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The effect of blood serum on the size and stability of phospholipid liposomes

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Liposomes have been prepared by sonication (SUV) and reverse phase evaporation (REV) from dipalmitoylphosphatidylcholine (DPPC) and its mixtures with phosphatidylinositol (PI), stearylamine and cholesterol. The effect of rat and human blood serum on the liposomes has been investigated by measurement of the particle size in the serum-liposome mixtures by photon correlation spectroscopy in the range of serum protein concentration up to approx. 25 mg ml^{-1}. At low serum protein concentrations the measured particle sizes exceed those calculated from the known sizes and concentrations of liposomes and serum particles in the mixtures: a result consistent with serum-induced aggregation of the liposomes, but the aggregates dissociate at higher serum protein concentration. The effect of serum on the release of encapsulated [^{14}C]glucose from REV liposomes has been investigated over a range of serum protein concentration by gel filtration. At low serum concentration a proportion of the liposomes remain intact but as the serum concentration is increased the size of the liposomes decreases with concomitant release of encapsulated glucose. At high serum concentrations (approx. 24 mg protein per ml) the larger liposomes in the distribution are disrupted and some of the liposomal lipid becomes associated with serum protein. The results are discussed with reference to the effect of blood on the uptake of liposomes by rat liver.

Introduction

The potential use of liposomes as delivery systems of therapeutic agents by intravenous injection into the circulation is limited by the stability of liposomes in the blood and their rapid removal by the mononuclear phagocyte cells of the reticuloendothelial system, particularly by the liver and spleen. The problem of interaction of liposomes with blood components and their stability in whole blood, blood plasma and serum has been extensively investigated and reviewed [1–6]. From these studies it is possible to make some generalisations regarding the stability of liposomes in blood; specifically it has been found that plasma high density lipoproteins (HDL) destabilise phospholipid liposomes possibly by removal of phospholipid from the liposomal bilayer [7]; that cholesterol-rich liposomes are more stable than cholesterol-free liposomes [8] and that small unilamellar liposomes (SUV) have longer clearance rates in the circulation than larger liposomes (i.e. multilamellar (MLV) or liposomes produced by reverse phase evaporation (REV)) [9]. Liposomes also appear to be more stable in whole blood than in blood serum [10,11], possibly due to the HDL–erythrocyte interactions predominating over HDL–liposome interactions or the transfer of cholesterol from erythrocytes to liposomes [5]. Liposome stability in blood can also be enhanced by incorporation of gangliosides [12], structural modification of the phospholipid, by replacing ester linkages with ether and/or carbamyl bonds [13], or by lipid polymerisation [14].

Despite extensive investigations the mechanism(s) which result in liposome destabilisation on interaction with blood components are not clearly understood. This is in part due to the overall complexity of the problem and the difficulties of studying liposomal instability in vivo. Numerous investigators have followed

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permeability changes in liposomes by monitoring the release of encapsulated quenched carboxyfluorescein (CF) on exposure to biological fluids [5] but it is not always possible, using this technique, to distinguish between release of CF arising from interactions leading to bilayer membrane porosity from overall liposome disruption, although there is evidence that SUV are completely disrupted by transfer of phospholipid to lipoprotein [15]. In a recent study on the effect of blood on the uptake of liposomal lipid by perfused rat liver, it was found that very low levels of blood (0 to 8% haematocrit) had a significant effect on the rate of uptake of liposomes covering a range of size by the liver [16]. At blood serum levels corresponding to such low blood haematocrits it is possible to directly monitor changes in liposome size and size distribution on exposure of liposomes to blood serum. In the present study the size distribution of a range of diplamitoylphosphatidylcholine (DPPC) liposomes and its mixtures with phosphatidylinositol (PI), stearylamine and cholesterol have been investigated after exposure to rat and human blood serum by using photon correlation spectroscopy (PCS).

Materials and Methods

L-α-Dipalmitoylphosphatidylcholine (DPPC) product No. P-0763 and cholesterol product No C-8253 were obtained from Sigma (London) Chemical Company. Phosphatidylinositol (PI) from wheat germ (as the sodium salt, molecular weight 846 [17]) was Grade I from Lipid Products, South Nutfield, U.K. Stearylamine (m.p. 49–56 °C, 90% C_{18} chains, 98% NH_2) was obtained from Koch-Light, Haverhill, Suffolk, U.K. [^{3}H]DPPC (tritiated in the N-methyl position, specific activity 76 Ci/mmol) and [^{14}C]glucose (uniformly labelled, specific activity, 270 mCi/mmol) were obtained from Amersham International U.K. All inorganic reagents were of analytical grade and aqueous solutions were made up with doubly distilled water.

