Biochemical Pharmacology, Vol. 29, pp. 877–886.

0006-2952/80/0315–0877 $02.00/0

INTERACTION OF LIPOSOMES WITH CULTURED CELLS: EFFECT OF SERUM

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(*Received* 8 *August* 1978; *accepted* 28 *September* 1979)

Abstract—The binding of sonicated liposomes (small, mainly unilamellar) by cultured rat fibroblasts and mouse L1210 cells, in the presence or absence of serum, determined as μmoles phospholipid/mg cell protein, depends on the composition of the liposomes. Negatively charged solid liposomes (phospholipids below their phase transition temperature) were bound by cells several times more than fluid liposomes were under the same conditions. There was less binding, for each type of liposome studied, in the presence of serum. Time, liposome concentration and temperature effects suggest that liposomes interact with the cultured cells studied by a two-stage process: an initial rapid step, probably adsorptive, followed by a second slower step. Cell fractionation studies showed that solid and fluid liposome phospholipids were treated similarly by the cells and equilibrated mainly at densities similar to those of plasma membrane marker enzymes. Further, after incubations with inulin entrapped in liposomes, there was no accumulation of [^{3}H]inulin in lysosomal fractions. Whereas the data indicated that the liposomes studied were not endocytosed intact, there was also no evidence that 'fluid' liposomes rapidly fused with cells with their contents found in soluble fractions. Studies on liposomal permeability indicated, as has been suggested previously, that cholesterol is a key component for reducing the leakiness of liposomes in the presence of significant concentrations of serum. For short-term studies liposomes lacking cholesterol can be used for studies on cell binding or uptake of entrapped species, in the presence of serum, but in longer term studies the effects of serum make interpretation of data difficult in that type of liposome.

Liposomes (phospholipid vesicles) have been suggested as an *in vivo* drug delivery system, acting by targeting to specific sites [1, 2] and/or as a 'depot' or time release system [3, 4]. It is of interest to determine how liposomes interact with environmental constituents such as serum and cells in conditions approximating the *in vivo* situation. Several (not necessarily mutually exclusive) mechanisms of interaction between liposome components and cells *in vitro* and *in vivo* have been proposed: adsorption [5, 6], phospholipid exchange [7, 8], endocytosis [9–11] and fusion [8, 9, 12, 13]. At present there does not seem to be consensus as to which of these is the most important mechanism of liposome–cell interaction. Here we have compared the binding of several different types of liposomes with two quite different cultured cell types, mouse leukemia L1210 cells and rat fibroblasts, under standardized conditions. As it has been shown that the presence of serum may have marked effects on liposomes [3, 14–18], and thus on the possible interactions of liposomes and their constituents with cells, we have investigated the effects of serum on liposome integrity and on liposome interactions with cells.

We have also made studies on the subcellular localization of liposome constituents and of inulin entrapped inside several different types of liposomes. The aim of these experiments was to determine whether the intracellular distribution of liposomes is dependent on the liposomal lipid composition and whether inulin entrapped in different types of liposomes is distributed in cells differently from free (non-entrapped) inulin under similar experimental conditions.

MATERIALS AND METHODS

Phospholipids

Phosphatidyl serine (PS) (bovine brain) and phosphatidyl choline (PC) (egg lecithin) were obtained from Lipid Products Ltd. (South Nutfield, Surrey, England). These were >99 per cent pure as assayed by thin-layer chromatography (S. Frøkjaer, Novo Industri, Bagsvard, personal communication). Cholesterol (Chol) >99 per cent pure, synthetic distearoyl phosphatidyl choline (DSPC), dipalmitoyl phosphatidyl choline (DPPC) and dimyristoyl phosphatidyl choline (DMPC) were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). ^{14}C-Labeled-DSPC, specific activity 50 mCi/mmole, was obtained from Applied Science Laboratories (State College, PA, U.S.A.).

Markers for internal space

[^{3}H]Inulin, > 300 mCi/mmole, and [^{3}H]sucrose, 5 Ci/mmole, were obtained from the Amersham-Searle Co. (Amersham, Bucks, England).

Preparation of liposomes

Liposomes were prepared as described previously [4, 19, 20]. Five milliliters of aqueous solutions of [^{3}H]inulin or [^{3}H]sucrose in 1/10th strength PBS

(PBS: 136.8 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.2) were added to 100–300 μmoles dried lipids in 50 ml glass tubes. The molar ratios of the lipids used were as follows: PS/PC (1:4), PS/DMPC (1:4), PS/DSPC/DPPC (1:2:2), DSPC/DPPC (1:1) and PS/PC/Chol (1:4:5). [^{14}C]DSPC was used as a phospholipid marker at a molar ratio of labeled to unlabeled lipid of less than 1:100. The aqueous suspensions of lipids were vortexed for 10 min at 37° (PS/PC, PS/DMPC and PS/PC/Chol) or 50–54° (PS/DSPC/DPPC and DSPC/DPPC) and then were sonicated for twice the clearing time in a bath type sonicator (model G11 2 SPIT, Lab. Supplies Co. Inc., Hicksville, NY). After sonication, the molarity of the suspension was adjusted to normal strength PBS, and the suspensions were centrifuged at 130,000 *g* for 1 hr at 4°. Less than 10 per cent of the radioactivity pelleted, for all types of liposome. The supernatant fraction could be used directly as a liposome preparation where no internal marker had been entrapped; these liposomes are small, <50 nm in diameter, and mainly unilamellar (SUV) [18]. Separation of non-entrapped substances from liposomes was accom-

