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The absorption of phospholipid vesicles by perfused rat liver depends on vesicle surface charge

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Two types of sonicated vesicle have been prepared from dipalmitoylphosphatidylcholine (DPPC) by incorporation of phosphatidylinositol (PI) to give negatively charged vesicles and stearylamine to give positively charged vesicles. The absorption of the vesicles by rat liver has been investigated by perfusion techniques. A steady state of vesicle absorption is rapidly established in approx. 2 min and the initial rates of absorption decrease with PI content of the vesicles and increase with stearylamine content. In the steady state, the uptake of vesicles by the liver is similarly dependent on vesicle charge, being inhibited by PI and enhanced by incorporation of stearylamine in the vesicles. Fractionation of the liver into subcellular fractions following perfusion showed that most of the vesicular lipid could be found associated with a nuclear (plus plasma membrane) fraction. The suppression of vesicle absorption by PI may be of value as a means of bypassing the liver in relation to the use of vesicles as a delivery system.

Introduction

The potential use of liposomes as a means of delivering drugs and genetic material to cells has led to numerous studies concerned with the interaction between liposomes and cells [1–3]. Of particular interest in this area is the uptake of liposomes by the liver and spleen, which are the organs largely responsible for the clearance of liposomes from the blood [1,4]. The relationship between liposome properties (i.e., composition, size and charge) and liposome-cell interaction has been investigated for numerous cellular systems [5–10]. It has often been found that negatively charged liposomes are more readily endocytosed than neutral or positively charged liposomes. This observation has been reported to hold for the uptake of liposomes by cultured Kupffer cells [10] and hepatocytes and Kupffer cells in vivo [7]. In contrast to these studies, the introduction of positive charge (e.g., by the incorporation of stearylamine) into liposomes has been shown to increase liposome interaction with cultured rat peritoneal macrophages [6], HeLa cells [12], rabbit thymocytes [13], murine L1210 cells [14], and erythrocytes [15].

These conflicting observations relating to the effect of liposome surface charge on liposome-cell interaction have led us to investigate the uptake of lipid from vesicles prepared from mixtures of dipalmitoylphosphatidylcholine (DPPC) with phosphatidylinositol (PI) (negatively charged) and DPPC with stearylamine (positive charged) by perfused rat liver. Phosphatidylinositol (PI) is an interesting membrane phospholipid in that its degradation and resynthesis are stimulated by a variety of hormones and neurotransmitters [20]. It has also been shown to inhibit liposome fusion when incorporated in phosphatidylserine liposomes [21], and liposomes incorporating PI have

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been reported to suppress phagocytic function and phospholipid in macrophages [22].

Two procedures have been used to determine the uptake of vesicles by perfused rat liver: a flow-through procedure, which gives initial rates of lipid uptake, and a recirculating procedure, which gives uptake in the steady state. Both procedures demonstrate that when PI is incorporated into DPPC vesicles it inhibits the uptake of the vesicles by rat liver, in marked contrst to stearylamine, which increases vesicle uptake.

Materials and Methods

Materials

L-α-Dipalmitoylphosphatidylcholine (DPPC) was obtained from Sigma (London) Chemical Company approx 99% pure (product number P0763). L-α-Dipalmitoylphosphatidyl[*N-methyl*-^{3}H]choline (code TRK 673) was obtained from Amersham International. Phosphatidylinositol (PI) from wheat germ (as the sodium salt, molecular weight 846 [23]) was Grade I obtained 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. Sodium pentobarbitone ('Sagatal'), concentration 60 mg · ml^{-1}, was obtained from May & Baker (Dagenham). Heparin from porcine intestinal mucosa (as the sodium salt) was obtained from Sigma London Chemical Company (product number H 7005).