Liposome preparation

Labelled ([^{3}H]DPPC) liposomes were prepared in Krebs-Henseleit buffer, pH 7.4 (120 mM NaCl/5 mM KCl/1.2 mM KH_2PO_4/1.2 mM $MgSO_4$/2.5 mM $NaHCO_3$) by either sonication (SUV) as previously described [18] or by reverse phase evaporation (REV) by a modification of the method of Szoka and Papahadjopoulos [19] as previously described [20]. Calcium chloride was added to the preparations to give a final concentration of 2.6 mM. Some preparations were made with encapsulated [^{14}C]glucose (used as supplied, 270 mCi/mmol) which was added during rehydration of the lipid film (SUV) or at the initial stage of the REV preparation. These preparations initially contained 10 μCi (REV) or 25 μCi (SUV) [^{14}C]glucose.

Blood serum preparation

Rat serum was obtained by cardiac puncture from ad libitum fed Sprague-Dawley rats (200–500 g) anaesthetised by inhalation of halothane. The withdrawn blood was clotted and centrifuged at $3500 \times g$ for 2 min to obtain the serum. Human serum was obtained from clotted transfusion blood obtained from the North West Regional Transfusion Centre, Manchester. Before use the serum was clarified by centrifugation for 2 min in an Eppendorf microfuge.

Effect of serum on liposome size

After preparation the liposome size distributions were measured by photon correlation spectroscopy (PCS) using a Malvern autosizer in which the laser light scattering data are fitted to an equivalent normal weight distribution of particle sizes [20] to give the weight-average diameter ($\bar{d}_w$) of the liposomes and the standard deviation (σ_w) of the distribution. The effect of serum on the liposome size distribution was obtained by addition of the required aliquots of serum (0–500 μl) to 2 ml suspensions of liposomes (total lipid concentration in the range 0.08–0.11 mg ml^{-1}) in the PCS cuvette. The serum concentration was expressed in terms of total serum protein [21] and/or by measurement of the haemoglobin concentration of the whole blood from which the serum was derived by the Drabkin method [22]. To determine the weight-average diameters ($\bar{d}_w$) of the liposomes in the presence of serum, from the diffusion coefficients (D) measured by PCS, it was necessary to know the viscosity (η) of the suspensions in the relationship,

$$\bar{d}_w = \frac{kT}{3\pi\eta D} \quad (1)$$

where k and T are the Boltzmann constant and absolute temperature, respectively. Relationships were obtained between the relative viscosity ($\eta_r = \eta/\eta(\text{water})$) and concentrations of serum at various dilutions in PBS, as expressed in terms of total protein concentration [P] (mg ml^{-1}) by use of a Ubbelohde dilution viscometer. The following expressions were derived for rat and human serum, respectively.

$$\eta_r = 1.020 + 6.775 \cdot 10^{-3}\,[\text{P}] \quad (2)$$

$$\eta_r = 1.002 + 1.030 \cdot 10^{-2}\,[\text{P}] \quad (3)$$

These expressions apply up to a total protein concentration of 15 mg ml^{-1} and were used in conjunction with the viscosity of water ($8.904 \cdot 10^{-4}$ N m^{-2} s at 25 °C) to obtain η for use in Eqn. 1 for each liposome-serum mixture.

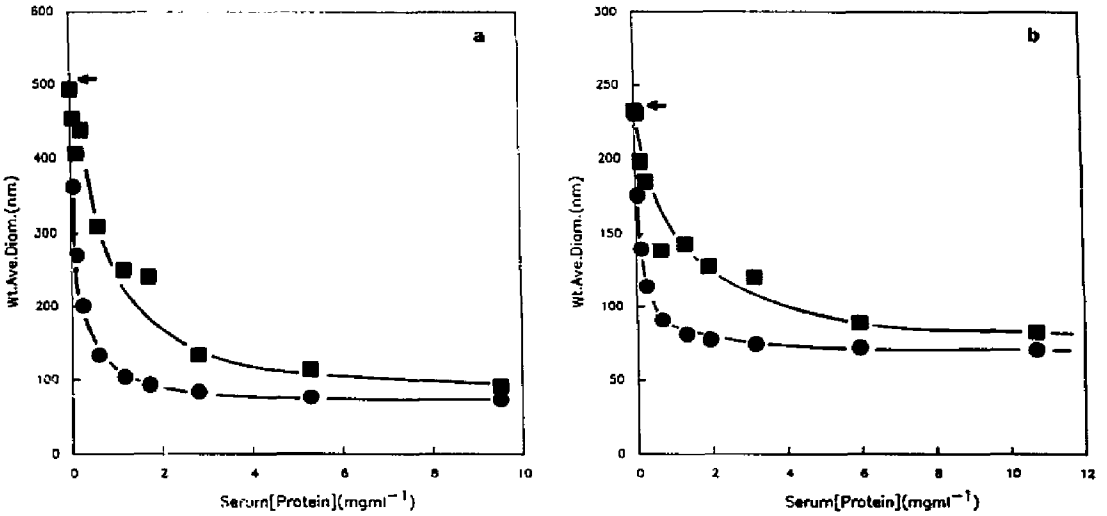

Fig. 1. Particle weight-average diameters ($\bar{d}_w$) in liposome-rat serum mixtures in Kreb-Henseleit buffer as a function of total serum protein concentration. (a) DPPC-PI (50:50 wt%) REV 0.1061 ± 0.0072 mg ml^{-1}. (b) DPPC-stearylamine (90:10 wt%) REV 0.1001 ± 0.0064 mg ml^{-1}. ■ Experimental points. ●, Theoretical points calculated as described in the text. The arrows denote the liposome sizes in the absence of serum.

