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SERUM-INDUCED LEAKAGE OF LIPOSOME CONTENTS

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Summary

Efflux of contents from small unilamellar vesicles of various compositions, containing a highly quenched fluorescent compound (calcein, 175 mM) was determined as a function of temperature in the presence and absence of human serum. Efflux of calcein from the liposomes was monitored as an increase in fluorescence as calcein became dequenched upon release from the liposomes. The presence of serum significantly increased liposome leakage in all cases. Incorporation of increasing molar ratios of cholesterol into liposomes reduced leakage of calcein from liposomes incubated with buffer and with serum. Leakage was significantly faster from liposomes with an osmotic gradient across the membrane (higher inside) than from equiosmolar liposomes. The leakage of [^{14}C]sucrose from egg lecithin liposomes at 37°C was also dramatically increased in the presence of serum.

An understanding of the processes which may affect liposome integrity in vivo is important for the effective use of liposome-encapsulated drugs in the treatment of disease.

Sweet and Zull [1] have reported that serum albumin enhances diffusion of [^{14}C]glucose from phosphatidylcholine-cholesterol-dicetylphosphate multilamellar vesicles at pH 3.5, but not at pH 7.0. Zborowski et al. [2] have reported enhanced leakage of sucrose from phosphatidylcholine liposomes induced by heparinized blood or by serum albumin. Scherphof et al. [3] have shown that during incubation with 50—66% plasma, phosphatidylcholine from liposomes was transferred to high density lipoproteins in a one-way process,

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Abbreviations: HDL, high density lipoproteins; Tes, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid.

with resulting release of entrapped ^{125}I-labelled albumin from the liposomes. More recently, Yatvin et al. [4] have reported that the presence of 10% fetal calf serum dramatically increased release in the region of the phase transition from dipalmitoylphosphatidylcholine-distearoylphosphatidylcholine (3 : 1) vesicles. The presence of 10% fetal calf serum had little effect at temperatures below the phase transition. Chobanian et al. [5] have shown that small unilamellar vesicles prepared from egg lecithin, when incubated in vitro with human plasma lipoproteins, with high density lipoprotein (HDL) or with apo-HDL were rapidly transformed into a smaller particle similar in size and density to HDL. Increasing concentrations of apolipoprotein C III (apo C-III) have been found to cause increasing amounts of release of quenched calcein from egg lecithin small unilamellar vesicles (Allen, T. and Segrest, J., unpublished results).

The experiments reported above have examined the effect on liposomes of either diluted serum or isolated serum components. Our present experiments were designed to look at leakage of contents from a variety of phospholipid vesicles in the presence of undiluted human serum or plasma at 37°C. These conditions approximate in vivo conditions making this system an appropriate in vitro model for predicting liposome leakage in vivo.

Leakage from liposomes was examined using a modification of the technique of Weinstein et al. [6]. In their technique liposomes are prepared containing a highly quenched fluorescent compound, 6-carboxyfluorescein (a charged analog of fluorescein). As 6-carboxyfluorescein leaks from the liposomes it becomes diluted and therefore dequenched, and an increase in fluorescence is seen.

The advantages of this method are 2-fold. Firstly, a very large amplification in fluorescence upon release of 6-carboxyfluorescein from the liposomes allows use of a very small liposome sample resulting in negligible dilution of the serum. Secondly, the fluorescent signal, and therefore leakage, can be monitored continuously with time, allowing very rapid leakage processes to be measured. We have used the fluorescent compound calcein (I) in our studies which has an advantage over 6-carboxyfluorescein in that the fluorescence is practically pH-independent over the pH range 6.0—8.5 [7] as compared to 6-carboxyfluorescein which shows considerable pH dependence of fluorescence in this pH range [8].