Vesicle preparation

Vesicle suspensions were prepared by adding the required amounts of phospholipid stock solutions (concentrations 2–4 mg · cm^{-3}) in chloroform/methanol (4:1, v/v) plus approx. 2 μCi [^{3}H]DPPC together with 50 cm^3 of chloroform/methanol to a 1 litre round-bottomed flask. The resulting solution was rotary evaporated at 60°C to form a lipid film to which was added 10–15 cm^3 of Krebs-Henseleit bicarbonate buffer [24] (pH 7.4) (120 mM NaCl/5 mM KCl/1.2 mM KH_2PO_4/1.2 mM $MgSO_4$/2.5 mM $NaHCO_3$) previously purged with nitrogen gas. The resulting suspension was sonicated for 1 h in a Decon FS100 frequency-sweep sonicator at 60°C. The sonicated vesicles were cooled to 37°C and gassed with oxygen/carbon dioxide (95% : 5%) to give a pH of 7.4. Calcium chloride was then added to a final concentration of 2.6 mM. It should be noted that vesicles designated as 'pure' PI also contained [^{3}H]DPPC label; however, the molar ratio of PI to [^{3}H]DPPC was in the range $5 \cdot 10^5$ to $1 \cdot 10^6$ and hence the effect of the labelled lipid on vesicle properties was considered negligible.

Perfusion of rat liver

Perfusion of the liver of Sprague Dawley rats (180–250 g) was carried out using the method of Berry and Friend [25] as modified by Elliot et al. [26]. The rats were anesthetized by intraperitoneal injection of sodium pentobarbitone (60 mg plus 650 units of heparin per kg body weight). The abdomen and chest were opened and the inferior vena cava was cannulated with a heparinized 16-gauge needle. The hepatic portal vein was cannulated with a 19-gauge needle. Both cannulae were ligated. The liver was perfused at a rate of 25 cm^3/min in the physiological direction using a peristaltic pump (Watson-Marlow 501) with a combined bubble trap-depulser located between the pump and the portal cannula. Two methods of mesuring liposome uptake were used, a single flow-through method (i) and a recirculating method (ii).

(i) Flow-through perfusion. Reservoirs containing vesicle suspensions of the required composition and concentration (total volume 100 cm^3) in Krebs-Henseleit buffer and a reservoir of buffer were maintained at 40°C and gassed with oxygen/carbon dioxide mixture. The liver was initially flushed with 100 cm^3 of buffer. The contents of each vesicle reservoir were passed through the liver and for each vesicle suspension samples (100 μl) of the outflow were taken at 3 s intervals over a period of 3 min. Between perfusing the liver with each vesicle suspension the liver was washed by perfusion with 100 cm^3 of buffer. The 100 μl samples were added to 2 cm^3 of scintillant (PPO/POPOP cocktail T, BDH Chemicals) and counted on a Beckman LS 9800 scintillation counter.

(ii) Recirculating perfusion. Two reservoirs, one containing 170 cm^3 of vesicle suspension in Krebs-Henseleit buffer and a second containing only buffer, were maintained at 40°C and con-

tinuously gassed with oxygen/carbon dioxide. The liver was initially flushed with 100 cm^3 of buffer before perfusing with vesicle suspension in a closed loop. The perfusion was continued for 1 h, during which 100 μl aliquots of liposome suspension were removed from the reservoir at 5-min intervals. Finally, the liver was flushed with 50 cm^3 of buffer, removed from the carcass, rapidly frozen at $-80°C$ and stored for subsequent analysis.

In the perfusion experiments the glass, vesicle resevoirs and the silicone rubber tubing were treated with dimethyldichlorosilane solution (2% in 1,1,1-trichloroethane (BDH)). Control experiments showed that the treatment reduced loss of lipid due to adsorption on the glass and tubing to neglible levels.