Liposome integrity in the presence of serum

To investigate the integrity of liposomes in the presence of rat serum, SUV and REV liposomes were prepared encapsulating [^{14}C]glucose. Extraliposomal glucose was removed by gel filtration on a Sephadex G200 column and the liposome fractions mixed with rat serum incubated for 30 min at room temperature (approx. 23° C), and again subjected to gel filtration. The fractions eluted from the column were analysed for lipid ([^{3}H]DPPC), [^{14}C]glucose and for protein, and where possible particle sizes in the fractions were measured.

Results

The particles observed by photon correlation spectroscopy (PCS) in mixtures of liposomes and serum arise from the liposomes and their products of interaction with serum components, and from the particles present in the serum, principally proteins and lipoproteins. In order to evaluate the effects of serum on the particle sizes in mixtures of liposomes and serum, for each system studied, a theoretical curve of particle size (weight-average diameter ($\bar{d}_w$)) as a function serum concentration, as expressed in terms of total protein

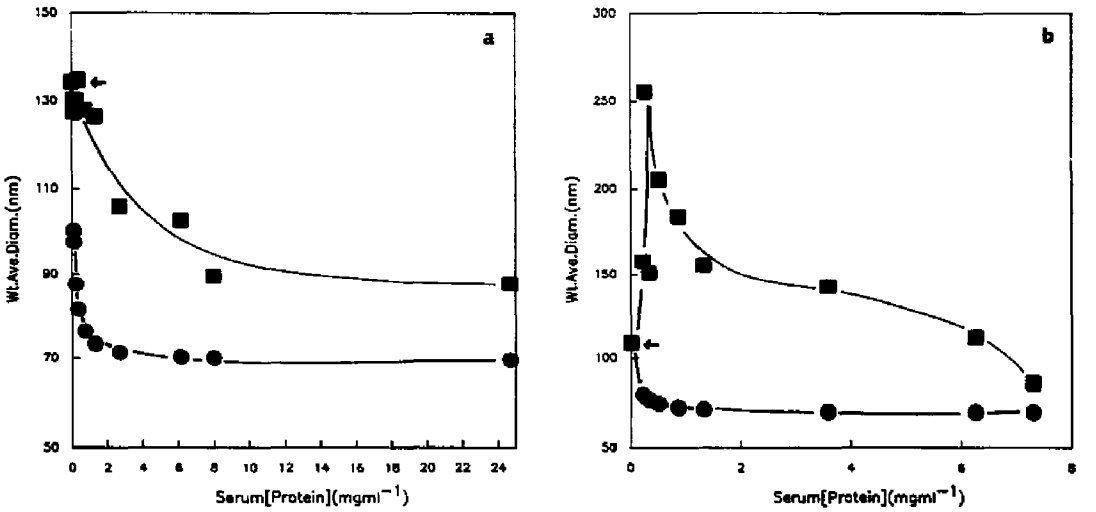

Fig. 2. Particle weight-average diameters ($\bar{d}_w$) in liposome-rat serum mixtures in Kreb-Henseleit buffer as a function of total serum protein concentration (a) DPPC SUV 0.0899 mg ml^{-1}. (b) DPPC-PI (50:50 wt%) SUV 0.0825 mg ml^{-1}. ■, Experimental points. ●, Theoretical points calculated as described in the text. The arrows denote the liposome sizes in the absence of serum.