Liposomes containing entrapped calcein were prepared by the following procedure. Organic solvent was removed from 20 mg of chromatographically pure phospholipid by rotary evaporation under vacuum. 1 ml of 200 mM calcein (pH 7.4) in 2 mM Tes, 2 mM histidine, 150 mM NaCl buffer (pH 7.4) buffer was added to the dried phospholipid. The mixture was vortexed and subsequently sonicated to clarity at 20°C in a bath-type sonicator (Lab Supplies Co., Hicksville, NY). Liposomes prepared in this fashion were subsequently found to be osmotically unstable (200 mM calcein in buffer is 541 mosM, buffer is 273 mosM) which affected leakage from liposomes (see Results). Liposomes were subsequently prepared using a solution of 175 mM calcein in water (pH 7.4, 280 mosM). Calcein-containing liposomes were separated from free calcein by chromatography on a 1×40 cm Sephadex G-50 column in buffer (273 mosM) at 20°C and leakage experiments were started as soon as the

liposome fractions were collected. Liposomes were made from either egg phosphatidylcholine, dipalmitoylphosphatidylcholine or distearoylphosphatidylcholine. Liposome preparations were made containing 0, 0.1, 0.2 or 0.5 mol cholesterol/mol phosphatidylcholine. Cholesterol-containing vesicles were also prepared containing either 10% stearylamine, which imparts a positive charge to the liposomes, or 10% dicetylphosphate which imparts a negative charge to the liposomes.

Efflux from the vesicles was monitored on a Perkin-Elmer MPF-4 spectrofluorimeter with attached temperature control and recorder. Excitation was at 490 nm, and emission was monitored at 520 nm. At the start of each experiment 10 μl of calcein-containing liposomes (approx. 0.2 μM phospholipid) was pipetted into 2 ml of either buffer or human serum. Change in fluorescence was monitored with time at constant temperature. The half-time for efflux of liposome contents was calculated from the percent leakage noted in the first few minutes of incubation of each sample using as 100% efflux the fluorescence reading obtained when the sample was lysed with 100 μl of 1.3% deoxycholic acid in buffer, pH 7.4. Percent leakage for samples with very low leakage was calculated from percent leakage noted after an overnight incubation. The presence of deoxycholic acid did not significantly change the fluorescence of the samples. A plot of log[% entrapped calcein] versus time was linear until approx. 20—30% calcein remained trapped in liposomes, indicating that the initial 70—80% of leakage is a single exponential process. Estimates of $t_{1/2}$ were made from the initial linear portion of the curve.

Table I gives the half-time ($t_{1/2}$) for leakage of calcein from osmotically unstable and osmotically stable sonicated egg lecithin vesicles at three different temperatures in the presence of either buffer or serum. As was expected, the $t_{1/2}$ decreased with increasing temperature. Incubation of the liposomes in serum resulted in a significant decrease in $t_{1/2}$ at all three temperatures. No differences were noted between serum, plasma or heat-inactivated serum. It was observed that if osmotically unstable liposomes, which were stored and passed over Sephadex G-50 to separate free calcein at room temperature were mixed directly with buffer or serum at a higher or lower temperature, a rapid

TABLE I

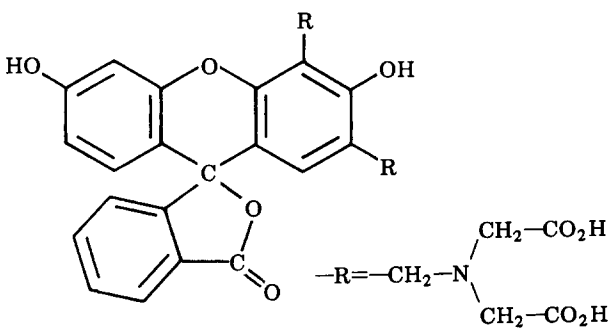
HALF-TIME OF LEAKAGE OF CALCEIN FROM OSMOTICALLY UNSTABLE AND OSMOTICALLY STABLE PHOSPHATIDYLCHOLINE LIPOSOMES

Osmotically unstable liposomes 541 mosM inside, 273 mosM outside. Osmotically stable liposomes: 280 mosM inside, 273 mosM outside. n.c., no change.

