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Effect of the Cholesterol Content of Small Unilamellar Liposomes on their Stability *in vivo* and *in vitro*

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Small unilamellar neutral, negatively and positively charged liposomes composed of egg phosphatidylcholine, various amounts of cholesterol and, when appropriate, phosphatidic acid or stearylamine and containing 6-carboxyfluorescein were injected into mice, incubated with mouse whole blood, plasma or serum or stored at 4°C. Liposomal stability, i.e. the extent to which 6-carboxyfluorescein is retained by liposomes, was dependent on their cholesterol content. (1) Cholesterol-rich (egg phosphatidylcholine/cholesterol, 7:7 molar ratio) liposomes, regardless of surface charge, remained stable in the blood of intravenously injected animals for up to at least 400 min. In addition, stability of cholesterol-rich liposomes was largely maintained *in vitro* in the presence of whole blood, plasma or serum for at least 90 min. (2) Cholesterol-poor (egg phosphatidylcholine/cholesterol, 7:2 molar ratio) or cholesterol-free (egg phosphatidylcholine) liposomes lost very rapidly (at most within 2 min) much of their stability after intravenous injection or upon contact with whole blood, plasma or serum. Whole blood and to some extent plasma were less detrimental to stability than was serum. (3) After intraperitoneal injection, neutral cholesterol-rich liposomes survived in the peritoneal cavity to enter the blood circulation in their intact form. Liposomes injected intramuscularly also entered the circulation, although with somewhat diminished stability. (4) Stability of neutral and negatively charged cholesterol-rich liposomes stored at 4°C was maintained for several days, and by 53 days it had declined only moderately. Stored liposomes retained their unilamellar structure and their ability to remain stable in the blood after intravenous injection. (5) Control of liposomal stability by adjusting their cholesterol content may help in the design of liposomes for effective use in biological systems *in vivo* and *in vitro*.

An important prerequisite for the effective use of liposomes as a drug carrier is control over their stability (Gregoriadis, 1979). Defined here as the extent to which the carrier retains its drug contents *in vitro* or *in vivo*, stability is influenced not only by the biological environment with which liposomes come into contact, but also by their structural characteristics as well as those of the associated drugs (Gregoriadis, 1979; Kimelberg & Mayhew, 1978). For instance, liposomes containing charged lipids retain certain drugs through electrostatic bonding (Gregoriadis *et al.*, 1977), and lipophilic drugs (Gregoriadis, 1973; Juliano & Stamp, 1978; Dingle *et al.*, 1978) can be anchored into the liposomal lipid phase. On the other hand, entrapped macromolecules, such as enzymes (Gregoriadis & Ryman, 1972; Weissmann *et al.*, 1975), nucleic acids (Dimitriadis, 1978; Ostro *et al.*, 1978), polymers (Dapergolas *et al.*, 1976) and viruses (Wilson *et al.*, 1977) or viral components (Manesis *et al.*, 1979), are unable to cross the lipid bilayer because of their large size.

One variant in the liposomal structure that could control retention of drugs is cholesterol content. Indeed, numerous studies on the use of liposomes as membrane models have shown that cholesterol, by increasing the packing of phospholipid molecules (Ladbrooke *et al.*, 1968; Demel & de Kruyff, 1976), reduces bilayer permeability to non-electrolyte and electrolyte solutes (de Gier *et al.*, 1970; Demel *et al.*, 1972; Papahadjopoulos *et al.*, 1973). To our knowledge, there has been no investigation into a possible role for the sterol in improving retention of solutes by liposomes within a biological milieu. In the present report we have therefore studied *in vivo* and *in vitro* the effect of the cholesterol content of unilamellar liposomes composed of egg phosphatidylcholine on their stability. This was monitored by measuring

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changes in membrane permeability to 6-carboxyfluorescein entrapped at a concentration that, because of self-quenching, prohibits its fluorescence (Weinstein *et al.*, 1977). When, for any reason, liposomes become leaky to the dye, its escape and ensuing dilution in the surrounding medium enable it to fluoresce, thus providing an immediate and easily measurable index of membrane permeability (Weinstein *et al.*, 1977; Wreschner & Gregoriadis, 1978; Gregoriadis & Davis, 1979). Our results show that a high content of cholesterol in such liposomes is essential for the maintenance of their stability under a variety of experimental conditions. Stability is also promoted by the presence of blood cells and, to some extent, plasma.