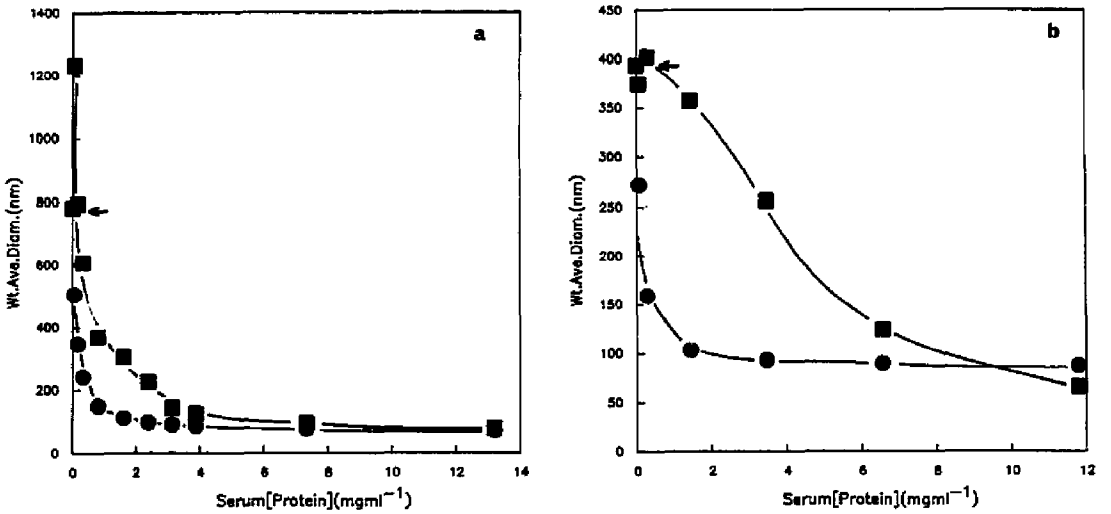

Fig. 3. Particle weight-average diameters ($\bar{d}_w$) in liposome-serum mixtures in Kreb-Henseleit buffer in the absence of calcium ions as a function of total serum protein concentration (a) Rat serum-cholesterol-rich REV 0.1044 ± 0.0064 mg ml^{-1} (DPPC-PI – cholesterol 47:6:47 wt%). (b) Human serum-DPPC-PI (50:50 wt%) REV 0.0881 ± 0.0065 mg ml^{-1}. ■, Experimental points. ●, Theoretical points calculated as described in the text. The arrows denote the liposome sizes in the absence of serum.

concentration in the serum, was calculated on the assumption that no interaction occurred.

Figs. 1 to 3 show typical data for the effects of serum concentration on the particle sizes in liposome suspensions covering a range of composition. Fig. 1 shows data for the effect of rat serum on negatively charged (DPPC-PI) and positively charged (DPPC-stearylamine) REV. Fig. 2 shows the effect of rat serum on DPPC and DPPC-PI SUV. The effect of rat serum on negatively charged cholesterol-rich (DPPC-PI-cholesterol) REV is shown in Fig. 3(a) and the effect of human serum on negatively charged (DPPC-PI) REV is shown in Fig. 3(b).

In each of the figures the particle size theoretically calculated for a mixture of liposomes and serum particles in the absence of interaction is also shown. In order to calculate the theoretical curves the particle size distribution in dilute serum was measured by PCS. Fig. 4 shows the typical particle size distributions for rat and human serum. For rat serum the weight-average particle diameter was found to be 69.3 ± 5.1 nm ($n = 6$) and for human serum 85.5 ± 3.7 nm ($n = 9$). In order to calculate the weight-average particle diameters in a mixture of liposomes, diameter $(\bar{d}_w)_L$, and serum particles, diameter $(\bar{d}_w)_S$, the weight concentrations of liposomes ([L]) and serum particles are required. The particles in serum include chylomicrons, very low, low and high-density lipoproteins together with serum proteins. Although protein is the major constituent by weight as the size measurements were related only to total serum protein ([P]) it is necessary to correct the protein weight concentration to the weight concentration of serum particles. For rat serum the weight concentration of lipoproteins is approximately 2.9 g l^{-1} and their protein content, taking into account the concentration of the different lipoproteins

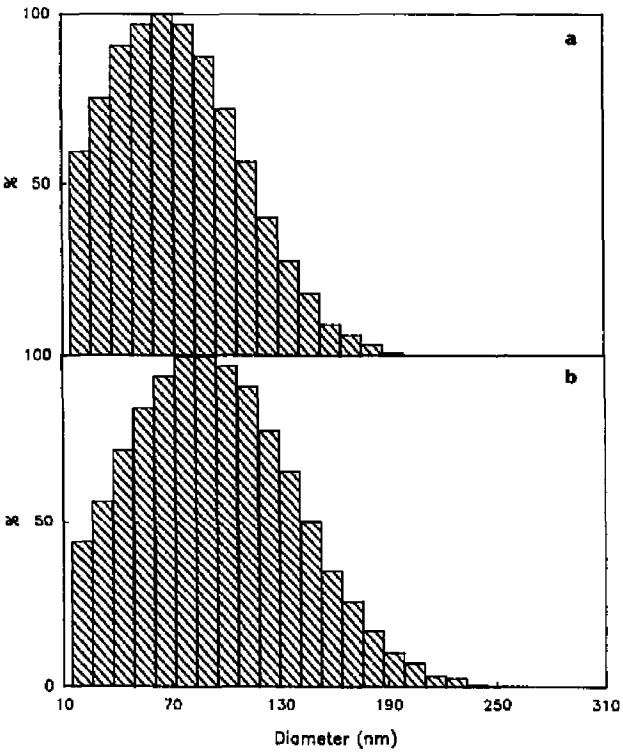

Fig. 4. Particle size distributions measured by photon correlation spectroscopy and fitted to an equivalent normal weight distribution for diluted rat (a) and human (b) blood serum. Total protein concentrations 2.37 mg ml^{-1} (rat) and 14.8 mg ml^{-1} (human).