	$T^{1/2}$ (h)					
	Osmotically unstable			Osmotically stable		
	6°C	22°C	37°C	22°C	37°C	50°C
Slow equilibration						
Buffer	14.5	8.4	1.1	202.5	23.7	5.34
Serum	1.1	0.4	0.37	5.42	1.06	0.288
Temperature shock						
Buffer	2.8	n.c.	0.77	n.c.	n.c.	n.c.
Serum	0.17	n.c.	0.13	n.c.	n.c.	n.c.

initial leakage took place, which we have attributed to an 'osmotic shock' to the liposomes. If, on the other hand, the liposomes were placed in buffer or serum at room temperature and slowly (over a period of approx. 5 min) equilibrated to either 6°C or 37°C, the initial leakage could be considerably reduced and the $t_{1/2}$ for leakage increased (Table I). Osmotically stable lipsomes had significantly longer $t_{1/2}$ for leakage and no effect of 'temperature shock' was noted in these liposomes. These results suggest that an effort should be made to ensure that liposomes destined for in vivo injection are osmotically stable or if they are osmotically unstable they should be slowly warmed to 37°C a few minutes prior to injection.

The activation energies for the diffusion of calcein from liposomes in buffer or in serum are, respectively, 14.4 kcal/mol and 6.3 kcal/mol in osmotically unstable and 24.6 kcal/mol and 19.8 kcal/mol in osmotically stable liposomes. The high energy of activation for calcein diffusion in the presence of buffer is compatible with the transfer of a large, charged molecule across the membrane [1,10]. The enthalpy of activation of calcein diffusion in the presence of serum approaches that for free diffusion in the osmotically unstable liposomes [10] and the simplest explanation for this observation is that serum lipoproteins are interacting with these liposomes and causing gross disruption in bilayer structure allowing free passage of calcein down the osmotic and concentration gradients through holes or discontinuities in the liposomes. The energy of activation for calcein diffusion in the presence of serum in osmotically stable liposomes is also lower than that in the presence of buffer. It would appear that the presence of serum lipoproteins is facilitating the diffusion of calcein from liposomes, probably by destabilizing the membrane through a phospholipid-exchange mechanism. This explanation is compatible with previous observations on the interactions of lipoproteins and apolipoproteins with liposomes (Refs. 3, 5 and 11; Allen, T. and Segrest, J., unpublished results). This phospholipid exchange is most likely mediated by apolipoproteins such as A-I present in the high density lipoproteins. Transfer of protein to liposomes has been observed by several authors [5,19].



I

Including increasing amounts of cholesterol in phosphatidylcholine liposomes decreased the leakage from liposomes with and without an osmotic gradient when incubated in buffer at 37°C or 22°C (Fig. 1) as has previously

been reported [9] and also decreased the leakage from liposomes incubated in serum. A number of recent reports have demonstrated that cholesterol will exchange rapidly with serum lipoproteins. There is evidence that liposome cholesterol will rapidly equilibrate with cells in vitro by exchange processes [15—17]. In addition Krupp et al. [17] have reported in vivo transormation in the rat of vesicles containing ^{14}C-labelled cholesterol oleate into particles resembling high density lipoproteins. This exchange also took place in vitro when liposomes were incubated with plasma [5]. Cholesterol oleate was transferred from vesicles to HDL more rapidly than was lecithin [5]. Jonas and coworkers [11,18] have shown that large amounts of solid dispersions of [^{14}C]-cholesterol can be taken up by human HDL_3 and by bovine HDL. The half-time for exchange of vesicle cholesterol with HDL is reported to be 1.1 h [11]. Cholesterol exchange processes, however, did not impair the protective effect which cholesterol had on egg phosphatidylcholine vesicles incubated in the presence of serum, although phosphatidylcholine exchange processes, on the other hand, appeared to destabilize the membrane and render it leaky. A