Subcellular fractionation

The frozen livers were thawed slowly on ice, chopped into small pieces and homogenized in a buffer (pH 7.4) containing 5 mM Tris, 0.25 M sucrose, 1 mM EDTA and 0.5% (w/v) Triton X-100 at a liver weight to buffer volume ratio of 1 g : 5 cm^3. The resulting homogenates were fractionated by differential centrifugation by a method based on that of DeDuve et al. [27,28]. Briefly, the initial homogenate was spun at 1900 rpm (MSE 6L centrifuge) for 10 min. The resulting pellet was redispersed in an equal volume of homogenizing buffer and spun again at 1900 rpm for 10 min to give a nuclei pellet (N). The supernatant was combined with the supernatant from the first centrifugation and spun at 8000 rpm for 3 min (MSE 18 centrifuge) to give a mitochondria pellet (M). The resulting supernatant was spun at 15000 rpm for 7 min (MSE 18 centrifuge) to give a lysosomal pellet (L) and a supernatant which was spun at 4000 rpm for 30 min (Beckman L5-65 centrifuge) to give a microsome pellet (P) and a final supernatant (S). The pellets (N, M, L and P) were finally resuspended in 0.5% Triton X-100 for assay.

The total volumes of the initial homogenates and the volumes of the resuspended pellets and supernatants were recorded throughout the fractionation so that the total radioactivity in each organelle fraction could be determined. During the fractionation, 1 cm^3 aliquots of each fraction were taken and stored at $-2°C$ for subsequent counting and protein assay by the Biuret method [29].

Results

The results of a typical flow-through experiment are shown in Fig. 1 for sonicated DPPC vesicles covering a concentration range. For a given vesicular lipid concentration a steady state of lipid uptake is established in approx. 90 s. In the steady state, the lipid concentration leaving

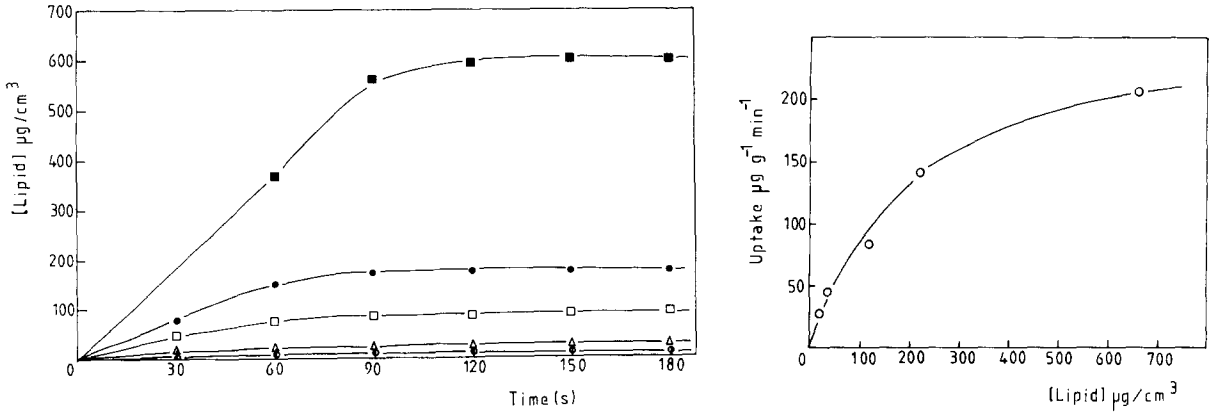

Fig. 1. (Left.) Initial uptake of lipid from sonicated dipalmitoylphosphatidylcholine vesicles by perfused rat liver. The lipid concentrations ($\mu g \cdot cm^{-3}$) entering the liver were as follows: ○, 18; △, 38; □, 118; ●, 217; ■, 666. The left-hand axis is the lipid concentration flowing from the inferior vena cava at a flow rate of 25 $cm^3 \cdot min^{-1}$.

Fig. 2. (Right.) Uptake of lipid from sonicated dipalmitoylphosphatidylcholine vesicles by perfused rat liver as a function of lipid concentration.