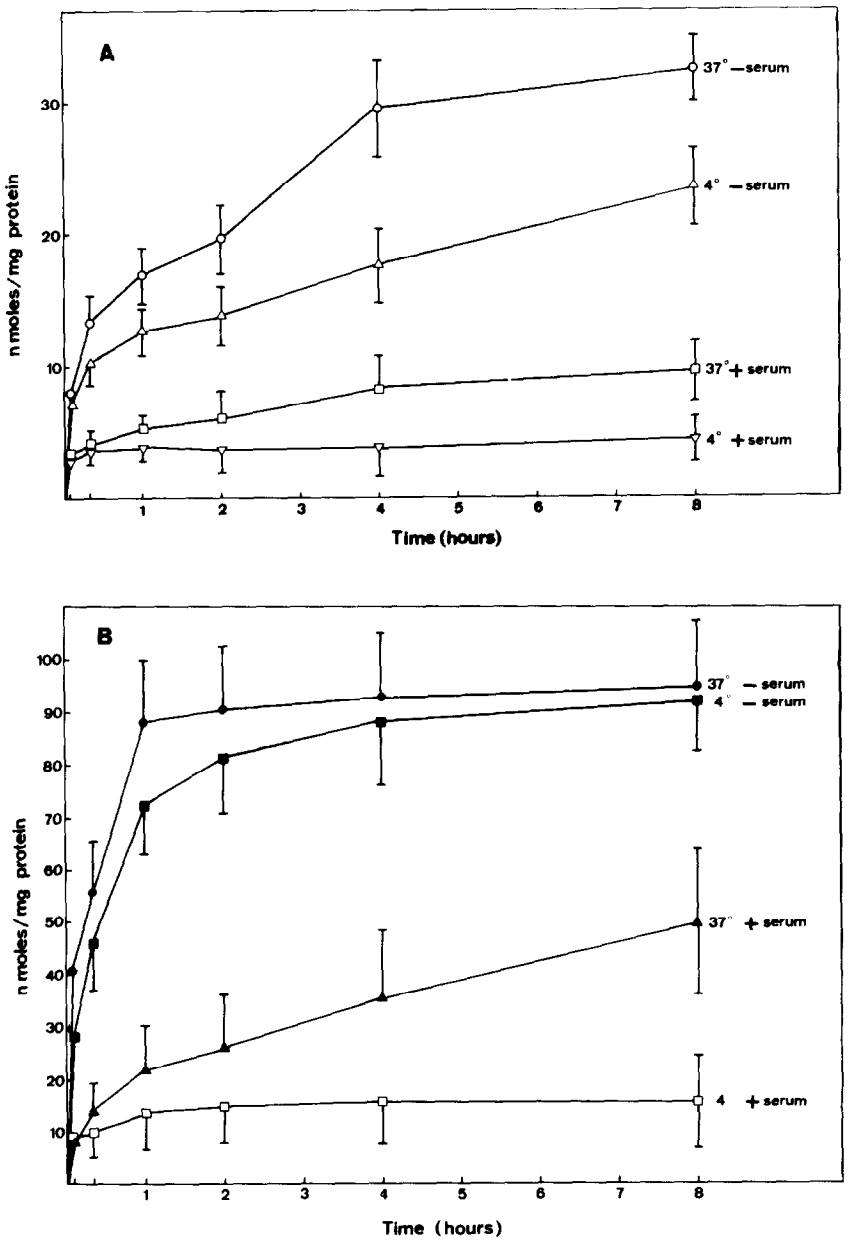

Fig. 1. Panel A: Binding of PS/PC liposomes by L1210 cells. Binding was carried out as described in Materials and Methods, except uptake was determined at 4° and 37°. The initial external concentration was 1 μmole phospholipid/ml. Vertical lines represent ± standard deviation. Key: (○——○) 37°, no serum; (△——△) 4°, no serum; (□——□) 37°, with 10% serum; and (▽——▽) 4°, with 10% serum. Panel B: Binding of PS/DSPC/DPPC liposomes by L1210 cells. Binding was carried out as described in the legend to panel A. Key: (●——●) 37°, no serum; (■——■) 4°, no serum; (▲——▲) 37°, with 10% serum; and (□——□) 4°, with 10% serum.

plished either by repeated dialysis against a large excess of PBS (sucrose-liposomes) or by passage of suspensions through Sephadex G-200 columns (inulin-liposomes). Liposomes eluted in the void volume, whereas non-entrapped solute eluted in later fractions. Liposome-containing fractions were pooled before use. Approximately 1–2 per cent of the original total sucrose or inulin was entrapped by SUV liposomes, indicating that these substances were probably trapped passively in the internal liposomal space.

Cells

L1210 cells. L1210 mouse leukemia cells were maintained in suspension culture in RPMI medium 1640, supplemented with 10% heat-inactivated fetal calf serum. Cells from the maintenance culture were pelleted at 250 *g* for 5 min and washed twice in fresh medium (without serum). The final pellet was resuspended in fresh medium with or without serum, depending on the experimental conditions, at 1–3 × 10^6 cells/ml in a volume of 0.5–2 ml. Liposomes and other substances were added in a maximum total volume of 0.1 ml. Cells were equilibrated for 10 min at the temperature of the experiment before addition of liposomes. After incubation, cells were washed by addition of 2.5 ml PBS (at 4°) and were centrifuged immediately at 1500 *g* for 5 min; the pellet was washed three times in 5 ml PBS by centrifugation in the same way. Finally, 1 ml of 0.5 N NaOH was added and aliquots were removed for protein [21] and radioactivity determinations. Dimuline (Packard, Downers Grove, IL, U.S.A.) was used as the counting fluid. All determinations in each experiment were made in triplicate.

Rat fibroblasts. Rat fibroblasts were isolated from 17-day-old rat embryos, as described by Tulkens *et al.* [22]. Primary cultures were set up in Roux bottles (~ 180 cm^2 surface area) in modified Eagle's medium supplemented with 10% newborn calf serum and were subcultured at confluency by trypsinization to 75 cm^2 or 150 cm^2 T flasks. Cells were used only from the first or second subculture, when they had just reached confluency but were not piled up. For liposome binding studies, cells grown in 75 cm^2 T flasks were washed several times with 10–5 ml of media with or without serum. Liposomes were then added to make a final concentration of 1 μmole/ml. of phospholipid. After incubation the cells were washed three times with PBS and dissolved in 1 or 2 ml of 0.5 M NaOH. Binding of liposomes by fibroblasts at various temperatures was studied by floating the T flasks on water in baths kept at the appropriate temperature. For cell fractionation studies, cells were washed three times with PBS, incubated for 10 min at 4° with 0.02% Na_2 EDTA (w/v) in PBS, detached by gentle scraping, collected by centrifugation, and resuspended in 0.25 M sucrose. Isopycnic centrifugation in sucrose density gradients of post-nuclear supernatant fractions, and enzymatic and chemical assays were performed as described by Tulkens *et al.* [22]. Other assays carried out were of cathepsin B [23] and phosphoglucomutase [24].