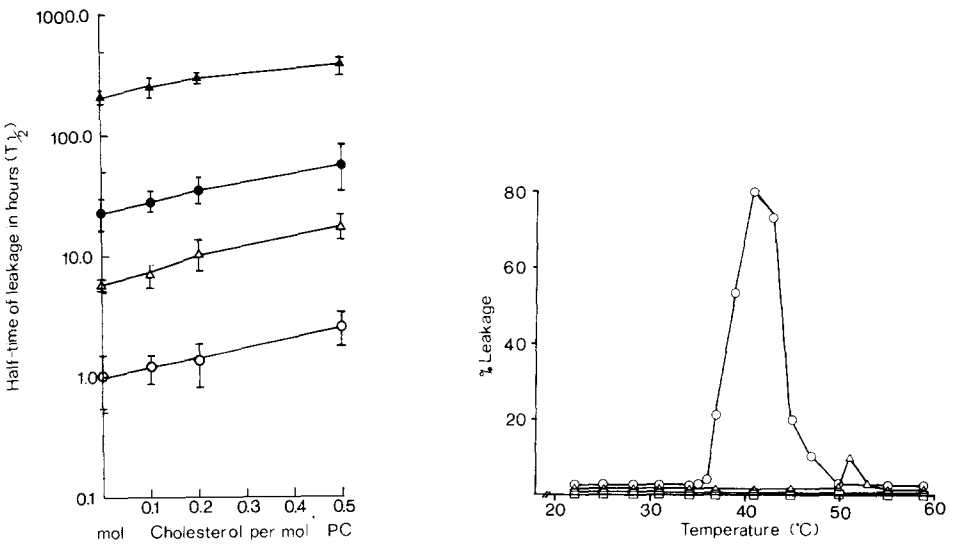


Fig. 1. Half-time for leakage ($t_{1/2}$) at 37° C and at 22° C of 175 mM calcein from egg phosphatidylcholine liposomes containing increasing mol fractions of cholesterol. 10 μl of calcein-containing liposomes at room temperature were diluted into 2.0 ml of either 2 mM Tes, 2 mM histidine, 150 mM NaCl buffer (pH 7.4), or human plasma or serum at room temperature or at 37° C. The increase in fluorescence was monitored with time. After a significant amount (approx. 10%) of the calcein had leaked out of the liposomes, the samples were lysed with 100 μl of 1.3% deoxycholic acid and the final reading was taken as 100% leakage and was used for calculating $t_{1/2}$ of leakage. Errors were calculated as the standard deviation of the mean. ○, serum, 37° C; △, serum, 22° C; ●, buffer, 37° C; ▲, buffer, 22° C.

Fig. 2. Permeability of dipalmitoyl phosphatidylcholine, (DPPC), distearoyl phosphatidylcholine (DSPC) and phosphatidylcholine (PC) liposomes in buffer to 175 mM calcein as a function of temperature. 10-μl samples of a stock liposome solution, maintained at 23° C, was diluted into 2 ml of buffer at 23° C and the cuvette was warmed over a few minutes to the temperature at which leakage was to be determined. Immediately the cuvette reached the assay temperature the fluorescence increase over a 4 min time period was measured. Leakage is plotted as percent increase in fluorescence as compared to 100% leakage determined by lysing each sample with 100 μl of 1.3% deoxycholic acid, pH 7.4. ○, DMPC; △, DSPC, and □, egg PC.

possible explanation is that the presence of cholesterol inhibits the phospholipid exchange process of liposomes with HDL. Chobanian et al. [5] report exchange with HDL in 1 h at 37°C of 50% of phosphatidylcholine from sonicated egg phosphatidylcholine liposomes containing 0.01 mol cholesterol oleate/mol phosphatidylcholine, while in a similar set of experiments Jonas and Maine [11] report exchange with HDL in 1 h at 37°C of only 21% phosphatidylcholine from sonicated egg phosphatidylcholine liposomes containing 0.5 mol cholesterol/mol phosphatidylcholine. Half-times for phosphatidylcholine exchange calculated from these figures for liposomes containing 0 and 0.5 mol cholesterol/mol phosphatidylcholine are 1 h and 2.9 h, respectively. Interestingly, and perhaps coincidentally, these figures are very close to the half-times for leakage of calcein from similar liposomes in the presence of serum at 37°C (Fig. 1, open circles). Pownall et al. [19] have observed that interaction of human plasma high density apolipoprotein A-I is inhibited at a mol ratio of cholesterol to dipalmitoyl phosphatidylcholine of 0.33. They postulate that defects in the lipid matrix are the sites of insertion of apo A-I. These defects would be most frequent at the phase transition and in the absence of cholesterol or in the presence of low levels of cholesterol. If the presence of high levels of cholesterol prevents or inhibits the association of apolipoprotein with membrane bilayers, and this association is the basis for the observed phosphatidylcholine exchange than decreased phosphatidylcholine exchange would be observed in the presence of increased mol ratios of cholesterol to phosphatidylcholine.

The presence of 10% negative charge somewhat prolonged the $t_{1/2}$ of leakage and the presence of 10% positive charge somewhat decreased the $t_{1/2}$.