TABLE I

RATE CONSTANTS (k_i) FOR THE INITIAL UPTAKE OF LIPID FROM SONCIATED VESICLES BY PERFUSED RAT LIVER AT 37°C

Vesicle composition	$10^3 k_i$ ($cm^3 \cdot g^{-1} \cdot min^{-1}$)	(No. expts.)
DPPC	60±23	(15)
DPPC + 10%(w/w)PI	34	(1)
DPPC + 25%(w/w)PI	19±5	(7)
DPPC + 50%(w/w)PI	24±9	(6)
DPPC + 75%(w/w)PI	24±12	(2)
PI	31±13	(8)
DPPC + 5%(w/w)stearylamine	86	(1)
DPPC + 15%(w/w)stearylamine	103	(1)

the liver remains constant at a value below that of the concentration entering the liver and the change in concentration (ΔC) increases with lipid concentration entering the liver. Fig. 2 shows the rate of uptake as a function of the entry concentration. The uptake rate is related to flow rate, F, and can be expressed per g of liver by the relation

$$\text{Uptake rate} = \frac{F\Delta C}{g} (\mu g \cdot min^{-1} \cdot g^{-1}) \quad (1)$$

Rate constants (k_i) can be determined from the initial slopes of plots such as Fig. 2 and these are given in Table I. The errors in the estimates of k_i for a given vesicle composition arise largely from variations between different livers. However the results clearly show that the introduction of PI into DPPC vesicles reduces the rate constant for initial uptake, while stearylamine increases the rate constant.

The kinetics of uptake of vesicular lipid in the steady state was investigated by the recirculating perfusion method which required only a single vesicle dispersion and allowed uptake to be monitored over a period of approx. 60 min, the uptake of lipid by the liver being determined from the loss of lipid in the reservoir.

If the flow rate is F ($cm^3 \cdot min^{-1}$), the volume of the reservoir is V_r (cm^3) and c_{in} and c_{out} are the lipid concentrations entering and leaving the liver (mass M (g)) from the reservoir, respectively, then the rate of change of lipid concentration in the reservoir and liver are given by

$$-\left(\frac{dc}{dt}\right)_{reservoir} = \left(\frac{dc}{dt}\right)_{liver} = \frac{F}{V_r}(c_{in} - c_{out}) \quad (2)$$

If the change in lipid concentration ($\Delta c = c_{in} - c_{out}$) is assumed to be directly proportional to the lipid concentration and the mass of the liver then

$$-\left(\frac{dc}{dt}\right)_{reservoir} = k_p \frac{FM}{V_R} c \quad (3)$$

where k_p is defined as a perfusion constant. Integration of Eqn. 3 gives

$$c = c_0 \, e^{-k_p \frac{FM}{V_r} t} \quad (4)$$

where c_0 is the initial lipid concentration in the reservoir. In order to test the applicability of Eqn. 4 to perfusion in the steady state, the data (i.e.,

TABLE II

PERFUSION CONSTANTS (k_p) FOR THE STEADY-STATE UPTAKE OF LIPID FROM SONICATED VESICLES BY PERFUSED RAT LIVER AT 37 °C

Vesicle composition (initial concentration, $\mu g \cdot cm^{-3}$)	c_0 ($\mu g \cdot cm^{-3}$)	$10^2 k_p$ (g^{-1})
DPPC (89.5)	85.98±1.25	3.668±0.073
DPPC + 2.5%(w/w)PI (81.0)	73.56±1.42	2.557±0.074
DPPC + 5.0%(w/w)PI (87.3)	77.87±1.83	1.658±0.082
DPPC + 10.0%(w/w)PI (92.5)	81.21±2.28	1.541±0.106
DPPC + 25.0%(w/w)PI (90.5)	81.12±1.13	0.396±0.038
DPPC + 50.0%(w/w)PI (91.0)	83.04±2.01	0.440±0.067
PI (90.0)	83.57±1.14	0.456±0.038
DPPC + 5%(w/w)stearylamine (103.0)	95.47±3.07	4.299±0.179
DPPC + 10%(w/w)stearylamine (95.0)	93.66±3.34	5.227±0.229
DPPC + 15%(w/w)stearylamine (94.0)	95.82±7.47	7.302±0.637