RESULTS AND DISCUSSION

Kinetics of uptake and concentration dependency

The data shown in the charts and tables were calculated as μmoles phopholipid bound/mg cell pro-

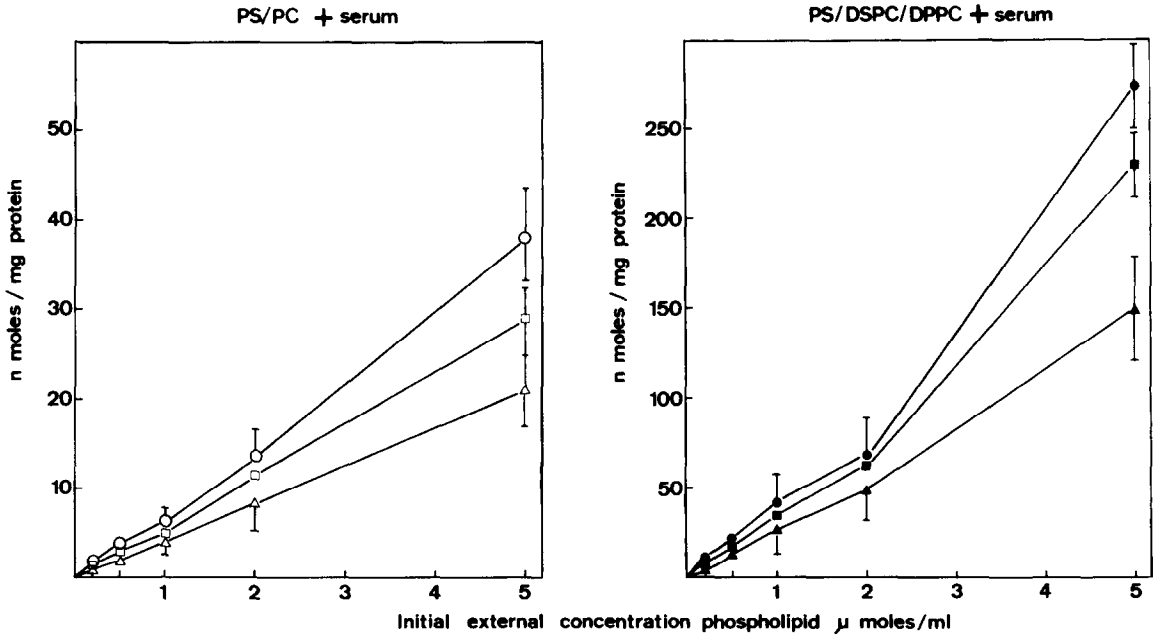

Fig. 2. Effects of variation in external concentration of liposomes on binding by L1210 cells. The temperatures of incubation was 37° in the presence of 10% serum. Key: (○——○) PS/PC liposomes, 4 hr incubation; (□——□) PS/PC liposomes, 2 hr incubation; (△——△) PS/PC lipsomes, 1 hr incubation; (●——●) PS/DSPC/DPPC liposomes, 4 hr incubation; (■——■) PS/DSPC/DPPC liposomes, 2 hr incubation; and (▲——▲) PS/DSPC/DPPC liposomes, 1 hr incubation.

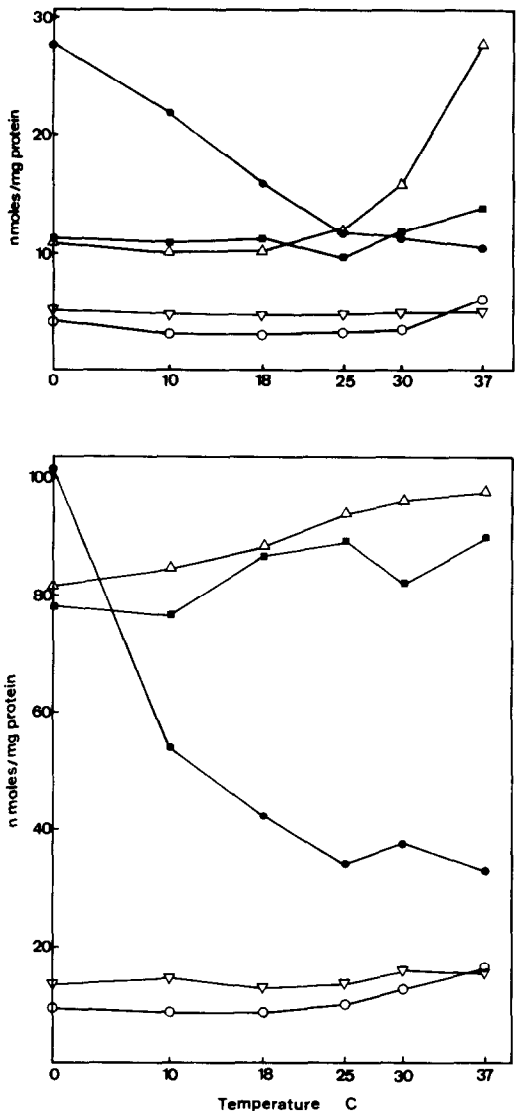

Fig. 3. Top panel: Effect of temperature on binding of liposomes by L1210 cells (with 10% serum). Binding was carried out as described in Materials and Methods. The initial external concentration of liposomes lipid was 1 μmole/ml. Key: (△) PS/DSPC/DPPC; (■) DSPC/DPPC; (●) PS/DMPC; (○) PS/PC; and (▽)PS/PC/Chol.
Bottom panel: Effect of temperature on binding of liposomes by L1210 cells (without serum). Binding was carried out as described in Materials and Methods. The initial external concentration of liposomes lipid was 1 μmole/ml. Key: (△) PS/DSPC/DPPC; (■) DSPC/DPPC; (●) PS/DMPC; (○) PS/PC; and (▽) PS/PC/Chol.

