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Drugs-in-cyclodextrins-in liposomes: a novel concept in drug delivery

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Abstract

Inclusion complexes of tritiated dehydroepiandrosterone (DHEA), retinol (R) and retinoic acid (RA) were formed with ^{14}C-labelled 2-hydroxypropyl-β-cyclodextrin (HPβCD) or unlabelled β-cyclodextrin (βCD) polymers 2009 and 2010 (Mol. Wt 4000–4500 and 8700, respectively) at various molar ratios. Formation of inclusion complexes was confirmed by the complete or partial solubilization of the drugs used and by the simultaneous elution of drug and HPβCD radioactivities following molecular sieve chromatography of the complex solutions. Inclusion complex solutions (also containing 'void' cyclodextrins) were subsequently entrapped into dehydration-rehydration vesicles (DRV liposomes). Ratios of entrapment values (% of amounts used) for drugs and cyclodextrin (HPβCD) approximating unity were taken to denote entrapment that did not discriminate between complexes and void cyclodextrin. Near unity ratios and highest entrapment values (e.g., up to $32.3 \pm 11.9\%$ (DHEA) and $31.9 \pm 11.8\%$ (HPβCD) of the materials used; distearoyl phosphatidylcholine (DSPC) DRV) were achieved with liposomes made of phospholipids with a high gel liquid crystalline transition temperature (T_c) or, when equimolar (to the phospholipid) cholesterol was also present, with all phospholipids, regardless of their T_c. When DSPC liposomes (without or with equimolar cholesterol) containing drug (DHEA, R or RA) complex solutions with cyclodextrins were exposed to rat blood plasma at 37°C for up to 60 min, cyclodextrin (HPβCD) retention was nearly complete (0.7–11.9% released at 60 min). However, release of drugs was considerable with values being significantly greater for DHEA (60.2–62.0%) than for R or RA (26.6 and 26.8%, respectively). Experiments with DRV containing both carboxyfluorescein (CF) (as a marker of vesicle stability) and inclusion complex solutions revealed that entrapped cyclodextrins do not destabilize liposomes. Instead, data suggest that during or after the entrapment of complex solution into liposomes, some of the included drug is displaced from the cyclodextrin cavity by phospholipid and/or cholesterol (to a degree probably dependent on the stability constant of the complex) to end up in the lipid bilayer in a state which, on incubation, ensures rapid release into the media. Results suggest that entrapment of water-insoluble (or certain soluble drugs) in the form of cyclodextrin-inclusion complexes into the aqueous phase of liposomes may circumvent some of the problems associated with their entrapment as such.

Keywords: Liposome; Cyclodextrin; Cyclodextrin polymer; Inclusion complex; Liposomal stability; Drug entrapment

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1. Introduction

Liposomes (Gregoriadis, 1993) and cyclodextrins (Uekama and Otagiri, 1987) have been investigated extensively in the last two decades as 'containers' of molecules in drug delivery and a variety of other uses. However, the structural characteristics of the two systems, the way by which they encapsulate and retain molecules as well as their behaviour in vivo differ radically. Cyclodextrins, for instance, a family of hydrophobic cavity-forming water-soluble oligosaccharides that can accommodate water-insoluble drugs ('guests') in their cavities to form water-soluble inclusion complexes (Frank, 1975), can have their guest molecules replaced by other molecules in the biological milieu with greater affinity for the cavity (Uekama and Otagiri, 1987), can be haemolytic (Irie et al., 1982) or removed rapidly from the blood circulation through the kidneys with ensuing local toxicity (Frank et al., 1976). Thus, whilst cyclodextrins are useful in improving drug formulations (e.g., increased drug solubility, suppressed volatility, masking of taste, etc.) (Szejtli et al., 1983; Gal-Fuzy et al., 1984; Stadler-Szoke et al., 1985) and in many instances, the pharmacological action of included agents (Bootsma et al., 1989; Hassan et al., 1990; Puglisi et al., 1991), their behaviour in vivo is far from ideal (Uekama and Otagiri, 1987). Liposomes, on the other hand, can retain drug contents en route to their destination and, when appropriately tailored, exhibit predermined rates of clearance and tissue distribution (Gregoriadis, 1988). However, accommodation of water-insoluble drugs in the lipid bilayers of liposomes is often limited in terms of drug to lipid mass ratio and drug choice, can be detrimental to bilayer formation and stability and requires the use of organic solvents (Gregoriadis et al., 1993).