Materials and Methods

The sources and grades of egg phosphatidylcholine, cholesterol, phosphatidic acid, stearylamine and 6-carboxyfluorescein have been described elsewhere (Gregoriadis, 1976; Gregoriadis & Davis, 1979). [1-^{14}C]Cholesteryl oleate (34 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Preparation of small unilamellar liposomes

For the preparation (Gregoriadis, 1976) of small unilamellar liposomes bearing neutral, negative or positive net surface charge, egg phosphatidylcholine (20μmol) and, when appropriate, cholesterol, tracer [^{14}C]cholesteryl oleate and a charged amphiphile (see the Results section) were dried under a stream of O_2-free N_2 and then disrupted with 2 ml of 0.1 M-sodium phosphate buffer supplemented with 0.8% NaCl and 0.2% KCl (phosphate buffer), pH 7.4, and containing 0.25 M-6-carboxyfluorescein. The suspension was sonicated for a total of 10 min (1 min sonication with 30 s cooling periods) at 4°C using a titanium probe (1.9 cm tip diameter), and subsequently centrifuged at 100000 *g* ($r_{av.}$ 8.6) for 60 min. Small unilamellar liposomes with entrapped 6-carboxyfluorescein were separated from the unentrapped dye by passing the supernatant through a Sepharose CL-6B column (1 cm × 25 cm) equilibrated with phosphate buffer and subsequently dialysed against the same buffer until their use, at most within 1 h. Liposomal 6-carboxyfluorescein in appropriately diluted samples was measured in the absence (free dye) and presence (total dye) of Triton X-100 (1% final concentration) on a Perkin-Elmer 204A fluorimeter, by using excitation and emission wavelengths of 490 and 520 nm respectively. The lowest concentration of 6-carboxyfluorescein that could be determined accurately was 4 ng/ml. Latent 6-carboxyfluorescein was estimated as the difference in dye values obtained with and without Triton X-100 and expressed as percentage of total dye in the sample.

Electron microscopy

A drop of the liposomal suspension was placed on an ionized carbon-coated grid and immediately washed off with several drops of aq. 1% uranyl acetate. The excess fluid was removed with filter paper, and the grid allowed to dry before electron microscopy.

Experiments in vivo

Liposomal 6-carboxyfluorescein was injected into T.O. mice, weighing 20–25 g, by the intravenous (tail vein, 0.2 ml), intraperitoneal (0.5 ml) and intramuscular (hindleg, 0.2 ml) routes. In some experiments, mice were injected intravenously with free 6-carboxyfluorescein in 0.2 ml of phosphate buffer (for details see the Results section). At time intervals, 25–50μl of blood was collected in micro-pipettes and rapidly mixed with 2.0 ml of phosphate buffer in conical glass tubes. Immediately before fluorescence measurements, the tubes were centrifuged at 3000 rev./min for 5 min and the supernatants analysed for 6-carboxyfluorescein in the absence and presence of Triton X-100 (1% final concentration). Latent 6-carboxyfluorescein in the blood, derived as above from the difference in fluorescence values obtained with and without Triton X-100, was determined as the percentage of the total dye in the sample. Unless otherwise stated, these values were subsequently expressed as percentages of the latency in the liposomal preparation used. It was established that the amount of latent 6-carboxyfluorescein in the diluted blood was not altered upon standing for at least several hours. In experiments with liposomes labelled with [^{14}C]cholesteryl oleate, portions of the supernatants were used for the assay of ^{14}C radioactivity (Gregoriadis *et al.*, 1977).