The leakage of calcein from sonicated dipalmitoyl phosphatidylcholine, distearoyl phosphatidylcholine and egg phosphatidylcholine liposomes is shown in Fig. 2. The rate of release of trapped compound becomes very rapid at 37°C for dipalmitoyl phosphatidylcholine, at a temperature about 5°C below the reported phase transition for multilamellar dipalmitoyl phosphatidylcholine vesicles. Small unilamellar vesicles have been reported to have a broad phase transition a few degrees below that of multilamellar vesicles [12,13] and the increased leakage at 37°C in our preparation is a reflection of this lowered phase transition (T_c = 36.4°C ± 0.5 for small unilamellar vesicles, Ref. 13). The rapid leakage of contents from dipalmitoyl phosphatidylcholine liposomes at 37°C ($t_{1/2} < 5$ min) suggest that liposomes made of this phospholipid would not be suitable as a drug carrier for in vivo use in a homeothermic animal. Similar results have recently been reported by Yatvin et al. [4].

Liposomes made from distearoyl phosphatidylcholine (T_c = 51.3°C ± 0.5 for small unilamellar vesicles, Ref. 13) showed a similar leakage to egg phosphatidylcholine liposomes at 37°C (Fig. 2). At this temperature distearoyl phosphatidylcholine liposomes are below their phase transition temperature. This suggests that distearoyl phosphatidylcholine liposomes (or a distearoyl phosphatidylcholine : dipalmitoyl phosphatidylcholine combination (see Ref. 4) would be practical choices for drug delivery in vivo where a solid-crystalline liposome is desired. Distearoyl phosphatidylcholine liposomes show increased leakage at 50°C (Fig. 2).

Leakage of liposomal contents induced by serum was maximal for 100%

TABLE II

LEAKAGE OF CALCEIN FROM EGG PHOSPHATIDYLCHOLINE LIPOSOMES IN THE PRESENCE OF INCREASING CONCENTRATIONS OF SERUM AT 22°C

% serum	$T^{1/2}$ of leakage (h)
0	202.5 ± 19.1
10	9.73 ± 0.42
20	7.57 ± 0.45
50	7.53 ± 0.57
70	6.44 ± 0.59
100	5.50 ± 0.69

serum (the dilution of the serum by the liposomal aliquot, 10 μl in 2 ml, is negligible). Mixtures of serum and buffer showed less leakage as the proportion of buffer to serum increased (Table II). The most dramatic increase in leakage occur with the inclusion of 10% serum. This observation undoubtedly relates to the lipoprotein to liposome phospholipid ratios (which were very high in our experiments). Chobanian et al. [5] report maximum phospholipid exchange at high HDL to phospholipid ratios. At HDL to phosphatidylcholine ratios of 0.74 exchange of phosphatidylcholine decreased markedly while at ratios of 0.19 no exchange occur within 1 h at 37°C. In our experiments, even in the presence of only 10% serum we were at high lipoprotein to phosphatidylcholine ratios (approx. 0.15 mg phosphatidylcholine and 0.4 mg HDL). Decreasing the lipoprotein to liposome ratio would no doubt lead to intermediate leakage values. In this set of experiments we were interested in conditions which would apply when small liposome samples are injected in vivo and in this case, lipoprotein to liposome phospholipid ratios would be high. Our results may, therefore, more closely predict the leakage properties of liposomes in vivo, than in studies where diluted serum, serum components or low lipoprotein to liposome phospholipid ratios are used.

When free calcein was incubated with serum and the mixture passed over a Sephadex G-50 column, a portion of the fluorescence appeared in the void volume with the serum proteins, indicating that calcein is binding to serum proteins. It is unlikely, however that this binding affects the initial rates of leakage of calcein from liposomes. Calcein will bind to calcium and this could alter its charge and thus change its leakage rate, however, incubation of calcein-containing liposomes in buffer or serum containing calcium was found to have no effect on leakage rates. Binding of calcium to calcein does not affect its fluorescence in the range pH 6.5—9.0.