tein, enabling comparisons to be made between different experimental conditions, assuming that the association of [^{14}C]DSPC is a valid marker of phospholipid binding by the cells [13]. Figure 1 shows the time course of binding of phospholipid by L1210 cells. For both PS/PC and PS/DSPC/DPPC liposomes, in the presence or absence of serum, there was a rapid initial binding phase. Several times more phospholipid was bound in the absence of serum than in its presence, for both types of liposome [16, 17]. There was also several times more binding of PS/DSPC/DPPC phospholipid than of PS/PC, under similar conditions. These differences were maintained over a more prolonged time span. Since these types of liposomes have a similar amount of PS imparting a similar negative charge, the key difference in cell binding properties is probably related to the difference in the median phase transition temperature (T_m) of the lipids, the PS/PC and the PS/DSPC/DPPC lipids being, respectively, fluid and solid between 4 and 37° (Ref. 9, S. Frøkjaer, personal communication). The time course experiments suggest that for both types of liposome after the initial rapid binding phase there followed a slower phase which continued for at least 8 hr. It can also be seen that there was an initial rapid binding of phospholipid at 4° similar to that occurring at 37° in both the presence or absence of serum. In the absence of serum, the binding of PS/DSPC/DPPC liposomes was very similar at 37° or 4° after a few hours of incubation, whereas with PS/PC liposomes the difference in binding between 37 and 4° was maintained. In the presence of serum for PS/PC and PS/DSPC/DPPC liposomes, the binding appeared to reach a plateau at 4°, whereas at 37° the binding continued to increase. The difference between binding at 37° and 4° was particularly strong for PS/DSPC/DPPC liposomes in the presence of serum after several hours of incubation. Figure 2 shows that, after incubation with PS/PC and PS/DSPC/DPPC liposomes in the presence of serum, the binding of radioactive phospholipid was directly proportional to the initial external concentration, and no overall saturation was observed under the conditions used. These data suggest that the net phospholipid binding is not saturable for L1210 cells at the concentrations used.

Similar studies have been carried out for rat fibroblasts. In general, the time course studies, and the variation of external concentration and temperature studies made with rat fibroblasts showed the same uptake patterns as for L1210 cells, although the binding was less under the same experimental conditions.

Five different liposome compositions were tested under similar conditions (PS/PC, PS/PC/Chol, PS/DMPC, PS/DSPC/DPPC and DSPC/DPPC liposomes), and the relative uptake rates in L1210 cells and rat fibroblasts were much the same, solid liposomes being taken up to a higher degree than fluid ones.

With fibroblasts, the binding of these liposomes to the cells was reduced by the presence of serum in much the same way as with L1210 cells. One of the possible factors with regard to the quantitative differences between the two cell types could be the inaccessibility to liposomes of the fibroblast plasma membranes facing the support in contrast to the full accessibility of L1210 membranes.

Temperature effects

Lowering of temperature may affect association of different liposomes to cells in two ways, first by inhibiting metabolism-dependent uptake mechanisms (i.e. endocytosis) and second by reducing fluidity of both liposome and cell membranes. Here we compared the binding of five different types of lipo-

Table 1. Effects of trypsin and EDTA on liposome binding by L1210 cells*

		PS/DMPC			DSPC/DPPC	
		nmoles phospholipid/mg protein				
	Treatment	1	2	Decrease (%)	nmoles phospholipid/mg protein	Decrease (%)
No serum	Control†	30.4	27.8		104.4	
	EDTA‡	18.2		40	71.8	31
	Trypsin§		15.3	45	22.3	79
10% serum	Control	11.2	12.8		16.7	
	EDTA	9.4		16	12.3	26
	Trypsin		11.8	8	7.1	57

* L1210 cells were incubated in RPMI 1640 in the presence or absence of 10% serum for 1 hr with [^{14}C]PS/DMPC or [^{14}C]-DSPC/DPPC liposomes at 37°. The cells were washed three times in PBS and were resuspended in 1 ml PBS. One ml of PBS (controls, †), 0.02% EDTA in PBS (‡) or 0.02% Trypsin (GIBCO) in PBS (§) was added and the suspension incubated with continuous agitation for 5 min. The cell suspensions were centrifuged at 250 *g* for 5 min and washed three times in PBS. Determinations of radioactivity and protein in the cell pellet were made.

somes in the range of 0–37°, after a 1-hr incubation in the presence or absence of serum. Without serum (Fig. 3, bottom panel), the cellular uptake of PS/DSPC/DPPC, DSPC/DPPC, PS/PC and PS/PC/Chol liposomes as a function of increasing temperature, if changed at all, showed a small increase. In contrast, PS/DMPC liposomes showed a significant decrease in binding with increasing temperature [6].

In the presence of serum (Fig. 3, top panel), there was a more marked increase in binding of PS/DSPC/DPPC phospholipid with increasing temperature, while binding of PS/DMPC liposomes decreased with temperature as in the absence of serum. The temperature-dependent increase of PS/DSPC/DPPC binding and the different clearance rates, i.e. slopes for rate of uptake, at 4 and 37° (Fig. 1) could suggest a metabolically linked effect on the extent of binding.

The much higher binding of 'solid' liposomes (DSPC/DPPC and PS/DSPC/DPPC) in comparison with 'fluid' liposomes (PS/PC and PS/PC/Chol) throughout the temperature range studied suggests an important role of liposome membrane fluidity in the initial binding to cells. This is further substantiated by the behavior of the PS/DMPC liposomes, which were the only type of liposomes used with a phase transition temperature between 0 and 37°. These liposomes showed increased binding with increasing solidity, i.e. decreasing the incubation temperature below 21°, the phase transition temperature of DMPC [18, 25] which is the highest melting component.