Experiments in vitro

Liposomal 6-carboxyfluorescein was incubated at 37°C with fresh mouse blood mixed with the anticoagulant sodium citrate (0.63% final concentration), with fresh mouse plasma obtained from such blood or with fresh mouse serum. The volume ratio of biological fluid/liposomes was 10 (whole blood) and 5 (plasma or serum) i.e. similar to that expected upon intravenous injection of mice with 0.2 ml of liposomes (2 ml and 50% assumed blood volume and haematocrit respectively). At time intervals, 25μl samples were obtained and assayed for 6-carboxyfluorescein as for the experiments *in vivo*. Latent 6-carboxyfluorescein in the blood was again expressed as percentage of that in the preparation used. In control experiments 6-carboxyfluorescein latency was measured in liposomes incubated for 90 min in the

presence of 1% NaCl containing sodium citrate (0.63% final concentration).

Stability experiments

Immediately after their preparation, neutral and negatively charged liposomes labelled with [^{14}C]-cholesteryl oleate and containing 6-carboxyfluorescein were passed through Millipore filters (0.22 μM pore diameter), and distributed under sterile conditions into glass vials. These were then flushed with N_2, sealed and kept in the dark at 4°C. At time intervals, vial contents were analysed for 6-carboxyfluorescein latency and ^{14}C radioactivity. In addition, portions (0.2 ml) were injected intravenously into mice as above, and total and latent 6-carboxyfluorescein as well as ^{14}C radioactivity were subsequently measured in blood samples.

Results

Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo

Intravenous injection of mice with free 6-carboxyfluorescein was followed by a rapid linear clearance of the dye from the circulation with less than 5% remaining in the blood after 60 min (Fig. 1). There was only a minor reduction in the rate of clearance when 6-carboxyfluorescein was entrapped in cholesterol-free (egg phosphatidylcholine) or cholesterol-poor (egg phosphatidylcholine/cholesterol, 7:2 molar ratio) liposomes (about 6 and 9% of the dose respectively at 60 min). With both these preparations, clearance rates were non-linear and consistent with the behaviour expected from solutes that are released from their liposomal carrier in the circulation (Gregoriadis, 1973). Such release of 6-carboxyfluorescein was reflected in: (*a*) the very rapid (within 2 min) reduction of its latency to 45% (cholesterol-poor liposomes; results not shown) and 30% (cholesterol-free liposomes) of the values in the injected preparations (Fig. 2) [however, with cholesterol-free liposomes latency was subsequently increased to higher values (see the Discussion section)]; (*b*) the increase in the liposomal lipid ^{14}C/6-carboxyfluorescein ratio from the arbitrary value of 1.0 in the injected preparations to 9.2 (cholesterol-poor) and 14.3 or 18.3 (cholesterol-free liposomes) 150 min after injection (Table 1). In contrast, 6-carboxyfluorescein entrapped in cholesterol-rich (egg phosphatidylcholine/cholesterol, 7:7 molar ratio) liposomes exhibited a slow linear clearance, with about 70% of the dose being present in the blood at 60 min, suggesting that the dye was eliminated from the blood in association with its carrier. This was confirmed by findings (Fig. 2) showing complete (100%) maintenance of 6-carboxyfluorescein latency in these liposomes for at least 400 min after injec-