Here we report on a novel concept (Gregoriadis and McCormack, 1993, 1994) in drug delivery which, by taking advantage of certain properties of cyclodextrins and liposomes, combines them into a single system to circumvent problems associated with both systems. The concept, entailing entrapment of water-soluble cyclodextrin inclusion complexes in liposomes, would allow passive accommodation of insoluble drugs in the aqueous phase of vesicles to an extent that is dependent on the concentration of the complexes in the solution during vesicle formation and the method of entrapment used. This would potentially increase the drug to lipid mass ratio to levels above those attained by conventional drug incorporation into the lipid phase, enlarge the range of insoluble drugs amenable to encapsulation to include, for instance, membrane destabilizing agents, allow targeting of complexes to specific sites and reduce toxicity. In the present work, water-soluble inclusion complexes of cyclodextrins with dehydroepiandrosterone, retinol and retinoic acid were prepared and entrapped into multilamellar liposomes by the dehydration-rehydration procedure. Complex-containing liposomes were then exposed to blood plasma and the retention of drug and cyclodextrin by the vesicles was monitored. Results show that complex entrapment into liposomes depends on the lipids used and conditions of entrapment. Nearly all of the cyclodextrin and considerable portions of the drugs were found to remain associated with the carrier in the presence of plasma.

2. Materials and methods

2.1. Materials

The sources and grades of egg phosphatidylcholine (PC), dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), hydrogenated egg phosphatidylcholine (HPC), sphingomyelin (SM), phosphatidic acid (PA), cholesterol (CHOL) and carboxyfluorescein (CF) have been described elsewhere (Kirby and Gregoriadis, 1984). Dehydroepiandrosterone (DHEA), retinol (R), retinoic acid (RA) and 2-hydroxypropyl-β-cyclodextrin (HPβCD) were purchased from Sigma Chemical Co. (Poole, U.K.). [1,2-^{3}H(N)]-Dehydroepiandrosterone ([^{3}H]DHEA; 37 MBq), [11,12,^{3}H(N)]retinol (9.25 MBq) ([^{3}H]R) and [11, 12-^{3}H(N)]retinoic acid (3.7 MBq) ([^{3}H]RA) were from Du Pont (U.K.) Ltd, New England Nuclear Products (Stevenage, Herts, U.K.). ^{14}C-labelled

DSPC ([^{14}C]DSPC) (100 μCi/ml) was from Amersham International (Amersham, U.K.) and ^{14}C-labelled 2-hydroxypropyl-β-cyclodextrin ([^{14}C]HPβCD; 200 μCi/ml) from the University of Florida, Nuclear Sciences Centre (Gainesville, FL). β-Cyclodextrin (βCD) polymers 2009 (Mol. Wt 4000–4500) and 2010 (Mol. Wt 8700), each containing 52% of their weight βCD, were purchased from Cyclolab (Budapest, Hungary).