Fluidity seems also to determine the uptake after the initial association, as seen from Fig. 1. After prolonged incubations of cells in the presence of liposomes at 37° and 4°, small differences 1 hr after incubation become larger differences, as metabolically dependent binding continues. These data show that the apparently conflicting results obtained previously [6, 9] can be reconciled to some extent. It is possible for different temperature effects to be observed for different liposomes, but only when the data are expressed in a similar manner can the effects be seen to be clearly due to changes in solidity and fluidity of the liposomes [6]. The present results with L1210 cells do not show a sharp temperature-dependent increase in binding for fluid liposomes at around 20° in the absence of serum, as reported for 3T3 cells [9] and murine lymphocytes [8].

Effects of EDTA and trypsin on cell-bound liposomes

Table 1 shows that a significant proportion of bound phospholipid or cell-associated radioactive label was susceptible to removal from cells by EDTA and trypsin concentrations that would not have damaged the cells. For DSPC/DPPC liposomes, more material was susceptible to trypsin than EDTA in the presence or absence of serum, whereas for PS/DMPC liposomes approximately similar amounts were removed by both agents. In the presence of serum quite small amounts of PS/DMPC liposomes were removed by either agent; most bound radioactive material was inaccessible to the agent. These results are similar to those found by Pagano and Takeichi [6] and support the idea that there are at least two types of initial binding for liposomes at cell surfaces, i.e. EDTA/trypsin sensitive and insensitive binding.

Permeability of liposomes in the presence of serum

Different substances trapped in liposomes leak out at different rates depending on the type of substance and the composition of the liposome [18, 26, 27] and on the presence of serum [15, 28]. However, for studies on cellular uptake of liposomes it is important to check whether the entrapped marker molecules stay inside the liposome present in the culture medium. For three liposome compositions used later on, we studied the effects of serum on the leakage of untrapped [^{3}H]sucrose by equilibrium dialysis.

Figure 4A shows the effects of different concentrations of serum on sucrose efflux from PS/PC, PS/DSPC/DPPC and PS/PC/Chol liposomes. At 2 hr, virtually all 'free' sucrose had leaked out of the dialysis bags. The loss of [^{3}H]sucrose from Triton X-100-treated liposomes was the same as for 'free' sucrose, indicating that the integrity of the liposomes

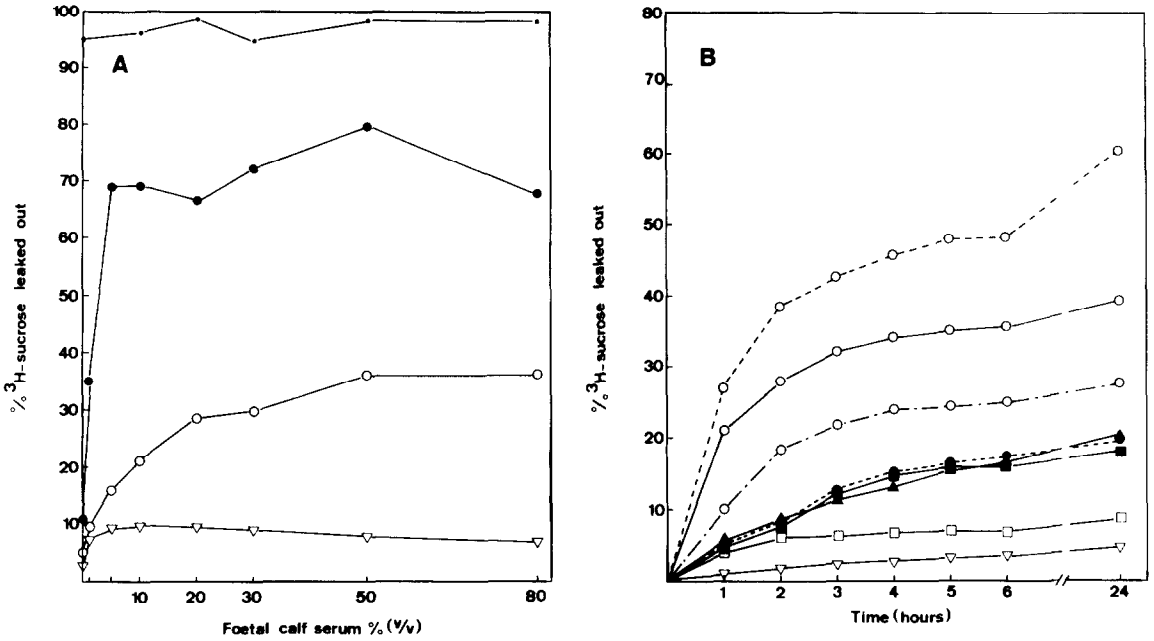

Fig. 4. Panel A: Sucrose leak from liposomes in presence of serum (variation of serum concentration). Liposomes with entrapped sucrose were prepared as described in Materials and Methods. Before incubation with serum, liposomes were dialyzed against PBS for 1 hr at 37°. A series of dialysis bags (0.5 cm diameter) was set up in 15 ml tubes containing 10 ml PBS at 37°. Into bags were added 0.1 ml liposome and varying amounts of heat deactivated fetal calf serum and PBS, or PBS and 1/1000 Triton X to give a final total volume of 1 ml. The final liposome concentration varied from 1 to 2 μmoles phospholipid/ml. As a control, some bags received non-entrapped [^{3}H]sucrose and serum. The tubes were incubated at 37° with continuous agitation. At 2 hr samples were taken from the dialysate, and the radioactivity was determined. The free sucrose was calculated assuming a total volume of 11 ml. Knowing the original radioactivity added to the dialysis bags, the amount leaked out of the liposomes could be calculated. The sucrose leak from up to twenty dialysis bags could be determined in one experiment. The serum concentration indicated in the figure is the final concentration (v/v). Key: (·——·) free sucrose; (●——●) PS/DSPC/DPPC–sucrose liposomes; (○——○) PS/PC–sucrose liposomes; and (▽——▽) PS/PC/Chol–sucrose liposomes.
Panel B: Sucrose leak from liposomes in the presence of serum (time course). The experiments were carried out as described in the legend to Fig. 4A, except that samples (0.1 ml) were taken from the dialysate at various times for up to 24 hr. Results were calculated in the same way. Key: (○- - -○) PS/PC–sucrose liposomes, 80% serum; (○——○) PS/PC–sucrose liposomes, 30% serum; (○-·-○) PS/PC–sucrose liposomes, 10% serum; (□——□) PS/PC–sucrose liposomes, 0% serum; (●- - -●) PS/PC/Chol–sucrose liposomes, 80% serum; (■——■) PS/PC/Chol–sucrose liposomes, 30% serum; (▲——▲) PS/PC/Chol–sucrose liposomes, 10% serum; and (▽——▽) PS/PC/Chol–sucrose liposomes, 0% serum.

was completely disrupted by this detergent. When the three types of liposomes were incubated with serum, PS/DSPC/DPPC liposomes were the most susceptible to serum, PS/PC liposomes showed intermediate effects and PS/PC/Chol liposomes were the least affected.