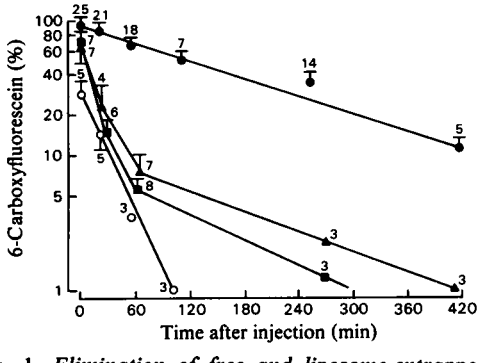

Fig. 1. *Elimination of free and liposome-entrapped 6-carboxyfluorescein from the blood of injected mice*
Mice were injected intravenously with 6-carboxyfluorescein, free (50–200 μg) or entrapped in small unilamellar liposomes (50–200 μg of 6-carboxyfluorescein, 1.5 mg of egg phosphatidylcholine). In individual experiments, equal amounts of free and liposomal 6-carboxyfluorescein were given. 6-Carboxyfluorescein values are percentages of the injected dose recovered in total blood at time intervals, and are means ± S.D. Numbers near symbols denote numbers of animals used. O, Free 6-carboxyfluorescein; liposomes containing 6-carboxyfluorescein were cholesterol-free (■), cholesterol-poor (▲) and cholesterol-rich (●).

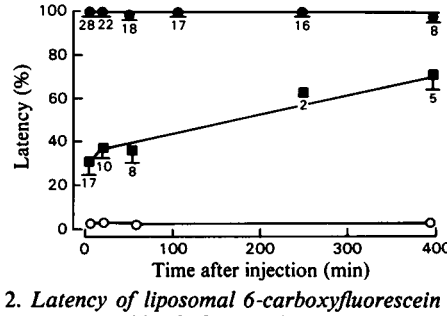

Fig. 2. *Latency of liposomal 6-carboxyfluorescein in the blood of injected mice*
Mice were injected intravenously with 6-carboxyfluorescein, free or entrapped in small unilamellar liposomes as in Fig. 1. 6-Carboxyfluorescein latency values in blood at time intervals are percentages of latencies in the respective injected preparations (means ± S.D.). These were 93.3 ± 2.4 (ten preparations) for cholesterol-rich liposomes and 77.6 ± 6.3 (six preparations) for cholesterol-free liposomes. Numbers near symbols denote numbers of animals used. For explanation of symbols and other details see legend to Fig. 1.

tion. Quantitative retention of 6-carboxyfluorescein by intact cholesterol-rich liposomes was further supported by results in Table 1: the ratio of the liposomal lipid ^{14}C/6-carboxyfluorescein markers retained its initial value of 1.0 for at least 150 min. At later time intervals radioactivity values were too low for the accurate determination of ratios.

The introduction of a net negative or positive charge on the surface of cholesterol-rich liposomes altered 6-carboxyfluorescein latency only slightly after intravenous injection (Table 2): for up to 300 min, the latency range was about 92–97% (negative) and 103–108% (positive liposomes). Furthermore, clearance rates were again linear, although, as expected (Gregoriadis & Neerunjun, 1974; Juliano & Stamp, 1975; Tagesson *et al.*, 1977), positive and neutral liposomes were removed less rapidly than were negative ones (Fig. 3).

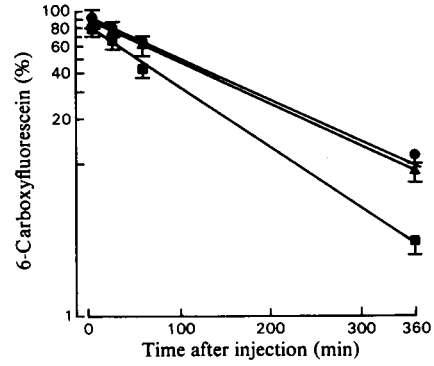

Fig. 3. *Effect of liposomal surface charge on the elimination of entrapped 6-carboxyfluorescein from the blood of injected mice*

Mice (eight in each group) were injected intravenously with 6-carboxyfluorescein (15–200 μg) entrapped in neutral (●), positively (▲) and negatively (■) charged small unilamellar cholesterol-rich liposomes (1.3–1.5 mg of egg phosphatidylcholine). Lipid composition of negative and positive liposomes was egg phosphatidylcholine, cholesterol and phosphatidic acid or stearylamine respectively (7:7:1, molar ratio). 6-Carboxyfluorescein values are percentages of the injected dose recovered in total blood at time intervals (means ± S.D.).

