dipalmitoylphosphatidylcholine/hydrogenated soy phosphatidylcholine/cholesterol and distearoylphosphatidylethanolamine derivatized with polyethylene glycol at a molar ratio of 100:50:30:6. In experiments designed to study the mechanism causing increased permeability of liposomes in bovine serum, we found two different distinct release patterns: a slow linear rise of rate of Dox release for fluid liposomes and fast exponential rise reaching plateau within 5 minutes for solid phase (rigid) liposomes. This release of Dox from rigid but not fluid liposomes was inhibited by pre-heating serum at 55°C for 30 minutes or by addition of EDTA (but not EGTA) or antiserum to the C3 component of complement.

Conclusions. A formulation of sterically stabilized liposomes with the proper thermal sensitivity in human plasma has been obtained. In addition, the results suggest that complement may play an important role in the interaction of rigid but not fluid liposomes with bovine serum. Human plasma did not show this effect.

KEY WORDS: liposomes; doxorubicin; drug carrier; thermosensitivity; hyperthermia; bovine serum; human plasma; complement.

INTRODUCTION

Local hyperthermia is currently receiving increased attention as a therapeutic tool for use either alone or in conjunction with radiation or drugs [1]. In addition to older methods of heating (e.g., in a water bath or with warmed perfusate), microwaves and ultrasonic energy are now being investigated, especially for the local heating of deeper structures [1]. Since many normal mammalian cells begin to show damage at about 42°C, the aim has been to achieve therapeutic results just a few degrees above physiological temperature.

Liposome-encapsulated drugs appear to represent an increasingly useful method for delivery of chemotherapeutic agents [2]. The combination of liposome-mediated drug delivery and hyperthermia treatment is an attractive approach, and synergistic effects have been demonstrated on tumor models with encapsulated cisplatin [3] or bleomycin [4]. In one of the *in vivo* experiments [5], liposomes showing a phase transition a few degrees above physiological temperature delivered more than four times higher dose of methotrexate to murine tumors heated to 42°C compared to unheated ones. To evaluate the rate of drug release at the tumor and maximal drug targeting after administration of thermosensitive liposomes with hyperthermia, a theoretical and experimental method was derived [6] assessing the fraction of drug released from liposomes in a single pass through the heated tumor, and the drug targeting index. The results confirmed earlier findings [7] that large unilamellar vesicles release cisplatin almost completely at the heated tumor and this was due to their high heat sensitivity and small systemic clearance. In another study [8] several lipid mixtures were tested under defined thermal conditions *in vitro* in a medium containing 15% fetal calf serum at 43°C. Despite extensive investigations [9] not only the mechanism responsible for

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ABBREVIATIONS: PC, Phosphatidylcholine; DPPC, Dipalmitoylphosphatidylcholine; DSPC, Distearoylphosphatidylcholine; HSPC, Hydrogenated soy phosphatidylcholine; CHOL, Cholesterol; EPC, egg phosphatidylcholine; PEG-PE, Distearoyl phosphatidylethanolamine derivatized at the amino position with polyethylene glycol; HEPES, N-[2-Hydroxyethyl] Piperazine-N-[2-ethanesulfonic acid]; Dox, Doxorubicin.

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liposome destabilization upon interaction with blood components is not clearly understood, but the drug-release profile of liposomes in blood circulation has not been addressed. This is partly due to the overall complexity of the problem and the difficulties of studying liposomal instability *in vivo*. A recent study has demonstrated, however, that negatively charged liposomes activate the classical pathway of human or guinea pig complement system [10].

The potential benefit of a local hyperthermia treatment using liposomes has been enhanced by the development of sterically stabilized liposomes [2], which show increased circulation time in blood and also increased deposition in tumors. The study reported here has a dual purpose: to formulate a liposome composition that shows good differential thermal release for hyperthermia purposes as well as long circulation time in blood, and also to understand the mechanism of instability of various liposomes in bovine serum and human plasma. We have, therefore, evaluated the properties of a series of liposomes composed of DPPC, HSPC, CHOL and PEG-PE in order to optimize the thermosensitivity of liposomes with prolonged circulation time. The molar ratios of these components were adjusted in order to achieve a phase transition near 45°C in buffer, with liposomes of diameter in the range of 120-170 nm. In addition, we investigated the effect of bovine serum and human plasma on the stability of both rigid and fluid liposomes composed of PC, CHOL and PEG-PE at different molar ratios and at different temperatures (37°, 42°, 45°, 50°C). The stability of liposomes was studied by the rate of release of encapsulated doxorubicin (Dox), an anti-tumor drug, or calcein, a water-soluble fluorescent marker. Based on the differential release of liposome contents at different temperatures, and the effect of serum, plasma, EDTA, and anti C3-complement antibody, we have concluded that some components of complement interact preferentially with rigid liposomes and this effect is augmented in the presence of CHOL.

MATERIALS AND METHODS

Materials

Hydrogenated soy phosphatidylcholine (HSPC) of >98% purity was obtained from Nattermann Phospholipid GmbH, Koln, Germany. Dipalmitoylphosphatidylcholine (DPPC) and egg phosphatidylcholine (EPC) of > 98% purity was purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama). Distearoyl-phosphatidylethanolamine derivatized at the amino position with a 1900 molecular weight segment of polyethylene glycol (PEG-PE) was synthesized as before [2]. Cholesterol (CHOL) of >99% purity was obtained from Calbiochem (La Jolla, California); Deferoxamine Mesylate was from Sigma Chemical Co (St. Louis, Missouri); Rabbit anti-bovine C3 antiserum from Organon Teknika Co. (Durham, North Carolina); Bovine calf serum from the cell culture facility of UCSF; Human plasma (using heparin as an anticoagulant) from the blood bank of Moffitt hospital (UCSF); EDTA (ethylenediamine tetraacetic acid) and EGTA (Ethylenebis-oxyethylenenitrillo tetraacetic acid), from Fisher Scientific Company (Fair Lawn, New Jersey); Doxorubicin(Dox) Hydrochloride from Cetus Corporation (Emeryville, California); Calcein (high purity) from Molecular Probes Inc. (Eugene, Oregon); Diethylether and Isopropylether (HPLC grade) from Aldrich Chemical Company (Milwaukee, Wisconsin).