The 'solid' liposomes were highly susceptible to interaction with serum, which caused a maximal effect at a 5% concentration. The high leakiness of the PS/DSPC/DPPC liposomes may be related to the presence of a small amount of their lipids being in the fluid state, since their phase-transition temperature is close to 37°. As shown before, liposomes at their phase-transition temperature are generally more leaky for passively trapped substances, even in the absence of serum [18]. On the other hand, PS/PC/Chol liposomes lost less than 10% sucrose in 80% serum in 2 hr and there was not very much difference between the effects of low and high serum concentrations, whereas the PS/PC liposomes showed serum concentration-dependent effects. The time dependence of the sucrose leakage is illustrated in Fig. 4B.

In similar studies made on [^{3}H]inulin trapped in the same three types of liposomes, essentially the same results were obtained, i.e. rapid loss of inulin from PS/DSPC/DPPC liposomes in the presence of serum, intermediate loss from PS/PC liposomes, and very little loss from PS/PC/Chol liposomes, even though inulin has about twenty times the molecular weight of sucrose.

Complement does not influence sucrose leakage since similar results were obtained using non-inactivated fetal calf serum and fresh rat serum. At first sight we cannot distinguish between leakage from still intact liposomes and destruction of liposomes. The latter mechanism seems the most probable for PS/PC liposomes, since studies using a separation of

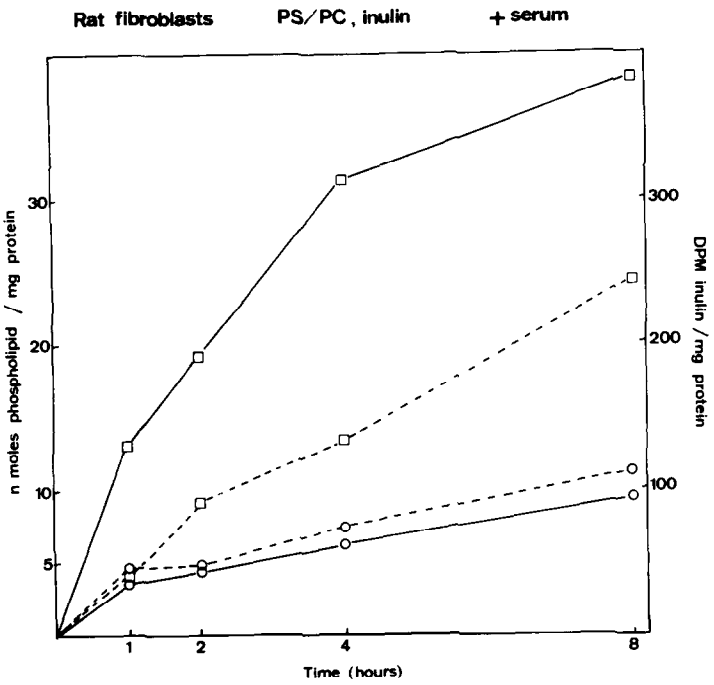

Fig. 5. Binding of 'free' inulin and inulin entrapped in PS/PC liposomes by rat fibroblasts. Ordinates are nmoles phospholipid/mg protein (left) and d.p.m. inulin/mg protein (right). Key: (□——□) [^{3}H]inulin (entrapped in PS/PC liposomes); (□----□) [^{3}H]inulin ('free') at same time as 'empty' PS/PC liposomes; (○----○) [^{14}C]PS/PC liposomes ('empty'); and (○——○) [^{14}C]PS/PC liposomes with entrapped [^{3}H]inulin.

the serum–liposome mixture on a Sepharose 4B column have shown a net transfer of phospholipids to a serum protein fraction concomitant with the release of internal marker (S. Frøkjaer, personal communication).

Uptake of liposomes with entrapped [^{3}H]*inulin*

In order to study cellular uptake of liposomes, we have used inulin as an internal marker because it is trapped by negative liposomes in the internal space and there is no evidence of external binding or association with the lipid bilayer [7]. Moreover, free inulin is taken up by cells by endocytosis [29]. Figure 5 shows that the uptake of inulin increased when rat fibroblasts were incubated with inulin trapped in liposomes, as compared to the uptake of 'free' inulin in the presence of 'empty' liposomes. Calculations of the lipid/inulin ratio shows that the cells bound in approximately the same ratio of [^{14}C]lipid to [^{3}H]inulin as was present in the original liposome preparation. This suggests that the increased binding of inulin by the cells was due to inulin being bound by the cells while still entrapped in the liposomes. Part of the cell-bound inulin could result, however, from an uptake of free inulin which had leaked out of the liposomes, because the data in the previous section shows that in the presence of 10% serum approximately 25 per cent of the entrapped inulin had leaked out of the PS/PC liposomes by 8 hr.

Subcellular localization of liposome phospholipid and free or entrapped inulin in rat fibroblasts

Fibroblasts were incubated for 2 or 24 hr at 37° with ^{14}C-labeled liposomes and [^{3}H]inulin (free or entrapped in the vesicles) and were then washed, homogenized and fractionated by isopycnic centrifugation. The density distributions of ^{14}C lipid and [^{3}H]inulin, and of phosphoglucomutase, 5′-nucleotidase, and cathepsin B, which are marker enzymes of, respectively cytosol, plasma membrane and lysosomes, are presented in Figs. 6 and 7. Other enzymes or constituents were also assayed (*N*-acetyl-β-glucosaminidase, cytochrome *c* oxidase, NADPH-cytochrome *c* reductase, RNA, protein), but their distributions were not significantly different from previous results [22].