2.2. Formation of inclusion complexes

DHEA (11.2–22.5 mg), R (10 mg) and RA (10 mg) were each dissolved into 2 ml chloroform and the solutions mixed respectively with tracer [^{3}H]DHEA (7×10^5 dpm), [^{3}H]R (7×10^5 dpm) and [^{3}H]RA (6.35×10^5 dpm). Following evaporation of the chloloform with a stream of oxygen-free nitrogen, deionized water (1–2 ml) containing 450 mg (for DHEA), 100 mg (for R) and 100 mg (for RA) HPβCD mixed with 7.25×10^5–2.4×10^6 dpm [^{14}C]HPβCD, was added to the dry materials. Initial molar ratios of drug to cyclodextrin were 1:8.52–1:4.26 (DHEA), 1:2.1 (R) and 1:2.1 (RA). Water-soluble inclusion complexes were formed within seconds (DHEA; 20°C) or after stirring for 2–3 days (R and RA; 30°C). The clear solutions of DHEA and R complexes formed were filtered through packed glass wool to remove non-solubilized matter. The RA complex suspension being milky, was centrifuged at 70000 $\times g$ for 60 min in a refrigerated Sorval Combi Plus ultracentrifuge and the pellet and clear supernatant were assayed for [^{3}H]RA and [^{14}C]-HPβCD radioactivity. Over 98% of the cyclodextrin was recovered in the filtrate and the supernatant. The final drug to HPβCD molar ratios in the clear solutions were lower than initially and estimated (on the basis of radioactivity measurements) as 1:8.25–1:5 (DHEA), 1:4.5 (R) and 1:5 (RA). In the case of R and RA, all steps described were carried out in the dark to avoid photodegradation of materials. In one experiment, DHEA inclusion complex with HPβCD was formed at an initial molar ratio of 1:1 by stirring the mixture for 2 days. The milky suspension formed was then centrifuged at 70000 $\times g$ for 60 min as above and the pellet and clear supernatant were assayed for [^{3}H]DHEA and [^{14}C]HPβCD radioactivity. Whereas practically all (over 98%) of the HPβCD was recovered in the supernatant, only 53% of DHEA became solubilized, giving a final molar ratio of 1:1.8. In other experiments, DHEA (2.24 mg) mixed with [^{3}H]-DHEA (3.77×10^6 dpm) as above was included in β-cyclodextrin (β-CD) polymers 2009 (90 mg) and 2010 (90 mg) by stirring the mixtures for 2 days at 30°C. Initial molar ratios, based on the β-CD content (52% w/w) of the polymers, were 1:2 (polymer 2009) and 1:1 (polymer 2010). The solutions formed were filtered as above to remove any non-solubulized matter. Because of the unavailability of radiolabelled polymers, it was not possible to estimate final molar ratios.

2.3. Entrapment of inclusion complexes into liposomes

The dehydration-rehydration procedure (Kirby and Gregoriadis, 1984) with modifications was used for the entrapment of inclusion complexes into liposomes. In brief, small unilamellar vesicles (SUV) (2 ml) prepared (Kirby and Gregoriadis, 1981) from a variety of phospholipids (32 μmol) without or with cholesterol (32 μmol), were mixed with doubly radiolabelled (3.5×10^4–1.75×10^5 dpm ^{3}H and 3.6×10^4–6×10^5 dpm ^{14}C) (see above) HPβCD-DHEA in 0.05 ml (22.5 mg HPβCD, 0.5–5 mg DHEA), HPβCD-R in 0.5 ml (25 mg HPβCD, 1.1 mg R) or HPβCD-RA in 0.5 ml (25 mg HPβCD; 1.06 mg RA) or with 1.0 ml tritiated DHEA complex with β-CD polymers 2009 (45 mg polymer, 1.12 mg DHEA) and 2010 (45 mg polymer, 1.12 mg DHEA). In one experiment, SUV made of DSPC (32 μmol) supplemented with 2×10^5 dpm [^{14}C]DSPC, were mixed with tritiated drug inclusion complex solution (HPβCD-DHEA) as above but devoid of [^{14}C]-HPβCD tracer. In other experiments, 0.2 M CF (0.2 ml) alone or together with tritiated DHEA inclusion complex solutions (HPβCD-DHEA, polymer 2009-DHEA or polymer 2010-DHEA as above) was mixed with 2 ml SUV made of DSPC (32 μmol). All mixtures were subsequently diluted to 3 ml and freeze-dried (Kirby and Gregoriadis, 1984) overnight as such or after further

dilution with deionized water to 10 ml. Controlled rehydration of the dried material to generate multilamellar (Gregoriadis et al., 1993) dehydration-rehydration vesicles (DRV) was carried out as described (Kirby and Gregoriadis, 1984) at temperatures above the gel-liquid crystaline transition temperature (T_c) of the phospholipids used. To separate liposome-entrapped from non-entrapped inclusion complex solutions, DRV suspensions, diluted to 10 ml with 0.15 M sodium phosphate buffer (pH 7.4) supplemented with 0.9% NaCl (PBS), were centrifuged at 36000 × g for 25 min at 4°C and the liposomal pellet suspended in 5 ml PBS and centrifuged again as above. The pellets containing entrapped inclusion complex solution were resuspended in PBS (1 ml final volume) and, together with the supernatants containing the non-entrapped complex solution, were kept at 4°C until further use.