Preparation of Liposomes and Drug Loading

Unilamellar vesicles composed of DPPC, HSPC with and without CHOL and PEG-PE, were prepared by membrane extrusion [2,11]. The molar ratio of the different components is indicated in parenthesis in the text. The lipids were mixed in chloroform and the solvent was removed under reduced pressure at 40°C. Multilamellar vesicles were formed by vigorous shaking of the lipid film in an aqueous solution of 250 mM ammonium sulfate and 1 mM Deferoxamine (pH = 4) at 55°C for five minutes and then the preparation was treated by freeze-thaw for 5 times. The resultant large oligolamellar vesicles were extruded under pressure (200-300 PSI) through double polycarbonate membranes (Poretics, Livermore, California) of 0.1 and 0.05 μm pore diameter five times each, using an extruder device (Lipex Biomembranes, Vancouver, British Columbia, Canada) heated at 55°C. The resultant liposomes after extrusion, were found to have a particle diameter between 120-170 nm as measured by dynamic light scattering apparatus (Coulter Model N4). The average size distribution for each preparation is given in Table I. Unentrapped ammonium sulfate was removed at room temperature by gel filtration through Sephadex (G-75) equilibrated with 20 mM HEPES buffer containing 0.9% NaCl at pH 7.4 and osmolarity of 290 mOs.

Doxorubicin (Dox) was encapsulated by the ammonium sulfate gradient method [12] as follows: Dox-Hydrochloride in a powder form was added to the liposome suspension described above at a concentration of 1 mg Dox/10 μmol phospholipid in 1 ml of buffered saline solution. The liposome-Dox mixture was incubated in a water bath for one hour at 55°C [12]. After incubation, unentrapped Dox was removed by passing through Sephadex (G-75) gel filtration column. The final concentration of liposomes was determined by phosphate assay [2]. The amount of drug trapped inside the liposomes was determined in a fluorometer (SPEX) using 470 nm as an excitation wavelength and 592 nm as an emission wavelength, after adding Triton X100 (50 μl of 10% v/v solution into 2 ml total) and heated at 55°C for 30 minutes.

Liposomes encapsulating calcein, were prepared following the same procedure, but instead of hydrating the lipids with ammonium sulfate solution, we used a 20 mM solution of calcein adjusted to pH 7.4 and 290 osmolarity with NaCl. The liposomes were extruded as described before. After extrusion the liposomes were passed through Sephadex (G-75) gel chromatography column, equilibrated with 20 mM HEPES/saline buffer, same osmolarity and same pH. The amount of encapsulated Calcein was determined by fluorimetry using 490 nm as an excitation wavelength and 520 nm as an emission wavelength. Table I shows the amount of phospholipid as determined by phosphate assay and the amount of encapsulated drug for each liposome composition.

For some experiments, as indicated in the text, we have used large unilamellar vesicles which were prepared by reverse-phase evaporation method [11]. The phospholipids were dissolved first in organic solvents such as diethylether

(for fluid lipids) or isopropylether (for rigid lipids). The ammonium sulfate solution was added directly to this phospholipid-solvent mixture, and the preparation was then sonicated for a brief period (2-3 minutes), forming a homogeneous emulsion. The organic solvents were removed under reduced pressure, resulting in the formation of a viscous gel-like intermediate phase, which spontaneously forms a liposome dispersion when residual solvent is removed by continued rotary evaporation under reduced pressure [11]. Unentrapped ammonium sulfate was removed by gel filtration and Dox was encapsulated as described above.

Method of Assay for Dox Release

We used the property of liposome-encapsulated Dox as a self-quenching material, to detect the drug in the medium following its release from the liposomes. We considered 100% release as the fluorescence intensity after the addition of detergent (Triton × 100, 50 μl of 10% v/v solution in 2 ml total), while 0% was the intensity after the injection of the liposomes (30-40 nmole phospholipid) in a cuvette containing 2 ml HEPES/saline buffer plus serum or plasma. In order to avoid the quenching the Dox in the medium we adjusted the final concentration of the drug to be near 1 μg/ml for all the different lysed samples. Corrections were made for the effects of temperature and serum or plasma on Dox fluorescence intensity. The curves shown in figures 2, 3, and 6 represent typical experiments and were obtained directly from the fluorometer. Repeat experiments with the same samples under the same conditions were well within 10% range of error.

Determination of the Phase Transition Temperature

The phase transition of the different liposome compositions was detected using differential scanning calorimetry [Perkin-Elmer DSC-4/Thermal Analysis Data Station system]. The rate of heating was 5°C/min, the samples were centrifuged at 150,000 g × 50 min using (Beckman) ultracentrifuge, the pellets were then collected and 7 mg from each sample was taken for the analysis.

RESULTS

Design of Thermosensitive Liposomes

Phase Transition Temperature

Earlier studies have already established a connection between liposome permeability, lipid phase transition and cholesterol content [13-15]. For this study we have examined the thermal behavior of DPPC/HSPC at 100:50 and 50:50 mole ratios, mixed with varying amounts of cholesterol for increasing stability in blood [9,16], and PEG-PE for increasing circulation time [2]. Figure 1 shows the thermotropic transitions for the different liposome compositions detected by differential scanning calorimetery (DSC). We found that at 1 to 2 or higher mole ratio of cholesterol to phospholipid, there is no clear phase transition and that the composition which seems to be optimal for hyperthermia exhibits a phase transition with a midpoint at about 48°C and a considerable broadness to the curve (38-57°C). We have also found that PEG-PE causes a small increase in the phase transition temperature, two degrees higher than the same composition without PEG-PE. However, encapsulation of Dox does not affect the phase transition of the lipid, since the same thermotropic behavior was observed before and after loading of the drug.

Thermosensitivity of Liposomes in Bovine Serum and Human Plasma

The time course for the release of Dox during incubation in HEPES/saline buffer and serum is shown in Figures 2 and 3 for liposomes composed of DPPC/HSPC/CHOL/PEG-PE at six different molar ratios (panels a to f) and at different temperatures (37°, 42°, 45°C). First we will discuss the behavior of various liposome compositions not containing PEG-PE (panels a to d) and will examine the role of both cholesterol and serum components in modulating thermal sensitivity.