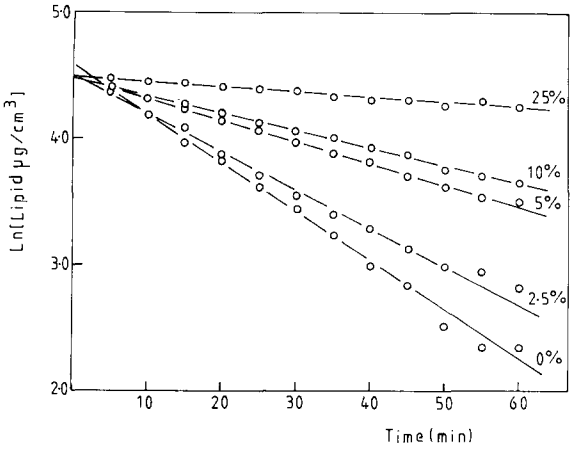

Fig. 3. Change in lipid concentration in the vesicle reservoir during recirculating perfusion of rat liver with sonicated dipalmitoylphosphatidylcholine (DPPC)-phosphatidylinositol (PI) vesicles. The figures denote the % (w/w) PI in the vesicles.

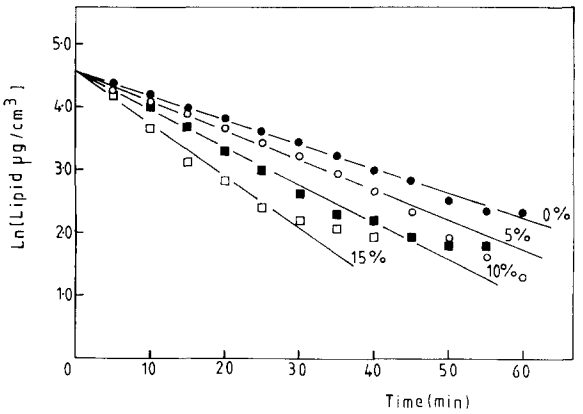

Fig. 4. Change in lipid concentration in the vesicle reservoir during recirculating perfusion of rat liver with sonicated dipalmitoylphosphatidylcholine (DPPC)-stearylamine vesicles. The figures denote the % (w/w) stearylamine in the vesicles.

values of c as a function of t) were fitted by non-linear regression using the method of Walmsley and Lowe [30] to obtain the best values of c_0 and k_p. The results of the computations are given in Table II, where the calculated values of c_0 are compared with the experimental values. The mean difference between the calculated and experimental values of c_0 is -6.9%. This deviation probably arises as a consequence of unavoidable leakage from the cannulae during 60 min of perfusion. It follows from Eqn. 4 that ln c should decrease linearly with time. Figs. 3 and 4 show the ln c vs. t plots for loss of DPPC plus PI and DPPC plus stearylamine vesicles from the reservoir during perfusion, respectively. The regression analysis and the linearity of the ln c vs. t plots suggest that Eqn. 4 is a satisfactory way to represent the data. For the DPPC/stearylamine vesicles some deviation from linearity of the ln plots was found after perfusion for more than 30–40 min for vesicles containing 10 and 15% stearylamine. Such an effect might arise should stearylamine have a toxic effect on liver function which results in inhibition of vesicle absorption after prolonged perfusion.

However, another possible source of error is the metabolic breakdown of lipid giving rise to water soluble (labelled) material [31,32]. Should

TABLE III

DISTRIBUTION OF LIPID (% w/w) IN SUBCELLULAR FRACTIONS OF RAT LIVER FOLLOWING PERFUSION WITH SONICATED VESICLES

Fraction designations: N + PM, nuclei plus plasma membrane; M, mitochondria; L, lysosomes; P, microsomes; S, supernatant.