2.4. Assay of radioactivity

Samples, including those from the suspended pellet of each of the liposomal preparations and the supernatants, were mixed with 4 ml Hisafe solution and assayed for ^3H and/or ^{14}C in a Wallac 1409 liquid scintillation counter appropriately programmed for the simultaneous assay of the two isotopes. Values obtained were used to monitor the presence of drugs (^3H), HPβCD (^{14}C) or DSPC (^{14}C) in various samples and, in the case of liposomes, for the estimation of percent entrapment of total cyclodextrin (HPβCD) and drug.

2.5. Assay of carboxyfluorescein

Measurement of CF in liposomal preparations, plasma, PBS and water in the absence and pres-

Table 1
Entrapment of DHEA-HPβCD inclusion complex solutions into liposomes

Liposomes	Freeze-dried volume (ml)	Complex solutions entrapped (% of amount used)		% ^3H/% ^{14}C ratio
		[^3H]DHEA	[^{14}C]HPβCD	
PC	3	3.0	0.7	4.28
PC	10	10.0	4.0	2.50
PC/CHOL	3	5.8	5.5	1.05
PC/CHOL	10	10.5	10.4	1.00
DSPC	3	11.7	10.2	1.14
DSPC	10	32.3 ± 11.9(7) [a]	31.9 ± 11.8(7) [a]	1.01
DSPC/CHOL	3	7.4	6.1	1.21
DSPC/CHOL	10	21.5 ± 9.1(5)	21.0 ± 7.8(5)	1.02
DMPC	10	14.7	1.0	14.70
DMPC/CHOL	10	6.5	6.7	0.97
DPPC	10	16.1	14.2	1.13
DPPC/CHOL	10	16.8	20.0	0.80
SM	10	7.9	0.9	8.77
SM/CHOL	10	17.0	18.4	0.92
HPC	10	34.4	38.6	0.90
HPC/CHOL	10	15.6	14.8	1.05
DSPC/CHOL/PA	10	37.0	35.0	1.05

[^3H]DHEA solubilized with [^{14}C]HPβCD was entrapped in DRV liposomes composed of a variety of phospholipids without or with equimolar cholesterol at temperatures above the T_c of the phospholipid. Dehydration (see section 2) was carried out at volumes of 3 or 10 ml. Final molar ratio of DHEA and HPβCD in the solutions used for entrapment were 1:5 unless otherwise stated. Values of complex solutions entrapped are % (±S.D. with number of preparations in parentheses when appropriate) of DHEA and total HPβCD (complexed and void) used for entrapment. For other details see section 2.

[a] Final molar ratios of DHEA and HPβCD used for entrapment in seven different DRV preparations ranged from 1:8.25 to 1:1.8. As percent entrapment values for each of the DHEA and HPβCD moieties were not significantly different, values were pooled.

ence of Triton X-100 was carried out as described elsewhere (Kirby and Gregoriadis, 1981).

2.6. Incubation of liposomes with blood plasma

Male rat (Wistar strain) fresh blood plasma (2 ml), PBS (2 ml) or deionized water (2 ml) prewarmed at 37°C were mixed with 0.3 ml of liposomes (9.6 μmol phospholipid) containing radiolabelled (approx. 10^4–5×10^4 dpm ^{3}H and 10^4–10^5 dpm ^{14}C) inclusion complex solution (approx. 1.5–2.5 mg HPβCD and 0.07–0.1 mg drug) and CF (when co-entrapped) or CF only and incubated at the same temperature. Samples (1.0 ml) were removed at time intervals and, after dilution to 5 ml with PBS, were centrifuged at $36000 \times g$ for 25 min at 4°C to separate liposomes (with entrapped materials) from the incubation medium. The pellets were then resuspended in 0.5 ml PBS and, together with the supernatants, assayed for ^{3}H, ^{14}C and CF as above to estimate released radioactivity and CF (when present).