During incubation of liposomes in buffer (Figure 2, a to d) the release of Dox was very small at 37°C and increased by raising the temperature, but the release from CHOL-containing liposomes was smaller at all the temperatures compared with liposomes without CHOL. This confirms earlier observations showing that liposomes with high cholesterol are more stable in buffer than liposomes without CHOL (9,16). When bovine serum was added to the incubation medium (50%) instead of buffer, liposomes generally showed increased leakage at all temperatures, (compare Figure 2 a to d with Figure 3 a to d). However, liposomes with high cholesterol content released more drug at 37°C than liposomes without CHOL (Fig. 3, a versus b) or liposomes with a low molar ratio of cholesterol (Figure 3, c versus d). We found also that the reaction is time-dependent, and that there is a certain time delay (about ten minutes) after which the rate of release increases. This delay time is longer with lower serum concentration (10-30%), which induces the reaction to start later, but the release ends later and gives a plateau at a lower level (data not shown).

The effect of 50% serum on CHOL-free liposomes was most clearly seen at lower temperature where the release of Dox was enhanced greatly compared to buffer alone (compare Figure 2a with Figure 3a). As expected, the plateau levels of release in buffer show clearly an increasing value as the temperature increases from 37° to 42° and 45°C. On the contrary, the plateau values for the CHOL-rich liposomes all show highest rate of release at 37°C with lower values at 42° and still lower at 45°C. Similar results were obtained when calcein was encapsulated passively in these liposomes instead of Dox, which had been loaded using an ammonium sulfate gradient. This enhanced effect of serum at 37°C with CHOL was unexpected, because there was no phase transition at this temperature range (Figure 1D) and decreased the value of these liposomes for hyperthermia.

In an attempt to reduce this serum effect we found that including 2% PEG-PE to the liposome composition reduced the leakage of these liposomes at 37°C, as shown from a comparison of Figure 3a with Figure 3e. The addition of PEG-PE was necessary not only for reducing the leakage of Dox produced by serum components at 37°C, but also for increasing the $T_{1/2}$ of these liposomes in the circulation, and

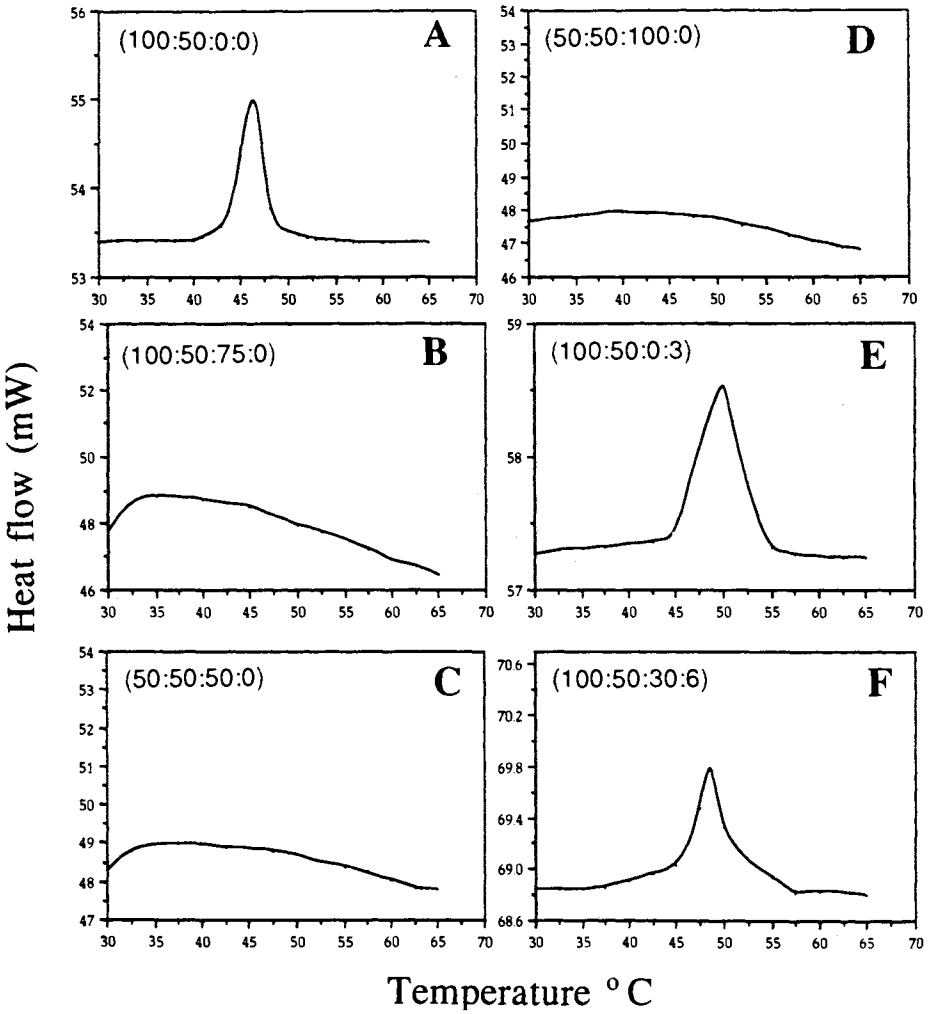

Fig. 1. Differential scanning calorimetry thermograms for different liposome compositions. The liposomes were composed of DPPC/HSPC/CHOL/PEG-PE at the indicated molar ratios. 100:50:0:0 (A); 100:50:75:0 (B); 50:50:50:0 (C); 50:50:100:0 (D); 100:50:0:3 (E); 100:50:30:6 (F).

thus enhancing their potential for extravasation into the tumor tissue, before [2] and during hyperthermia [17]. The effect of PEG-PE shown in Figure 3e is in the right direction, but with this composition (100:50:0:3) the release of Dox at 42°C was still low, and only slightly higher than at 37°. We, therefore, studied several other formulations varying the percent of CHOL and PEG-PE, in attempting to identify maximal thermal differential for Dox release between 37° and 42°. The optimal composition is described in Figures 2f and 3f showing a high release of 42° and a minimal release at 37°C.