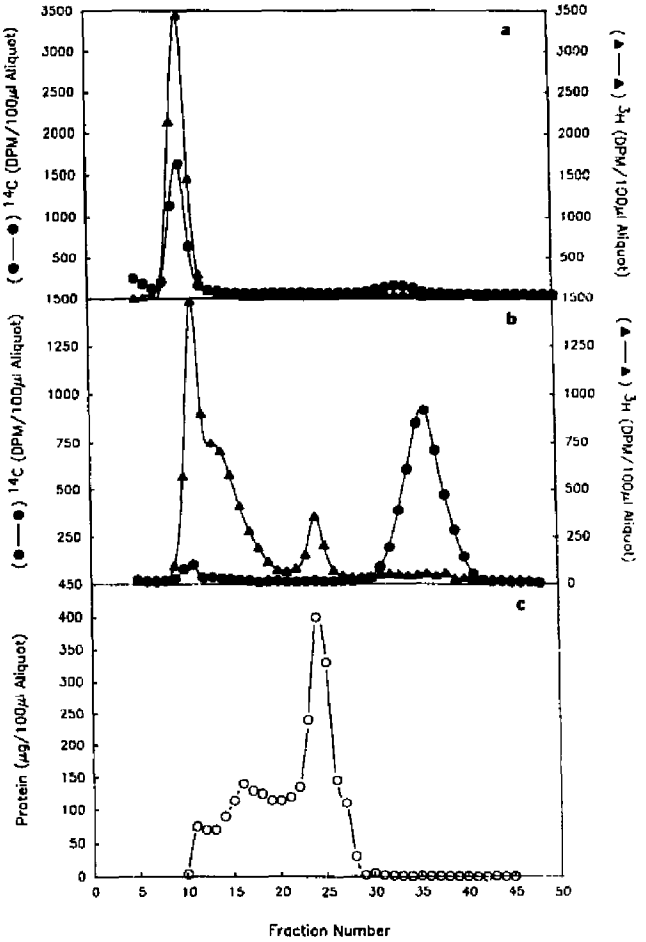

Fig. 5. The effect of rat serum on the elution profiles of DPPC-PI (50:50 wt%) REV encapsulating [^{14}C]glucose from a Sephadex G200 column. (a) Elution profile of REV ($\bar{d}_w$ = 421 nm); (▲) [^{3}H]DPPC; (●) [^{14}C]glucose. (b) and (c) Elution profile of REV fractions (9 + 10) from (a) plus rat serum (total protein concentration 23.4 mg ml^{-1}). ▲, [^{3}H]DPPC; ●, [^{14}C]glucose; ○, protein concentration.

present in serum and their respective protein levels, is 21% [23]. Our measurements of the total protein content gave 54 g l^{-1}. Thus, the weight concentration of particles in rat serum is given by 54 g l^{-1} plus 79% of 2.9 g l^{-1}, giving 56.3 g l^{-1} and hence the 'particle' weight concentration is 56.3/54 of the measured total protein concentration ([P]) or 1.043[P]. Hence the theoretical weight-average diameter ($\bar{d}_w$) of the liposome-rat serum mixture can be written

$$\bar{d}_w = \frac{\sum_i d_i w_i}{\sum_i w_i} = \frac{(\bar{d}_w)_L[L] + (\bar{d}_w)_S \cdot 1.043[P]}{[L] + 1.043[P]} \quad (4)$$

where w_i is the weight of species i. Similar calculations for human serum based on published data [23,24] gives

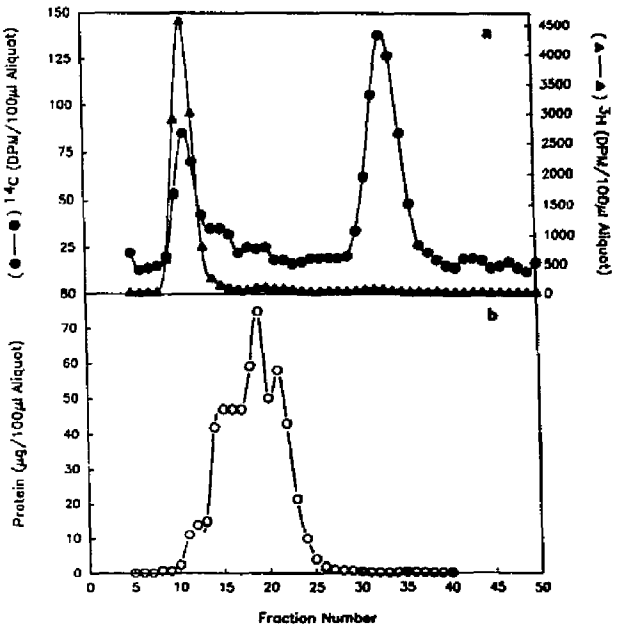

Fig. 6. The effect of rat serum (total protein concentration 2.37 mg ml^{-1}) on the elution profiles of DPPC-PI (50:50 wt%) REV encapsulating [^{14}C]glucose from a Sephadex G-200 column. (a) Elution profile of REV ($\bar{d}_w$ = 285 nm); ▲, [^{3}H]DPPC; ●, [^{14}C]glucose. (b) ○, Protein concentration.