Effect of the cholesterol content of small unilamellar liposomes on their stability in vitro

Exposure of cholesterol-rich liposomes to whole mouse blood, plasma or serum (Fig. 4) resulted in a minor loss of latency. This, however, was significant in the case of serum (down to 86% of that in the preparation after 90 min; Fig. 4c). In agreement with the experiments carried out *in vivo* (see above) latency

Table 1. *^{14}C/6-carboxyfluorescein ratios in the blood of mice injected with 6-carboxyfluorescein entrapped in ^{14}C-labelled liposomes*

Mice were injected intravenously or intraperitoneally with 6-carboxyfluorescein entrapped in cholesterol-rich (A), cholesterol-poor (B) and cholesterol-free (C) small unilamellar liposomes labelled with [^{14}C]cholesteryl oleate (3.9×10^4–4.1×10^5 d.p.m.). The ratio of ^{14}C/6-carboxyfluorescein in the injected preparations was taken as 1.0. Numbers in parentheses denote numbers of animals used, and results are means ± S.D. N.D., Not determined. For other details see legends to Figs. 1 and 5.

Treatment		^{14}C/6-carboxyfluorescein ratio				
		2 min	20 min	110 min	150 min	450 min
Intravenous	A	1.09 ± 0.08 (6)	1.09 ± 0.09 (6)	1.05 ± 0.0 (3)	1.02 ± 0.0 (4)	N.D.
	B	2.1, 2.3	5.5 ± 0.8 (3)	—	9.2 ± 3.1 (3)	—
	C	3.4, 6.0	11.5 ± 4.8 (3)	—	14.3, 18.3	—
Intraperitoneal	A	N.D.	1.01 ± 0.0 (4)	1.05 ± 0.1 (4)	1.06 ± 0.0 (4)	0.97 ± 0.0 (4)

Table 2. *Latency of 6-carboxyfluorescein in the blood of mice injected with 6-carboxyfluorescein entrapped in negatively or positively charged liposomes*

Mice were injected intravenously with 6-carboxyfluorescein entrapped in negatively (two experiments) or positively (two experiments) charged cholesterol-rich small unilamellar liposomes. Latency values in the blood of individual animals in the two experiments for each type of liposome were expressed as percentages of the respective latencies in the injected preparations (see Table) and then pooled to give means ± S.D. Values obtained at the various time intervals for negative or positive liposomes did not differ significantly. Numbers in parentheses denote numbers of animals used. For other details see the legend to Fig. 3.

Liposomes	Latency (%)	Latency in the blood (%)			
		6 min	27 min	65 min	300 min
Negative	88.9, 95.2	96.9 ± 1.9 (7)	95.6 ± 1.0 (6)	93.2 ± 5.9 (6)	91.9 ± 12.0 (7)
Positive	84.6, 88.4	107.9 ± 2.5 (6)	103.3 ± 6.4 (6)	102.9 ± 8.1 (6)	108.4 ± 2.1 (6)

of 6-carboxyfluorescein entrapped in cholesterol-poor liposomes was rapidly decreased (to about 55% within 2 min) in the presence of whole blood and reached a value of 13% after 90 min (Fig. 4*a*). As expected (see Fig. 2), latency of 6-carboxyfluorescein in cholesterol-free liposomes was reduced even more rapidly, with only 7% of its value being retained 2 min after mixing (Fig. 4*a*). Latency for both types of liposomes in the presence of plasma (Fig. 4*b*) was initially (2 min) reduced to the same extent as in the presence of whole blood but, subsequently, its rate of decrease became more pronounced. Interestingly, serum (Fig. 4*c*) had a greater detrimental effect on the stability of cholesterol-free or cholesterol-poor liposomes than did whole blood or plasma.