Thermosensitivity of Liposomes in Human Plasma

We have also tested the same liposome compositions in 50% human plasma at the same temperatures (37°, 42°, 45°C), and we found that human plasma was less active than bovine serum in releasing encapsulated Dox in the absence of PEG-PE, but more active in its presence. Figure 4 shows a comparison between the effect of bovine serum and human plasma on seven different liposome compositions at 37° and 42°C (upper and lower graphs respectively). The interesting and unexpected result shown in Figure 4 is that at 37°C, human plasma interacts preferentially with PEG-PE containing liposomes without cholesterol and causes their contents (Dox) to leak, while PEG-PE, protected these liposomes totally in the case of bovine serum. On the other hand addition of CHOL (between 20 to 30%) stabilized these liposomes to a certain extent in human plasma, while it caused their contents to leak in bovine serum (Figure 4). In both cases the differential thermal release between 37°C and 42°C in plasma and serum was low. A combination of 4% PEG-PE and 20% CHOL, showed high stability in both bovine serum and human plasma, and enhanced the differential thermal release of contents when heated at 42°C. These results indicate that both 4% PEG-PE and a 20 to 30% CHOL are required in order to achieve minimal release at 37°C, with concomitant high differential thermal release at 42°C.

Extended time-release experiments for up to 8 hours (as shown in Figure 5) in 50% human plasma at 37°C show that liposomes composed of DPPC/HSPC/CHOL/PEG-PE (100:50:30:6) give 10% release within 4 hours and then the rate of release is significantly diminished. An alternative composition, DPPC/HSPC (100:50) shows minimal release up to 4

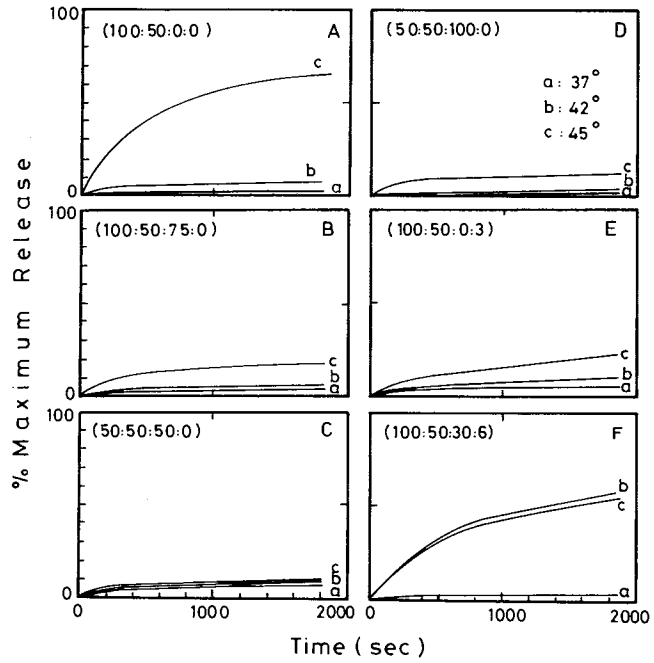

Fig. 2. Release kinetics in buffer solution of encapsulated self-quenched Dox from liposomes see Fig. 1 for molar ratios. Temperatures 37°, 42°, 45°C as shown on the graphs. The curves shown in Figures 2, 3 and 6 were obtained directly from the fluorimeter. Variation between runs with the same sample were well within the 10% range.

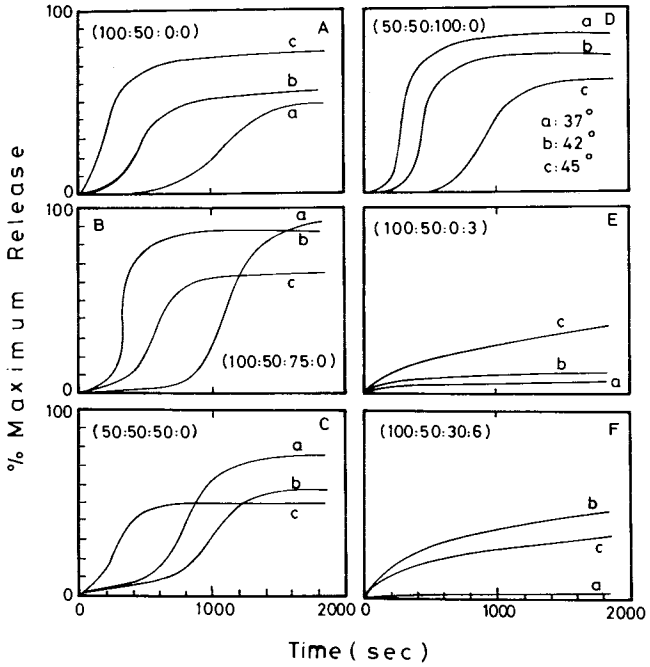

Fig. 3. Release kinetics in 50% bovine serum of encapsulated self-quenched Dox from liposomes composed of DPPC/HSPC/CHOL/PEG-PE at the indicated molar ratios see Fig. 1. Temperatures 37°, 42°, 45°C as shown on the graphs. Experiments done at 39°C and 41°C gave curves that were intermediate between 37°C and 42°C.