Liposomes were prepared containing [^{14}C]sucrose in addition to calcein and the leakage of both compounds from the liposomes was monitored with time. These experiments proved much more difficult to do, especially in the presence of serum which requires a rapid separation of liposomes containing entrapped sucrose from free sucrose and from serum components which themselves bind small amounts of [^{14}C]sucrose. The results are reported in Table III. At 22°C in buffer the liposomes were essentially impermeable to sucrose over a 48 h time period. The $t_{1/2}$ for leakage of [^{14}C]sucrose was somewhat higher than that of calcein in the presence of buffer. The presence of serum significantly

TABLE III

$T1/2$ OF LEAKAGE OF [^{14}C]SUCROSE AND CALCEIN FROM OSMOTICALLY STABLE EGG PHOSPHATIDYLCHOLINE LIPOSOMES

Data are in hours. n.d., not determined.

	[^{14}C]Sucrose		Calcein	
	22°C	37°C	22°C	37°C
Buffer	>60	60	202.5	23.5
Serum	n.d.	0.33	5.42	1.06

reduced the $t_{1/2}$ for leakage of sucrose (as well as for calcein) under the same conditions.

Liposomes appear to have a finite leakage to calcein, as compared to the less permeable sucrose molecule. This is not surprising as the calcein molecule has a partial polar nature due to its charged carboxyl groups and partial apolar nature because of its aromatic rings. It is similar in these characteristics to many drug molecules which one might wish to administer via liposome encapsulation.

Monitoring of liposome permeability by the calcein fluorscence method has proven to be simple, rapid and reliable. In addition, unlike 6-carboxyfluorescein it is pH independent over a wide range near physiological pH. Since very small, as well as large, liposome samples can be used the assays can be done using a large variety of serum concentrations or lipoprotein to liposome ratios. We therefore feel that this system is potentially useful for making predictions as to the permeability behavior of liposomes in vivo. Further experiments are currently underway which are designed to determine if it is possible to increase the latency of liposome-entrapped compounds in the presence of serum by using liposomes of varying head group, fatty acid composition or charge.

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References

1 Sweet, C. and Zull, J.E. (1969) Biochim. Biophys. Acta 173, 94—103
2 Zborowski, J., Roerdink, F. and Scherphof, G. (1977) Biochim. Biophys. Acta 497, 183—191
3 Scherphof, G., Roerdink, F., Waite, M. and Parks, J. (1978) Biochim. Biophys. Acta 452, 296—307
4 Yatvin, M.B., Weinstein, J.N., Dennis, W.H. and Blumenthal, R. (1978) Science 202, 1290—1293
5 Chobanian, J.V., Tall, A.R. and Brecher, P.I. (1979) Biochemistry 18, 180—187
6 Weinstein, J.N., Yoshikami, S., Henkart, P., Blumenthal, R. and Hagins, W.A. (1977) Science 195, 489—492
7 Diehl, H. (1964) in Calcein, Calmagite, and *0-0*-Dihydroxyazobenzene, G. Frederick Smith Chemical Company, Columbus, OH

8 Szoka, F.C., Jacobson, K. and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 551, 295—303
9 Papahadjopoulos, D., Nir, S. and Ohki, S. (1972) Biochim. Biophys. Acta 266, 561—583
10 Papahadjopoulos, D. and Watkins, J.C. (1967) Biochim. Biophys. Acta 135, 639—652
11 Jonas, A. and Maine, G.T. (1979) Biochemistry 18, 1722—1728
12 Van Dijck, P.W.M., De Kruijff, B., Aarts, P.A.M.M., Verkleij, A.J. and DeGier, J. (1978) Biochim. Biophys. Acta 506, 183—191
13 Lentz, B.R., Barenholz, Y. and Thompson, T.E. (1976) Biochemistry 15, 4521—4528
14 Kahane, I. and Razin, S. (1977) Biochim. Biophys. Acta 471, 32—38
15 Kirby, C.J. and Green, C. (1977) Biochem. J. 168, 575—577
16 Poznansky, M.J. and Lange, Y. (1978) Biochim. Biophys. Acta 506, 256—264
17 Krupp, L., Chobanian, A.V. and Brecher, P.I. (1976) Biochem. Biophys. Res. Commun. 72, 1251—2158
18 Jonas, A., Hesterberg, L.K. and Drengler, S.M. (1978) Biochim. Biophys. Acta 528, 47—57
19 Pownall, H.J., Massey, J.B., Kusserow, S.K. and Gotto, A.M. (1979) Biochemistry 18, 574—579