Free inulin after 2 hr of incubation (Fig. 6) showed a broad density distribution with slightly higher concentrations at densities with maximal 5′-nucleotidase activity than at densities for phosphoglucomutase and cathepsin B maximal activities. After 24 hr of incubation (Fig. 7), more inulin was found at densities for maximal cathepsin B activity. This shift in distribution to higher densities is compatible with an uptake by endocytosis [29]: initially, inulin associates with plasma membranes/phagosomes and is subsequently transferred to and accumulated in lysosomes.

The phospholipid radioactivity distribution after 2 hr of incubation with PS/PC liposomes, with or without inulin entrapped (Fig. 6), showed a major peak at densities equal to or slightly higher than those for the 5′-nucleotidase peak. Trapped inulin (Fig. 6, row B) distributed similarly to the phospholipids. Essentially similar results were obtained with PS/DSPC/DPPC liposomes containing entrapped inulin after 2 hr of incubation (Fig. 6, row C). Longer

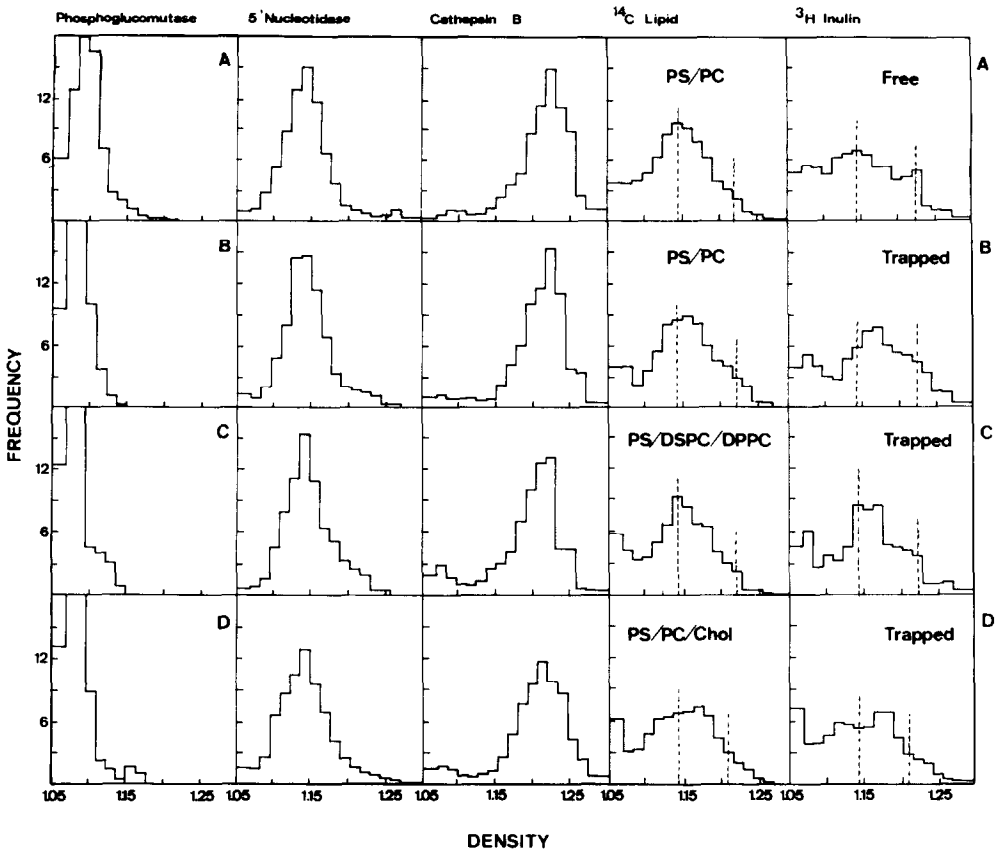

Fig. 6. Distribution patterns of enzyme markers, [^{14}C]lipid and [^{3}H]inulin of rat fibroblasts after 2 hr of incubation with liposomes. Cells were fractionated by isopycnic centrifugation in a linear sucrose gradient, and results were plotted as normalized histograms [30]. Abscissa: density scale divided into fifteen equal sections of density increment from 1.07 to 1.27. Ordinate: frequency ($\Delta Q/\Delta g$), where Q is the amount of constituent present in each fraction and g is the sum of the amounts found in all the subfractions. Column 1: phosphoglucomutase; 2: cathepsin B; 3: 5′-nucleotidase; 4: [^{14}C]phospholipid; and 5: [^{3}H]inulin. Cells were incubated for 2 hr with Row A: PS/PC liposomes plus free inulin; Row B: PS/PC liposomes with entrapped inulin; Row C: PS/DSPC/DPPC liposomes with entrapped inulin; and Row D: PS/PC/Chol liposomes with entrapped inulin.