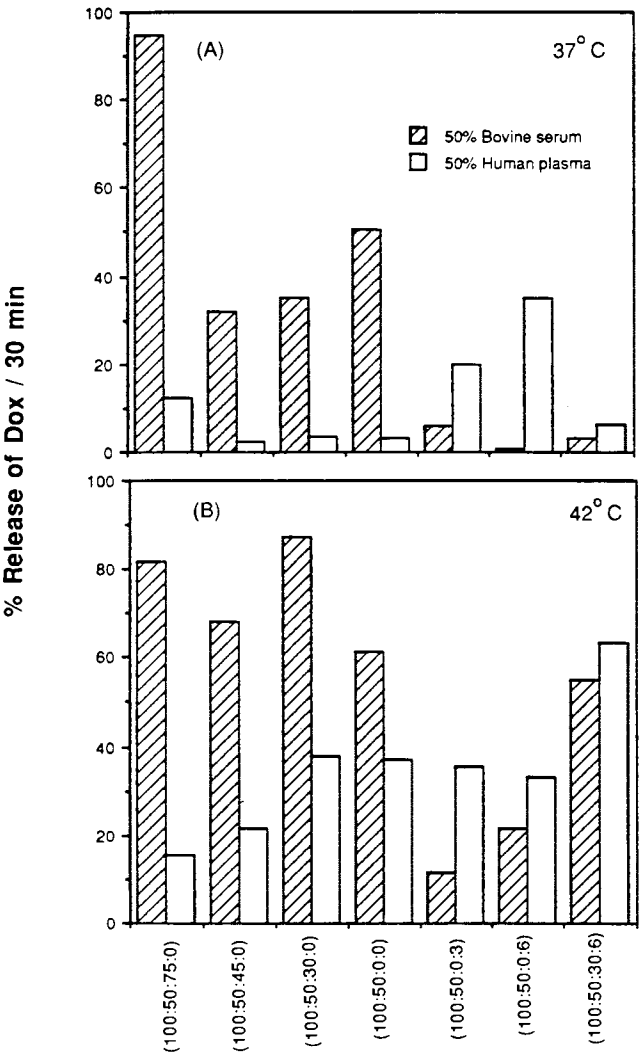

Fig. 4. A comparison between the effect of bovine serum and human plasma on the release of Dox at 37°C (graph A), 42°C (graph B) from liposomes composed of DPPC/HSPC/CHOL/PEG-PE, at the molar ratios indicated.

hours at 37°C, but increasing release after that. All other compositions show higher levels of release at these times. On the basis of the high differential thermal release shown by the 100:50:30:6 mixture, this formulation was chosen for further *in vivo* experiments involving hyperthermia in tumor-bearing animals [18].

Mechanism of Serum-Induced Release of Doxorubicin

The Role of Bilayer Fluidity and Cholesterol

In order to study the mechanism of the instability induced by various plasma or serum components we selected liposome compositions which showed relatively high instability against the most active reagent, bovine serum. Specifically we studied the rate of release of Dox from liposomes with different degrees of fluidity including those composed

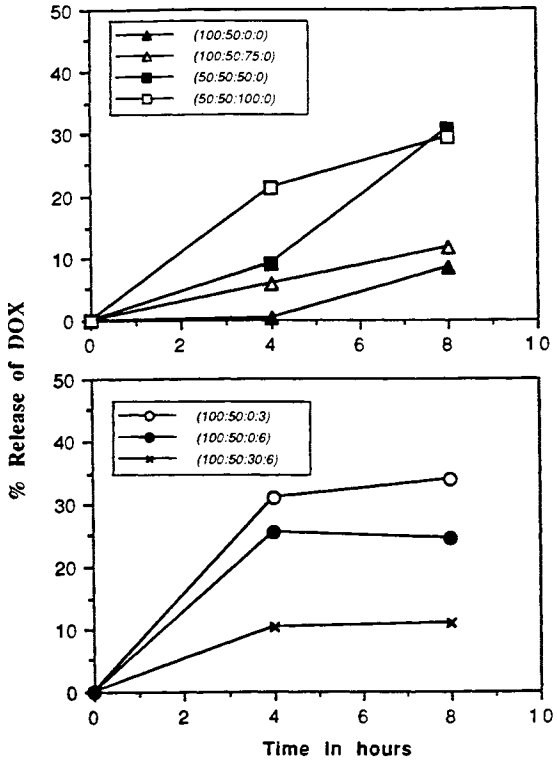

Fig. 5. Prolonged time release experiment for Dox released from liposomes composed of DPPC/HSPC/CHOL/PEG-PE at the molar ratio shown on the graph, in 50% human plasma at 37°C.

of EPC, DPPC, HSPC mixed with CHOL, in 50% bovine serum at 37°C. We found that the degree of fluidity affects not only the amount of released drug but also the pattern of the release versus time. As shown in Figure 6, fluid liposomes composed of EPC/CHOL (50:50) showed a slow linear rise of release, while rigid liposomes composed of DPPC/HSPC/CHOL at a molar ratio of 50:50:100, showed a fast

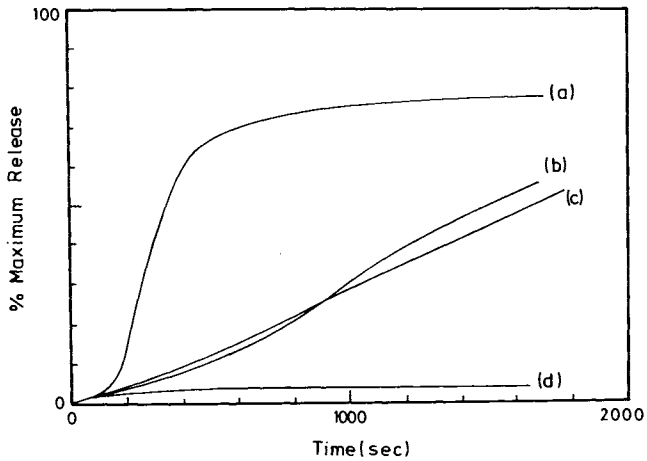

Fig. 6. The effect of serum and EDTA on the release of Dox from two liposome compositions. Rigid liposomes were composed of DPPC/HSPC/CHOL, at a molar ratio of 50:50:100 (panels a and d), and fluid liposomes were composed of EPC/CHOL, at a molar ratio of 50:50 (panels b and c). All experiments were done in 50% bovine serum at 37°C. Serum was added at time 0, in the absence (panels a and d), or presence (panels c and d) of 5 mM EDTA.

exponential rise reaching plateau within five minutes. Although both compositions shown in Figure 6 contain CHOL, similar results with respect to the relative effect of bovine serum on the release of Dox were obtained when using the same phospholipid without CHOL. The different kinetic profile for the release of Dox indicates different mechanism of action for bovine serum components on rigid versus fluid liposomes. This possibility is substantiated and elaborated below using a variety of treatments that distinguish the differences between rigid and fluid liposomes.