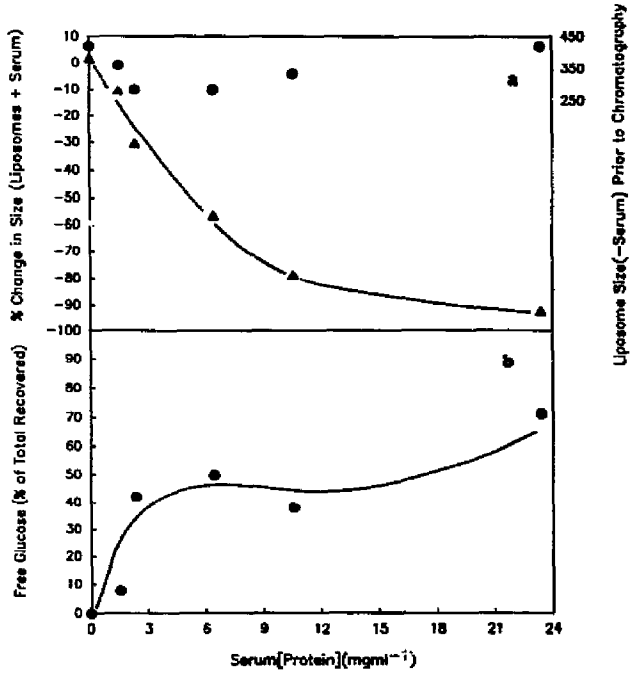

Fig. 7. (a) Percentage decrease in size (▲) of DPPC-PI (50:50 wt%) REV encapsulating [^{14}C]glucose after mixing with rat serum and eluting from a Sephadex G200 column. The initial sizes of the REV (●, $\bar{d}_w$ (nm)) before chromatography with serum are given on the right hand axis. (b) Percentage of total glucose released on addition of serum.

the following expression for liposome-human serum mixtures

$$\bar{d}_w = \frac{(\bar{d}_w)_L[L] + (\bar{d}_w)_S \cdot 1.029[P]}{[L] + 1.029[P]} \quad (5)$$

The numerical factors in Eqns. 4 and 5 are only best estimates, however, these corrections for non-proteinacious particulate material are only of the order of 3–4%.

From Figs. 1 to 3 it can be seen that for all the systems studied the measured weight-average diameters exceeded those calculated by the above method. For a few of the systems the addition of very low serum concentrations resulted in particle sizes exceeding those of the initial components (see, for example, Figs. 2(b) and 3(a)).

As Ca^{2+} ions were added to the liposome suspensions prior to size measurements to make up the Krebs-Henseleit buffer system and low levels of Ca^{2+} can lead to aggregation of DPPC-PI liposomes [25] some measurements were made in the absence of Ca^{2+} ions (see, for example, Fig. 3). The results were similar to those made in the presence of Ca^{2+} ions and confirmed that aggregation of the liposomes by Ca^{2+} ions was not the cause of the increases in particle sizes.

The effect of serum on the integrity of DPPC-PI (50 wt%) liposomes encapsulating [^{14}C]glucose was investigated by gel filtration. In these experiments the initial liposome preparations were divided into two aliquots and one aliquot was incubated with serum. Both aliquots were analysed by gel filtration (Sephadex G200) and the fractions analysed for lipid ([^{3}H]DPPC) and [^{14}C]glucose and in the case of the serum-treated aliquot for protein. Figs. 5 and 6 show the gel filtration profiles for DPPC-PI REV after incubation with high (Fig. 5) and low (Fig. 6) serum concentrations. Fig. 5(a) shows a typical preparation of REV encapsulating [^{14}C]glucose (i.e., coelution of liposomes and glucose). After incubation with a high concentration of serum (Fig. 5(b)) the [^{14}C]glucose is largely released and the lipid peak is shifted towards the inner volume, develops a shoulder and a smaller lipid peak develops between the remaining liposomal lipid and the released free [^{14}C]glucose. The protein profile (Fig. 5(c)) shows a major peak at the same elution volume as the small lipid peak suggesting that the lipid is associated with serum protein.

Fig. 6 shows the results of a similar experiment at a low concentration of serum protein. Fig. 6(a) shows that some liposomes encapsulating [^{14}C]glucose have remained intact and no small lipid peak in the inner volume is found. Similar experiments were carried out over a range of serum concentration and the size of the intact liposomes eluted from the Sephadex G200 column was measured together with the percentage release of [^{14}C]glucose. Fig. 7(a) shows the initial sizes (d_w(nm)) of the REV before addition of serum together with the percentage decrease in size after addition of serum and gel filtration. The corresponding release of encapsulated [^{14}C]glucose as a function of serum concentration is shown in Fig. 7(b). It is possible because of the low molar ratio of glucose to lipid ($\sim 10^{-5}$) in these experiments, that should the glucose adsorb to the liposomal bilayer the release of encapsulated glucose is underestimated although such adsorption is likely to be small.