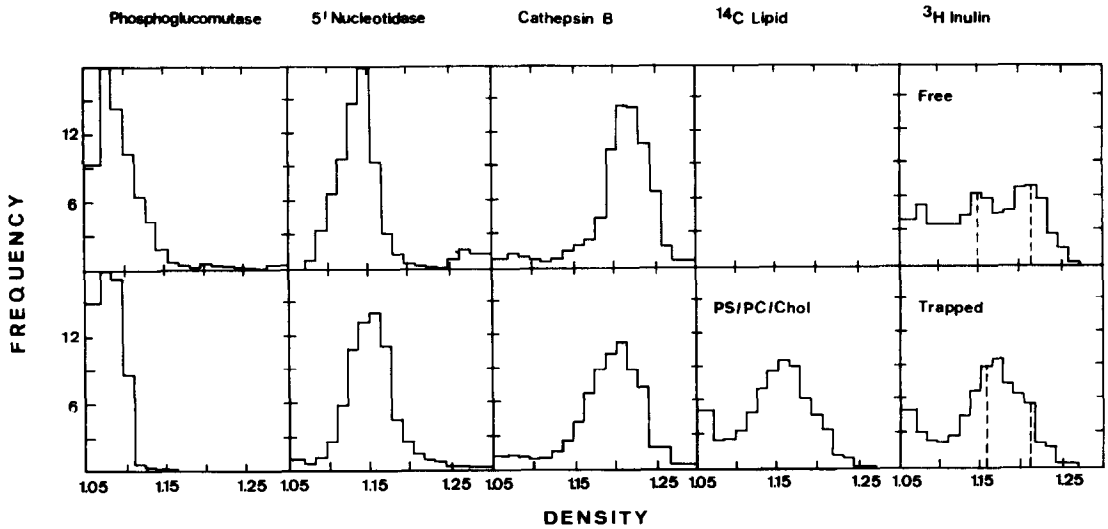

Fig. 7. Distribution pattern of enzyme markers, [^{14}C]phospholipid and [^{3}H]inulin of rat fibroblasts after 24 hr of incubation. Plots were made as described in the legend to Fig. 6. Top row : cells were incubated for 24 hr with free inulin; and bottom row cells were incubated for 24 hr with PS/PC/Cholesterol liposomes containing entrapped [^{3}H]inulin.

incubation periods with PS/PC and PS/DSPC/DPPC liposomes with inulin entrapped were not tried due to the serum-induced leakage of the internal marker.

The PS/PC/Chol liposomes containing inulin were incubated for 2 or 24 hr. At 24 hr and to a lesser extent at 2 hr the phospholipid distribution showed a broad peak at densities around the median 5'-nucleotidase activity; the distribution of inulin coincided with that of the lipid. Quantitatively, five times more liposomal lipid became associated with the fibroblasts after 24 hr of incubation, as compared to 2 hr. The entrapped inulin distributed differently from free inulin, and showed little accumulation at densities with maximal lysosomal enzyme activity.

Whereas uptake of free inulin could be due to endocytosis, this does not seem to be the case for the liposomes studied, because the initial association with plasma membranes/phagosomes was not followed by transfer to and accumulation in lysosomes of either liposomal lipid or entrapped inulin. In 24 hr the phospholipids could have been recycled from lysosomes into other membrane structures, thereby masking a possible endocytotic uptake; however, the inulin would in any case have been left in the lysosomes.

On the other hand, our results lend little support to the idea of an uptake by fusion, by which the initial plasma membrane association of lipid and internal marker would be followed by transfer of the entrapped compound into the cytosol. Though small amounts of the internal marker were found in the lightest fractions of the gradients, this was always balanced by similar amounts of lipid marker, and could be due to intact liposomes somehow being released from subcellular structures during the homogenization and fractionation procedures. Control experiments showed that intact liposomes did not adsorb to subcelluar structures and floated to the top of the gradient during centrifugation. This release could not be due to significant breakage of lysosomes since very little lysosomal enzyme (cathepsin B) remained at the top of the gradient. Leakage from broken, newly formed endocytotic vesicles cannot be excluded, since we had no marker for these structures. A third possibility could be release of intact liposomes adsorbed to plasma membranes. Quantitatively, this could only have been of minor importance since the most loosely bound liposomes (60–80 per cent) had already been removed by EDTA-treatment which detached the fibroblasts before the homogenization.

Because neither fusion nor endocytosis seems to have been a major importance in determining the total association of lipid and internal markers with the cells, stable adsorption may account to a large extent for the observed subcellular localizations.

Phospholipid exchange or leaky fusion [7] between liposomes and cells may contribute to the association of lipid marker with cells. Since in all of these cases the radioactive liposomal lipids may enter the phospholipid pool of the cells, studies are presently underway to determine the chemical nature after binding of the membrane lipid marker.

In conclusion, the results shown here indicate that cells interact differently with 'solid' and 'fluid' liposomes in the initial binding phase, resulting in increased binding of solid liposomes, in the presence or absence of serum, and that serum inhibits the initial binding of all types of SUV liposomes studied. The increased initial binding of solid liposomes is probably adsorptive since large amounts can be released again with trypsin or EDTA after short incubations.

Subcellular localization studies did no indicate that liposomes were endocytosed intact in rat fibroblasts in which endocytosis has been clearly shown to occur [31]. However, other cells, such as macrophages and liver cells, have been shown to endocytose liposomes [9, 32, 33]. The possible fusion between liposomes and plasma membrane lipids is not excluded by the phospholipid distribution data, but the key to proving this mode of interaction is a demonstration that the entrapped marker is initially free in the soluble cytoplasmic fractions. If endocytosis and/or fusion occur, the characteristics of these processes must be clearly distinguished from absorption of intact liposomes to cell surfaces with subsequent leakage of entrapped substances.

Since the main biological interest in liposomes is as drug carriers it is clear that the effects of serum on the integrity of liposomes, as far as leakiness to entrapped drugs is concerned, will be of major importance in determining their biological effects. One of the observations made here, that PS/DSPC/DPPC liposomes in the presence of serum were much leakier to sucrose and inulin than were cholesterol-containing liposomes or even PS/PC liposomes, has important consequences if drugs were to be entrapped inside these liposomes with the aim of using them chemotherapeutically. Unless liposomes found their 'target' cells rapidly, most of the drug (if it behaved like inulin or sucrose) would be lost into the plasma. Similarly, these liposomes would probably not be of much use as a circulating 'depot' system. 'Solid' liposomes with phase-transition temperatures well above 37° have been found less leaky than the PS/DSPC/DPPC liposomes used, in the presence of serum [34]. Also, PS/PC/cholesterol liposomes are much less leaky, and probably most of the drug would be retained inside the liposomes for considerable periods in full serum. Of course, to exert its action the drug must be able to escape from the liposome, irrespective of a targeting or depot mode of action.

Acknowledgements—We thank T. Aerts, K. Caruana, B. Hennau and O. Mairiaux-Hudsyn for excellent technical assistance. This work has been partially supported by a contract from NOVO Industri, Denmark, and in part by Grant CA 14405 from the National Institutes of Health, U.S.A.

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