It is generally recognized that the presence of CHOL decreases the permeability of liposomes and protects them from the destabilizing effect of plasma or serum proteins (9,16). However, as we have already discussed above, the effect of CHOL on the release of Dox from liposomes in the presence of serum or plasma depends on the physical state of the phospholipid bilayer, for example whether it is in gel (rigid) or liquid crystalline (fluid) phase. Figure 7 presents a summary of studies on the rate of release of Dox from fluid and rigid liposomes under different conditions. Figure 7 shows the effect of plasma and serum on liposomes composed of DPPC/HSPC (100:50) without and with CHOL (100:50:75) at three different temperatures: at 37°C (below the main thermotropic phase transition (Tc) when the bilayers are rigid), at 45°C (at the Tc when the bilayers are quite permeable even in buffer) and at 50°C (above the Tc, when the bilayers are fluid). Figure 7 also shows the behavior of liposomes composed of EPC with and without CHOL at 1:1 mole ratio, as an example of the release characteristics of a bilayer at a temperature (37°C) much higher than its Tc (−20°C for EPC). Panel A shows the release characteristics in buffer, where the protective effect of CHOL is seen at temperatures near and just above the Tc, when the liposomes without CHOL become quite leaky. Panels B and C show the release characteristics of the same liposomes in the presence of 50% bovine serum and human plasma respectively. In both cases, CHOL is having a protective effect only at temperatures at or above the Tc when the bilayers are fluid. Unexpectedly, the opposite effect is seen at temperatures below the Tc with rigid bilayers where the effect of serum or plasma in releasing Dox is enhanced in the presence of CHOL. These differences between fluid and rigid liposomes indicate different mechanism of action as will be discussed below.

Addition of Chelators and Dialysis

In order to further understand the mechanism of interaction between bovine serum and liposomes, we studied the effect of metal chelators and dialysis of serum. A concentration of 5 mM EDTA is generally considered to be sufficient for chelating both Mg^{2+} and Ca^{2+} present in plasma. We found that 5 mM EDTA inhibited the reaction (20 fold decrease in the release of Dox compared to the control) of bovine serum at 37°C with rigid liposomes composed of DPPC/HSPC/CHOL (50:50:100), but did not affect fluid liposomes composed of EPC/CHOL (50:50), as shown in Fig. 6. This inhibition effect was seen even when EDTA is added after the start of the reaction. However, EGTA (up to 20 mM) did not affect the release characteristics of either type of liposomes. In separate experiments we dialyzed the serum

Table I. Sample Characterization for Different Liposomal Preparations

Liposome composition DPPC/HSPC/CHOL/PEG-PE (Molar ratio)	Concentration of phospholipid determined by phosphate assay (μmol/ml)	Concentration of Dox encapsulated (μg/ml)	Ratio of Dox to phospholipid (μg/μmole)	Average particle diameter as calculated from the size distribution/ differential weight (nm)	Standard deviation of particle diameter (± nm)
100:50:0:0	3.51	216	61.54	123	40
100:50:75:0	4.3	213.25	49.6	170	57
50:50:50:0	2.41	64.56	144	62	62
50:50:100:0	2.9	105.1	36.24	147	64
100:50:0:3	9.3	443.2	47.65	99	26.3
100:50:0:6	8.2	560	68.3	98.3	30.6
100:50:30:6	3.36	99.5	29.7	168	47

against both HEPES/saline buffer and PBS buffer containing Ca^{2+} and Mg^{2+}, using 6000-8000 M_W cut-off dialysis bags. We then tested the activity of the dialyzed serum separately on liposomes composed of DPPC/HSPC/CHOL (50:50:100) at 37°C since these liposomes were the most sensitive to bovine serum among different liposomal preparations. We found that bovine serum dialyzed against HEPES buffer lost its activity (35 fold decrease in the release of Dox compared to control), while serum dialyzed against PBS containing Ca^{2+} and Mg^{2+} maintained most of its activity (1.5 fold decrease in the release of Dox compared to the control). The results of these experiments emphasize the importance of Mg^{2+} for maintaining the serum effect and raise the possibility that the destabilizing factor may be a complement component, which has been reported to interact with rigid liposomes [19].

Pre-Heating Serum at 55°C for 30 Minutes

Bovine serum was heated at 55°C for 30 minutes, then cooled to 37°C and its activity measured on two types of liposome compositions; one was rigid liposomes composed of DPPC/HSPC/CHOL (50:50:100) and the other was fluid liposomes composed of EPC/CHOL (50:50). We found that the drug release from the rigid liposomes at 37°C was completely inhibited (20 fold decrease in the release of Dox compared to the control), while the fluid phase ones were still leaky (2 fold decrease in the release of Dox compared to the control). These results suggested that some liposomes were destabilized by a serum component which was inactivated by heating at 55°C for 30 minutes, and this component affected rigid but not fluid liposomes. Since it has been known that the complement system is inactivated by heating at 56°C for 30 minutes, and that Ca^{2+} or Mg^{2+} are necessary for its activation [20], we conclude that the serum component which causes leakage of liposomal contents from rigid liposomes is probably related to complement. However, the participation of other proteins in inducing leakage is also possible.

Effect of Anti C3-anti Bovine Serum

In order to clarify the above experiment, we have tested the effect of antibodies against one of the complement proteins (anti bovine C3 anti serum) on both rigid liposomes composed of DPPC/HSPC/CHOL (50:50:100) and fluid liposomes composed of EPC/CHOL (50:50). We found that the release of Dox at 37°C was inhibited (7 fold decrease in the release of Dox compared to the control) in the case of rigid liposomes at an antibody concentration of 1 mg/ml, but not inhibited with the fluid liposomes, which showed same activity as the control experiment.