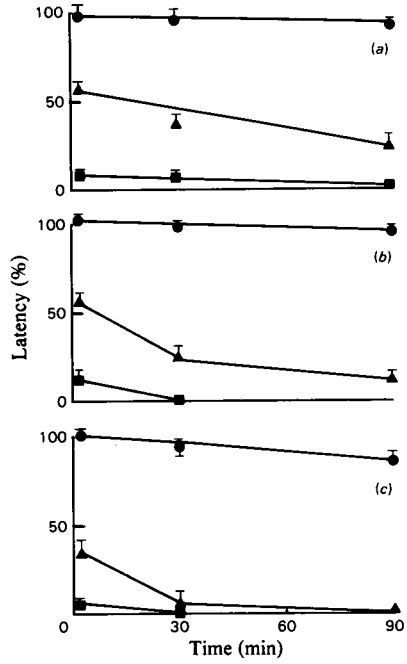

Fig. 4. *Latency of liposomal 6-carboxyfluorescein in the presence of whole blood, plasma or serum*
Small unilamellar liposomes containing 6-carboxyfluorescein were mixed with mouse whole blood (*a*), plasma (*b*) or serum (*c*) and incubated at 37°C. 6-Carboxyfluorescein latency values in samples obtained at time intervals have been corrected for the loss of latency incurred during incubation of liposomes in the presence of 1% NaCl containing 0.63% sodium citrate and are the means from four to six experiments. They are expressed as percentages of the latencies in the respective liposomal preparations. These were 92.7 ± 5.3 (four preparations) for cholesterol-rich (●), 93.2 ± 1.5 (three preparations) for cholesterol-poor (▲) and 83.8 ± 6.7 (three preparations) for cholesterol-free (■) liposomes.

Fate of cholesterol-rich small unilamellar liposomes administered to mice by the intraperitoneal and intramuscular routes

6-Carboxyfluorescein in cholesterol-rich liposomes given by the intraperitoneal route (Fig. 5) entered the circulation to a considerable extent, reaching a peak (30% of the injected dose) in about 150 min. Fig. 5 also shows that 6-carboxyfluorescein latency in the blood retained its initial value fully for at least 400 min, implying transport of intact liposomes from the peritoneal cavity into the blood circulation. This is further supported by the values of the liposomal lipid ^{14}C and 6-carboxyfluorescein marker ratios, which remained similar to that (1.0) in the injected preparation for up to 450 min (Table 1). After intramuscular injection of the same liposomes, entrapped 6-carboxyfluorescein also entered the circulation, albeit at a much lower rate (legend to Fig. 5). However, its latency was diminished to 64% 360 min after injection (legend to Fig. 5).

Stability of cholesterol-rich small unilamellar liposomes after storage

Latency of 6-carboxyfluorescein in neutral and negatively charged cholesterol-rich liposomes stored at 4°C for various periods of time (Table 3) remained relatively stable for several days, but was

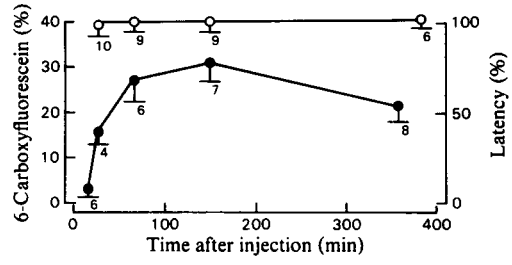

Fig. 5. *Latency and concentrations of liposomal 6-carboxyfluorescein in the blood of injected mice*
Mice were injected intraperitoneally with 6-carboxyfluorescein (55–304 μg) entrapped in small unilamellar cholesterol-rich liposomes (3.7 mg of egg phosphatidylcholine). 6-Carboxyfluorescein latency (O) and concentration (●) in total blood are expressed as percentages of the latency (90.7 ± 3.5; mean ± S.D. for five preparations) and the amount of the dye respectively in the injected preparation. Numbers near symbols denote numbers of animals used. For other details see the legend to Fig. 2. After intramuscular (hindleg) injection of five mice with the same liposomes, at most 2–4% of the dose could be recovered in the blood after 15, 70, 150, 360 min and 28 h. 6-Carboxyfluorescein latency values in the blood were 85.3 ± 13.5 and 64.4 ± 9.6% of that in the injected preparation (90.7 ± 3.5, five preparations) at 15 and 360 min respectively.