Discussion

Because of the size of the components in blood serum relative to the size of REV the addition of serum to liposomes will result in a decrease in the average size of the particles in the mixture. However for all the REV investigated here the particle sizes in REV-serum mixtures exceed the sizes theoretically calculated for a simple non-interacting mixture of species particularly at low concentrations of serum corresponding to protein levels in the range 0 to 10 mg ml^{-1}. Positive deviations from ideal mixing of particles occur for both REV and SUV, independent of whether the liposomes are negatively charged (DPPC-PI), positively charged (DPPC-stearylamine) or cholesterol-rich. However, the deviations are larger for SUV (Fig. 2). It follows from these measurements that the liposomes are initially aggregated by addition of serum. Following or concomitant with aggregation the liposomes become leaky to encapsulated [^{14}C]glucose as confirmed by the gel filtration studies. If it is assumed that the amounts of the serum components mediating the initial aggregation process represent only a small proportion of the total serum, then the measured particle sizes can be used in Eqns. 4 and 5 above to calculate the apparent weight-average diameters of the liposome aggregates which, when divided by the initial weight-average diameters of the liposomes in the absence of serum will give apparent average aggregation numbers. Figs. 8 and 9 show result of such calculations for REV and SUV, respectively. For REV the curves go through maxima as the serum concentration is increased; a result consistent with concomitant processes of aggregation, possibly disintegration and disruption. The apparent aggregation numbers are relatively small for REV in contrast to those for SUV (Fig. 9). Furthermore the DPPC SUV do not appear to disrupt at high serum concentrations (Fig. 9(a)) and most probably retain encapsulated glucose more effectively.

When liposome-serum mixtures are subjected to gel filtration, depending on the serum concentration, a proportion of the liposomes remain intact (Figs. 5 and 6) and from the serum protein elution profile they have a relatively small amount of protein co-eluting with

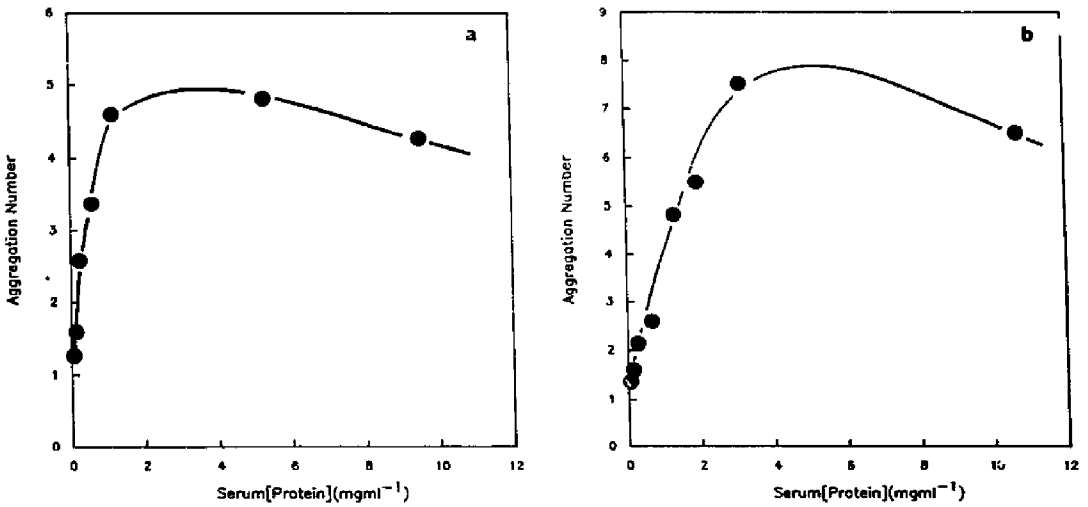

Fig. 8. Apparent aggregation numbers of liposomes as a function of rat serum concentration. (a) DPPC-PI (50:50 wt%) REV. (b) DPPC-stearylamine (90:10 wt%) REV.