Pre-Treatment of Serum with Control liposomes

In an attempt to study the mechanism of interaction of bovine serum with both rigid and fluid liposomes, we performed competition experiments by adding a three-fold excess of control liposomes (same composition, without Dox). We found that the effect of serum on the release of encapsulated Dox from rigid liposomes composed of DPPC/HSPC/CHOL (50:50:100) was inhibited to a large extent (7 fold decrease in the release of Dox compared to the control) by 100 nmole of control liposomes (extruded through 0.05 μm membrane) composed of the same lipid composition or composed of DPPC/HSPC (50:50), but was not affected by similar size fluid liposomes (extruded through 0.05 μm membrane) composed of EPC/CHOL (50:50) at the same concentration. On the other hand, we found that the effect of serum on the release of Dox from small (extruded through 0.05 mm) fluid phase liposomes composed of EPC/CHOL (50:50), was not affected by either rigid liposomes composed of DPPC/HSPC/CHOL (50:50:100) or by fluid phase liposomes composed of EPC/CHOL (50:50) all of similar size. This experiment provides clear evidence that the interaction of bovine serum with rigid liposomes is different from the interaction with fluid liposomes.

Mechanism of Human Plasma-Induced Release of Doxorubicin

The destabilizing effect of human plasma at 37°C on liposomes of different compositions without PEG-PE was lower than that with bovine serum (4 bars on the left, Figure 4A). However the destabilizing effect of human plasma increased, when PEG-PE was incorporated in the liposomes. Figure 4A shows that, the most sensitive composition to human plasma is the one which has DPPC:HSPC:CHOL:

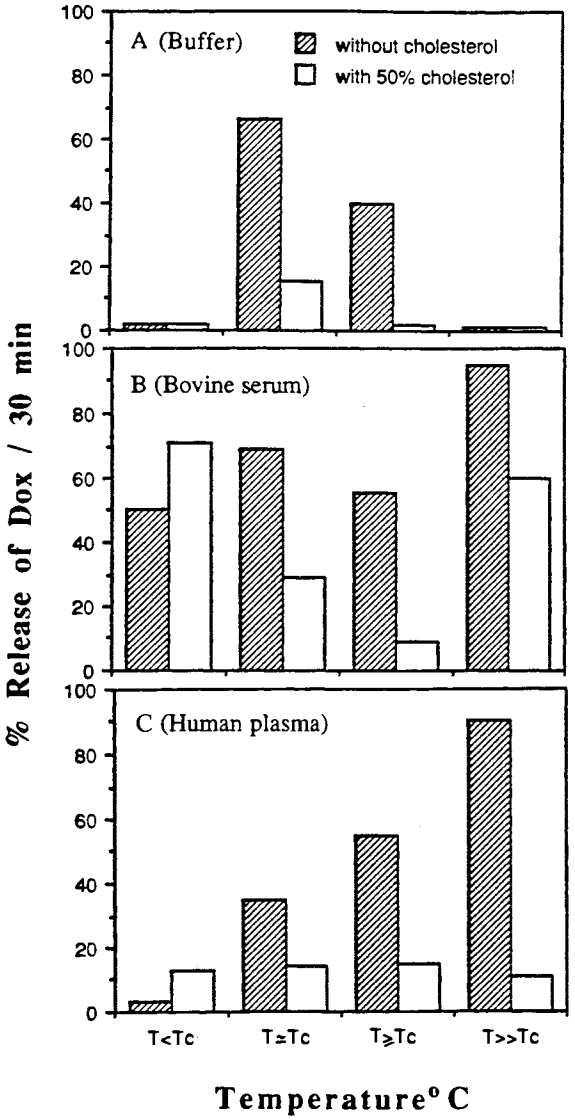

Fig. 7. The effect of CHOL on the stability of liposomes in buffer (graph A), bovine serum (graph B), and human plasma (graph C) at temperatures related to the phase transition temperature (Tc). The columns designated as T<Tc refer to liposomes composed of DPPC/HSPC (100:50), or DPPC/HSPC/CHOL (100:50:75) at 37°C. The columns designated as T≃Tc refer to the same liposome composition at 45°C. T≥Tc refers to the same liposome composition at 50°C. T≫Tc refers to liposomes composed of EPC or EPC/CHOL (50:50) at 37°C. It should be noted that the effects of serum described here refer to conventional liposome compositions with and without cholesterol. When such liposomes are stabilized by the addition of PEG-PE, the effects are quite different, as can be seen in figures 2 and 3.

PEG-PE at a molar ratio of (100:50:0:6). Using this composition, we have performed experiments involving a variety of pre-treatments to human plasma. We found that, pre-heating human plasma at 55°C for 30 minutes, addition of chelators and antibodies against the complement component C3, did not inhibit the reaction of human plasma against the PEG-PE containing liposomes. Therefore, it seems that complement is not responsible for the effect of human plasma on these liposomes.

DISCUSSION

The combination of local hyperthermia with liposome-mediated drug release is an attractive approach for tumor chemotherapy. Local hyperthermia has been used to enhance the effectiveness of chemotherapy [21], generally by increasing blood flow, membrane permeability, local metabolism and drug efficacy [22].

It has been known for some time that there is a maximal release of low molecular weight liposome contents at a temperature close to the gel-to-liquid crystalline transition [13]. It is also known that the transition is affected by the liposome composition, and that it is abolished completely by high mole ratios of cholesterol [14]. In order to obtain maximal release of liposome contents at 42°-45°C from liposomes which are stable at 37°C, the phase transition should be starting at temperatures >40°C. Mixtures of DSPC and DPPC have been used for hyperthermia experiments [5]. The phase diagram of this mixture of phospholipids has been studied in detail [15]. HSPC has been known to contain mostly DSPC after hydrogenation and this lipid (HSPC) has already been used extensively in drug delivery with liposomes [2].

The first thermosensitive formulation of liposomes was proposed in 1978 [5], and the idea was to design liposomes with a gel-liquid crystalline transition temperature above physiological temperature but in a range attainable by mild local hyperthermia. This was based on the earlier observation that liposomes show an enhanced release of contents at the transition temperature [13]. Liposomes prepared with DPPC/DSPC (7:1) showed *in vitro* release kinetics demonstrating a rapid release of their contents at 42°C in 15% mouse plasma [3]. Since cholesterol is known to play an important role in the stability of liposomes in serum or biological fluids [9,16], a modified formula composed of DPPC/HSPC/CHOL (5:4:2) was used recently [8], and a differential thermal stability of 72% between 37°C and 42°C was reached in 15% fetal calf serum, conditions useful only for *in vitro*, cell culture study.