Table 3. *Effect of storage on the stability of liposomes in vivo and in vitro*

Neutral and negatively charged small unilamellar liposomes containing 6-carboxyfluorescein and labelled with [^{14}C]cholesteryl oleate were tested at time intervals after storage at 4°C (see the Materials and Methods section) for 6-carboxyfluorescein latency, and subsequently injected intravenously into mice. Injected preparations contained 63.0 (neutral) or 79.5 μg (negative) of 6-carboxyfluorescein and 1.38×10^5 (neutral) or 1.56×10^5 d.p.m. (negative). 6-Carboxyfluorescein latency and ^{14}C/6-carboxyfluorescein ratios were measured in the blood 2 and 50 min after injection. Latent 6-carboxyfluorescein is expressed here as percentage of the total dye in liposomes before injection (pre-injection values) or in the blood. To determine ^{14}C/6-carboxyfluorescein ratios in the preparations (taken as 1.0) or in the blood, entrapped (latent) 6-carboxyfluorescein values were used.

Storage (days)	Time after injection (min)	Neutral liposomes		Negative liposomes	
		Ratio	Latency	Ratio	Latency
4	Pre-injection		96.2		94.2
	2	1.03	88.2	1.00	90.4
	50	0.98	90.4	1.19	85.7
9	Pre-injection		93.8		93.9
	2	1.24	93.3	1.03	94.4
	50	1.23	94.6	1.20	89.2
13	Pre-injection		88.2		94.3
	2	1.13	88.7	1.18	91.2
	50	1.04	91.1	1.57	81.9
18	Pre-injection		89.0		93.5
	2	1.14	92.8	1.11	94.8
	50	1.20	92.5	1.50	80.0
33	Pre-injection		86.1		83.9
	2	1.03	94.7	1.02	94.7
	50	1.06	93.7	1.33	84.3
53	Pre-injection		79.8		75.3
	2	1.04	89.3	1.22	87.3
	50	0.94	91.8	1.20	87.2

subsequently decreased slowly from a value of about 96 (neutral) and 94% (negative liposomes) at 4 days to about 80 and 75% respectively in 53 days. Electron-microscopic studies revealed that storage for up to 53 days had no effect on the appearance of the unilamellar liposomes. Thus there was no aggregation of vesicles and their initial size (30–60 nm diameter) remained unaltered. In agreement with this, there was no measurable change in their turbidity. In addition Table 3 shows that storage did not alter significantly the ability of such liposomes to retain their 6-carboxyfluorescein latency *in vivo*: for both types of liposomes, absolute latencies in the blood remained uniformly high (88–94%, neutral and 82–95%, negative liposomes) at either 2 or 50 min after injection. Further evidence for the maintenance of the stability of stored liposomes *in vivo* was given by the finding that the liposomal lipid ^{14}C and (latent) 6-carboxyfluorescein marker ratio values in the blood 2 or 50 min after injection remained in most cases reasonably similar to that (1.0) in the injected preparations (Table 3).

Discussion

When expressed in terms of 6-carboxyfluorescein retention, stability *in vivo* and *in vitro* of small unilamellar neutral liposomes is dependent on their cholesterol content. Cholesterol-rich liposomes, for instance, remain stable in the blood of intravenously injected animals (Fig. 2) and *in vitro* in the presence of whole blood, plasma and, to a somewhat lesser extent, serum (Fig. 4). This is supported by the almost total retention of 6-carboxyfluorescein latency under all conditions mentioned (Figs. 2 and 4) and, for the work done *in vivo*, by the pattern of 6-carboxyfluorescein clearance (Fig. 1) and the maintenance of the lipid/water-phase marker ratios at approximately their initial level (1.0) in the preparations before injection (Table 1). The latter finding, indicating similar rates of elimination of the two labels from the blood (presumably as intact liposomes), rules out the possibility of significant leakage of 6-carboxyfluorescein and its subsequent rapid clearance. The stability of cholesterol-rich liposomes *in vivo* is also maintained when their net surface charge is negative or positive (Fig. 3 and Table 2). It would thus appear that interaction of liposomes with plasma proteins believed to occur *in vitro* (Black & Gregoriadis, 1976; Tyrrell *et al.*, 1977; Scherphof *et al.*, 1978) and *in vivo* (Krupp *et al.*, 1976) or their contact with circulating cells has no detrimental effect on the stability of liposomes when these are rich in cholesterol. Furthermore, our