Vesicle composition	Cellular fractions				
	N + PM	M	L	P	S
DPPC	55	4.5	7	7.5	26
DPPC + 10%(w/w)PI	67	5.5	6	6	15.5
DPPC + 25%(w/w)PI	75.5	4.5	5	4	11
DPPC + 50%(w/w)PI	65	4.5	7	6	17.5
PI	71.5	5	6	6.5	11
DPPC + 2%(w/w)stearylamine	33	11.5	14.5	15	26
DPPC + 5%(w/w)stearylamine	61	4	5.5	5	24.5
DPPC + 10%(w/w)stearylamine	5.6	3.5	5	6	29
DPPC + 15%(w/w)stearylamine	66.5	3.5	4.5	6	19.5

water-soluble label leave the liver then it could lead to an underestimate of lipid uptake from the vesicles. However, the time course for metabolic breakdown is realtively long, with a lag phase of about 15 min and for cultured Kupffer cells amounts to approx. 20% after 60 min for labelled egg phosphatidylcholine [31]. This would have no effect on the data obtained by flow-through perfusion, but could result in an upward curvature of the ln c vs. t plots at longer times if an appreciable fraction of water-soluble label diffused out of the liver. Thus for vesicles containing 10 and 15% stearylamine this could be another possible cause of the deviations from linearity.

The trend of the perfusion constants with vesicle composition in the steady state reflects the trend observed in the rate constants for initial uptake (Table I). Increasing the PI content of DPPC vesicles decreases the perfusion constants, while increasing stearylamine content increases them.

The distributions of lipid in subcellular fractions are given in Table III for DPPC plus PI and DPPC plus stearylamine vesicles. For both types of vesicle the lipid is predominantly associated with the nuclear fractions. The nuclear fraction obtained by differential centrifugation contains not only nuclei but also plasma membrane, cell debris and erythrocytes [33,34]. There is no systematic variation in the location of lipid in the subcellular fractions with vesicle composition. From over twelve fractionations the lipid distribution is as follows: nuclear (plus plasma membrane) fraction, 59.5 ± 10.5%; mitochondrial fraction, 4.9 ± 2.0%; lysosomal fraction, 6.8 ± 2.5%; microsomal fraction, 6.8 ± 2.7%; and supernatant, 21.8 ± 6.3%. It should be noted, however, that because of the duration of the vesicle preparation and perfusion experiments the livers had to be frozen before subcellular fractions. Although it would be preferable to fractionate fresh tissue, since most of the lipid was found to be associated with the plasma-membrane-containing fraction it is unlikely that this arose as a result of disruption of subcellular organelles due to freezing.

Discussion

Perfusion of the rat liver with sonicated vesicle suspensions at a flow rate of 25 $cm^3 \cdot min^{-1}$ establishes a steady state in less than 2 min. Uptake in the steady state is adequately described by a first-order equation (Eqn. 4). Both the initial rate constants (k_i) for uptake and the steady-state perfusion constants (k_p) are markedly dependent on the vesicle charge. For the DPPC/PI vesicles the values of k_p decrease by a factor of 8 from pure DPPC vesicles to 'pure' PI vesicles (Table II, Fig. 3); the decrease is not uniform over the whole composition range but is largely restricted to PI content from 0 to 25% (v/v). The size and the electrophoretic properties of sonicated DPPC/PI vesicles have been studied previously [35] to obtain the z-average diameters $(\langle d_z^{-1} \rangle)^{-1}$ by photon correlation spectroscopy and the electrophoretic zeta potentials ($\zeta°$) by microelectrophoresis. These parameters are shown as a function of vesicle composition in Fig. 5 together with the values of k_p. Although the solution conditions for the measurements of $(\langle d_z^{-1} \rangle)^{-1}$ and $\zeta°$ differ from those used in the perfusion experiments, the difference would not be expected to affect markedly $(\langle d_z^{-1} \rangle)^{-1}$ or the trend of zeta potential with vesicle composition. Fig. 5 illustrates that over the composition range 0–25% PI the decrease in k_p correlates with the increase in zeta potential and hence surface charge density, since vesicle size