Recent studies have shown that polyethylene glycol-derivatized phospholipids provide a hydrophilic surface coating (steric stabilization) on the liposomes, resulting in a pronounced increase in blood residence time with a remarkable decrease in uptake by liver and spleen [2,23] and an important increase in the uptake by tumors [2]. Encapsulation of Dox within liposomes is of great interest since it was demonstrated to reduced toxicity [24], while enhancing the efficacy against some tumors [2,24]. It is, therefore, interesting to encapsulate this compound in thermosensitive sterically stabilized liposomes, in order to take maximal advantage of both modes of treatment: liposomes and local hyperthermia. Little information is available on liposomes which have both thermosensitivity and prolonged circulation ability [25,26].

Our experiments were designed to evaluate a series of liposomes composed of DPPC, HSPC, CHOL and PEG-PE in order to optimize the thermosensitivity of sterically stabilized liposomes with prolonged circulation time. In addition, we have also performed a series of experiments with both bovine serum and human plasma, in order to clarify the mechanism of interaction of various blood components with

liposomes and the role of lipid composition in this interaction. We used Dox as a marker for studying the mechanism of interaction of liposomes with serum and plasma *in vitro*. Since this compound is self-quenched when encapsulated at high concentrations, its release can be detected by enhanced fluorescence intensity. This property of Dox enables continuous and sensitive monitoring of its release.

Our results have shown that in order to have liposomes that are stable at 37°C but release their contents at 42-45°C the phospholipid composition should be such as to undergo a phase transition around 45°C. Furthermore, in order to make these liposomes long-circulating and reduce their uptake by the liver [2] we have to include PEG-PE. Thus, in order to have liposomes with a long circulation in vivo, that are stable at 37°C in human plasma, and show increased release at 42-45°C, we have included 4% PEG-PE and about 20% to 30% CHOL to a phospholipid composition of DPPC/HSPC (100:50). This composition gives less than 10% drug release over 30 min at 37°C and 60% release at 42°C in human plasma, as shown in Figure 4. We found that PEG-PE protected the liposomes from interaction with bovine serum at the lower temperature without affecting the thermosensitivity. We also found that bovine serum is more active than human plasma in enhancing Dox release, which reduces the value of bovine serum for studying the release of drug from liposomes in vitro as a model to in vivo pharmacokinetic stability in humans. However, we have studied extensively the effect of bovine serum, as a means to understand the mechanism of liposome destabilization *in vitro*. These studies are discussed below.

Our results show that the effect of CHOL on liposome stability in both bovine serum and human plasma depends on the physical state of the lipid membranes, i.e. gel or fluid. This is clearly shown in Figure 7. Addition of CHOL to gel phase lipids destabilizes the liposomes, and increases the leakage of Dox in the presence of both bovine serum and human plasma. This result was somewhat unexpected, since it is well known that cholesterol added to fluid phase lipids stabilizes the liposomes and decreases the leakage of encapsulated material [9,16]. However, both effects are consistent with the earlier biophysical studies showing that CHOL affects the order parameter of the phospholipid acyl chains within the bilayer [13,14]. It is evident that the interaction of the bilayer with proteins from bovine serum and human plasma is very sensitive to these effects. In the presence of buffer only, CHOL shows a stabilizing effect by inhibiting the release of contents at temperatures equal or above Tc. The destabilizing effect at lower temperatures was only seen in the presence of plasma or serum. When the phospholipid is in the liquid crystalline state ($T \geq Tc$, Figure 7), CHOL increases the bilayer order by limiting the probability of trans-gauche isomerization of the phospholipid acyl chains [14]. It seems that bovine serum has comparatively a small effect on the bilayer when it is motion-restricted because of CHOL, but still in a fluid state, with consequently lower effect on the leakage from these liposomes at higher temperature such as 50°C. This contrasts with the much larger effect on the same bilayers also with CHOL at 37°C which is below the Tc of the phospholipids (Figure 7). It should also be noted that as shown in figure 3, liposomes containing PEG-PE are stabilized against the effect of serum with or without cholesterol, although cholesterol is still needed to stabilize them against human plasma at 37°C.

Earlier studies have demonstrated that various plasma proteins become associated with the lipid bilayer and can release entrapped contents from liposomes. For example, it was found that the destabilization of liposomes composed of EPC was due to transfer of phospholipid between liposomes and high density lipoproteins (HDL) [27] and that liposomes with high CHOL content are more stable than CHOL-free liposomes [9,16]. Furthermore, it has been suggested that liposomes composed mainly of PC are damaged by activation of the complement pathway, since serum activity was reduced when preheated at 56°C for 30 minutes [28]. Recent study [19] demonstrated that liposomes consisting of hydrogenated EPC, CHOL and dicetyl phosphate (4:4:1 molar ratio) were destabilized when incubated with plasma. This destabilization was completely inhibited by pre-heating at 56°C for 30 min or pretreating with EDTA but not with EGTA indicating that complement was involved. Indeed C3 was identified to be associated with liposomes incubated with fresh plasma [19]. In a more recent study [29], it was shown that the complement system activation, depends on the size of the liposome, and that small liposomes could not consume the opsonic activity, while the larger ones did so substantially.

Our results concerning the interaction of bovine serum with liposomes were similar to some extent with recent studies [19,29], in that serum activity was inhibited by preheating at 55°C for 30 minutes or treating with EDTA, but not with EGTA, and was inhibited by anti C3-anti bovine serum or large size control liposomes. However, our unique observation is that this inhibitory effect was found only with rigid liposomes and not with fluid liposomes. Moreover, we have established that the destabilization of PEG-PE containing liposomes by human plasma is not due to complement, since the reaction was not inhibited by preheating the plasma at 55°C, for 30 minutes or by addition of metal chelators or antibodies against C3. These and other results discussed above strongly suggest that the destabilizing factor in bovine serum is part of the complement system and interacts preferentially with rigid liposomes but not with fluid liposomes. The kinetics of the release of liposome contents by the bovine serum complement proteins are in accord with the formation of a pore through the liposome membrane as has been shown in the recent study with lipophilic peptides [30]. However, it is still not understood which components are responsible for the activity of bovine serum with fluid liposomes, and which components in human plasma are responsible for destabilizing liposomes containing PEG-PE without cholesterol.

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