results show beyond reasonable doubt that such liposomes survive in the peritoneal cavity to cross membranes and enter the circulation intact (Fig. 5). On the other hand, reduction in the liposomal cholesterol content leads to a very rapid (at most within 2 min) decrease of 6-carboxyfluorescein latency in the blood of injected animals (Fig. 2) and also *in vitro* in the presence of whole blood, plasma or serum (Fig. 4). Indeed, the loss of 6-carboxyfluorescein latency for cholesterol-free liposomes is so rapid (Fig. 4) that, *in vivo*, the very rapid clearance of the freed dye (Fig. 1) results in artificially high latency values for the 6-carboxyfluorescein that is still entrapped (Fig. 2).

The mechanism by which cholesterol controls liposomal stability within a biological milieu may be related to the finding that, upon contact with plasma, liposomes with little or no cholesterol lose some of their phospholipid to high-density lipoproteins (Krupp *et al.*, 1976; Scherphof *et al.*, 1978). According to Scherphof *et al.* (1978), this is associated with the liberation of previously entrapped agents. It is conceivable that excess sterol, by restricting the mobility of phospholipid (Ladbrooke *et al.*, 1968; Demel & de Kruyff, 1976), prevents the latter's subsequent loss to lipoproteins. Such an effect, expected to be proportional to the amount of cholesterol present, would thus explain the cholesterol-content-dependent latency that 6-carboxyfluorescein attains *in vivo* or *in vitro* (Figs. 2 and 4).

The presence of blood cells, presumably erythrocytes, reduces the rate of loss of 6-carboxyfluorescein latency seen upon mixing of cholesterol-free or cholesterol-poor liposomes with plasma or serum (Fig. 4). Two possible explanations can account for this finding: (*a*) erythrocytes are known to interact with lipoproteins in terms of phospholipid movement (Ladbrooke *et al.*, 1968; Bruckdorfer & Graham, 1976; Demel & de Kruyff, 1976), and it may be that such movement takes precedence over a similar one between liposomes and lipoproteins; (*b*) erythrocytes donate cholesterol to liposomes (Bruckdorfer *et al.*, 1968), and, if this takes place rapidly enough under the conditions described here, cholesterol-free and cholesterol-poor liposomes would be enriched in cholesterol and so less vulnerable to destabilization. It is of interest that loss of 6-carboxyfluorescein latency for cholesterol-poor or cholesterol-free liposomes in the presence of serum (Fig. 4*c*) is also reduced when this is replaced by blood plasma (Fig. 4*b*), although to a significantly lesser extent than that observed with whole blood (Fig. 4*a*). Plasma and serum differ by the absence of fibrinogen and some depletion of clotting factors in the latter. We are unable to explain at present how these differences contribute to the small but significant delay in the destabilization of such liposomes by plasma.

Regardless of the mechanism by which cholesterol and erythrocytes (and to some extent plasma) promote stability of liposomes, our data suggest that it may be possible to control drug release from liposomes. Such control is of particular relevance to chemotherapy, since in many instances it is desirable to transport entrapped drugs to target cells quantitatively, whereas in others a slow release is preferable (Gregoriadis, 1979; Kimelberg & Mayhew, 1978). From the practical point of view it is encouraging that the stability of cholesterol-rich small unilamellar liposomes observed after extended (at least 53 days) storage is also maintained in the intravascular space of injected animals (Table 3).

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