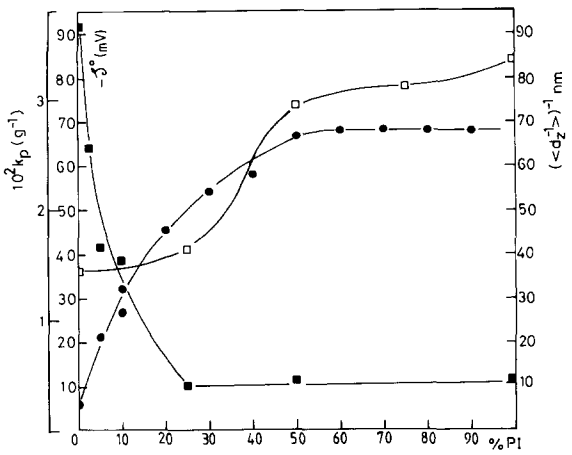

Fig. 5. Perfusion constant (k_p, ■), vesicle diameter ($(\langle d_z^{-1} \rangle)^{-1}$, □) and electrophoretic zeta potential ($\zeta°$, ●) for sonicated dipalmitoylphosphatidylcholine (DPPC)-phosphatidylinositol (PI) vesicles as a function of PI content. The vesicle dimeters and $\zeta°$ potentials were taken from Ref. 35 and refer to vesicles dispersed in water (pH 6.6) and phosphate-saline buffer (pH 7.4, ionic strength 0.0344 M) at 25°C, respectively.

changes only slowly over this part of the composition range.

In contrast to DPPC/PI vesicles, the perfusion constants for DPPC/stearylamine vesicles are larger and increase with stearylamine content. Taken together these results show that vesicle uptake by the liver is inhibited by negative charge and enhanced by positive charge, as found for several other cell types [6,12–15]. It follows that PI is a suitable inhibitor of vesicle uptake by the liver and might be successfully used to bypass the liver in vesicle delivery systems. PI has an advantage in this context over, for example dextran sulphate, which has been suggested as a liver blocking agent [36], in that it is a naturally occurring membrane component. It is not however, unique in its capacity to inhibit uptake by the liver, experiments using sonicated DPPC vesicles incorporating phosphatidylserine gave similar results (unpublished observations), however, the possibility that the effects observed here may not be entirely explicable in terms of the charge of PI and that PI specifically inhibits phagocytosis as observed for macrophages [22] cannot be eliminated. It should also be noted that our results apply to an essentially blood-free system and that in vivo interaction between the vesicles and blood plasma components could have a mediating effect on vesicle uptake. Such interactions might account for the differences between our results and those of, for example, Spanjer et al. [7] who found liver cells took up negatively charged vesicles more readily than neutral or positively charged vesicles.

There has been considerable discussion about the cells responsible for the uptake of vesicles by the liver, both parenchymal cells and Kupffer cells having been implicated [37]. Scherphof et al. [38] found that parenchymal cells had a preference for small vesicles (about 50 nm diameter) and that the size distribution of fenestrations in the endothelial cells determines the extent of vesicle uptake by parenchymal cells. It was further suggested that, while the initial site of uptake was Kupffer cells, a secondary translocation process occurred which resulted in the transfer of lipid to parenchymal cells. The fractionation studies here were not aimed at determining the cells responsible for uptake but at establishing the overall location of the lipid after perfusion. The highest levels of lipid label were found in the nuclear fractions which also contain plasma membrane [32]. Since 73.4% of the volume of plasma membrane in a liver homogenate can be attributed to parenchymal cells [39] and only 34% by volume of the nonparenchymal cells are Kupffer cells, parenchymal cell plasma membranes would predominate in the nuclear fraction and, although the possibility cannot be eliminated, it is probably unlikely that the vesicle lipid is exclusively associated with one cell type.

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