them. Fig. 7(a) shows that the liposomes which remain intact in the presence of serum are the smaller ones as the size of the eluted intact liposomes decreases with increasing serum concentration. The release of encapsulated [^{14}C]glucose increases most steeply at low serum concentrations indicative of the preferential disruption of the larger liposomes in the distribution, which encapsulate more glucose, when the concentration of serum protein is limiting. Under the conditions of gel filtration serum protein will be separated from the liposomes so that the aggregates seen by photon correlation spectroscopy may dissociate to some degree however they will have already become leaky to glucose. It is interesting that at the higher serum concentrations (Fig. 5) a lipid leak appears in the inner volume of the Sephadex G200 column which coincides with the major peak of serum protein and a shoulder appears at the higher elution volume side of the liposome peak which also has associated serum protein. On the basis of a globular protein calibration the molecular weights of the protein-containing particles associated with the shoulder on the liposome peak and the small lipid peak in the inner volume are estimated to be in the region $(0.7–1.0)\cdot 10^6$ and 160000, respectively. The latter molecular weight is close to the lower end of the molecular weight range of high density lipoproteins ($1.7\cdot 10^5$–$3.6\cdot 10^5$ [26]), suggesting that liposomal lipid has become associated with serum components in the process of leakage of encapsulated [^{14}C]glucose. Another possibility is that the small peak

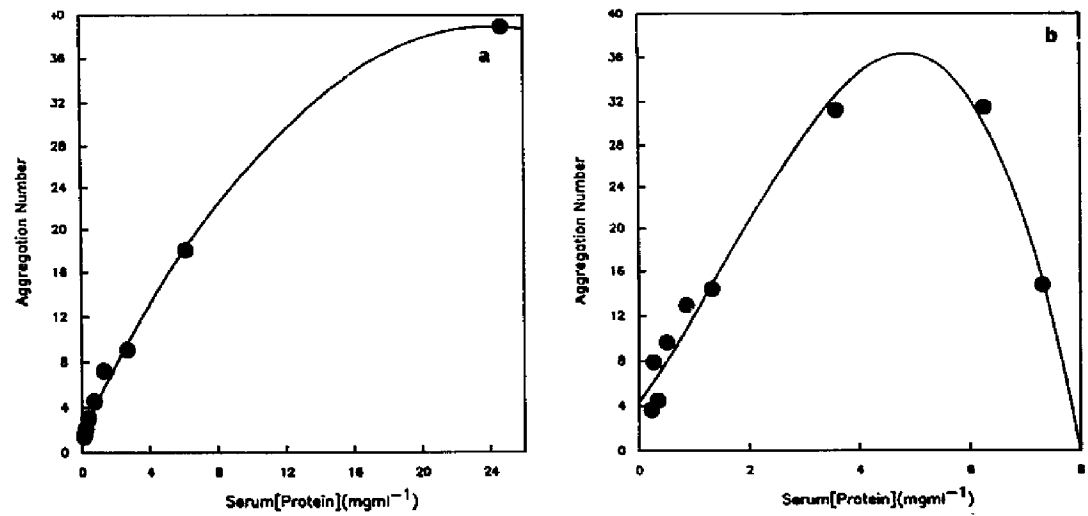

Fig. 9. Apparent aggregation numbers of liposomes as a function rat serum concentration. (a) DPPC SUV. (b) DPPC-PI (50:50 wt%) SUV.

arises from DPPC degradation to the lysophosphatidylcholine which binds to serum protein (specifically albumin) to form lipid-albumin complexes. It is significant, however, that even at the higher serum concentration a small proportion of liposomes remain intact (a small [^{14}C]glucose peak is still present in Fig. 5(b)) and that the bulk of the liposomal lipid elutes close to the void volume although it penetrates slightly more into the inner volume. The shoulder on the lipid peak reflects the change in the size distribution of the liposomes which accompanies loss of [^{14}C]glucose and possibly radioactive lipid that has exchanged with high density lipoprotein. While it is clear that serum components can remove some lipid from the liposomes and in so doing facilitate the release of encapsulated contents and that smaller liposomes are more stable to release of contents, it is unclear whether the release of contents from the larger liposomes in a distribution results in smaller intact liposomes or some other lipid state. Furthermore it is not possible to say whether interactions with serum components can cause release without total disruption although any such release if it occurs probably makes only a small contribution compared with release due to disruption [15].

Finally, in the context of our recent study of the effect of very low blood levels on the uptake of liposomes by rat liver [16] the perfusion constants for liposomes covering a range of lipid composition decrease rapidly at blood levels corresponding to haematocrits in the range 0 to 3% (serum protein levels in the range 0 to 3.5 mg ml^{-1}). In this serum protein range REV and SUV are aggregated by serum components and this work shows that REV, and to a lesser extent SUV, release encapsulated contents. However, the perfusion studies showed that there was no discernible dependence of perfusion constant on initial liposome size at least within the range of $\bar{d}_w$ from 40 to 400 nm. The rapid decrease in perfusion constant at low serum levels may arise from a reduced rate of uptake of liposomal aggregates which will pass more slowly through the liver sinusoids. Should this be the case higher perfusion constants might be expected at higher serum levels when the aggregates are dissociated, but we have found no evidence for such an effect. Alternatively the adsorption of serum components on liposomal surfaces may, to some degree, reduce their rate of uptake relative to 'naked' liposomes. The fact that the major changes in the rate of uptake by the liver occur at very low serum levels where liposome disruption is limited is consistent with uptake being mediated by adsorption of serum components to the liposomal surface.

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