3. Results and discussion

Formation of inclusion complexes with HPβCD or βCD polymers 2009 and 2010 and DHEA, R or RA was confirmed by the complete or partial solubilization of these highly insoluble drugs in the presence of the aqueous solutions of cyclodextrins. Further evidence for inclusion complex formation was supplied by molecular sieve chromatography on Sephadex G-10 (Pharmacia) of the doubly radiolabelled drug-HPβCD solutions (Gregoriadis and McCormack, 1993): a considerable proportion (75–96%, depending on the drug used) of ^{3}H radioactivity (drug) eluted and peaked together with ^{14}C radioactivity (HPβCD) (over 97% eluted) in identical patterns (results not shown). However, as HPβCD or βCD polymers were mostly used in excess of drugs in molar terms, and as in some cases drugs were solubilized only partially (see section 2), inclusion complexes and 'void' cyclodextrins were assumed to be both present in the solutions.

3.1. Entrapment of HPβCD-drug complexes into liposomes

Ratios of entrapment values (% of the amounts used) for drugs and cyclodextrin (HPβCD) approximating unity (1.00) were taken to denote entrapment that did not discriminate between complexes and void cyclodextrin. Table 1 shows that such entrapment ratios of near unity for DHEA were obtained with liposomes made of phospholipids with a high T_c (e.g., DPPC, DSPC or HPC) and, in the case of DSPC at least, regardless of the final molar ratios of DHEA and HPβCD in the solutions used (1:8.25 to 1:1.8; see legend of Table 1) or the presence of a negative (PA) charge on the bilayers. However, with liposomes made of SM with a T_c (around 37°C) slightly below that of DPPC (41°C) (New, 1990), or with 'low melting' phospholipids such as PC and DMPC, entrapment appeared to favour the DHEA, with drug to HPβCD entrapment

Table 2
Entrapment of R/HPβCD and RA/HPβCD inclusion complex solutions into liposomes

Liposomes	Complex solutions entrapped (% of the amount used)					
	[^{3}H]R/[^{14}C]HPβCD			[^{3}H]RA/[^{14}C]HPβCD		
	^{3}H	^{14}C	% ^{3}H/% ^{14}C	^{3}H	^{14}C	% ^{3}H/% ^{14}C
PC	5.7	1.4	4.07			
DSPC	17.9	18.3	0.98	24.7	26.8	0.92
DSPC/CHOL	18.2	22.9	0.80	13.9	15.1	0.92

[^{3}H]R or [^{3}H]RA solubilized with [^{14}C]HPβCD were entrapped in DRV composed of PC only or DSPC without or with equimolar cholesterol at temperatures above the T_c of the phospholipid. Dehydration (see section 2) was carried out at a volume of 10 ml. Final molar ratios of drug and HPβCD in the solutions used for entrapment were 1:4.5 (R) and 1:5 (RA). Values of complex entrapped are % of R or RA and total HPβCD (complexed and void) used for entrapment. For other details see section 2.

ratios reaching values of up to 14.7 (DMPC DRV) (Table 1). On the other hand, when cholesterol was also present, near unity values were achieved regardless of the phospholipid used (e.g., PC, DMPC, SM or DSPC) (Table 1). Moreover, percent entrapment values for both DHEA and HPβCD were generally higher (e.g., up to 32.3 ± 11.9 and 31.9 ± 11.8% respectively for seven DSPC preparations; Table 1) when dehydration of the mixture of SUV and complex solution (see section 2) was carried out after dilution from 3 to 10 ml (compare also entrapment values with the two volumes of 3 and 10 ml, for PC/CHOL, and DSPC/CHOL DRV; Table 1). This was anticipated from previous work (Kirby and Gregoriadis, 1984) showing that sugars (cyclodextrins in this instance) at certain concentrations can act as cryoprotectants during dehydration, thus preserving the structure of SUV and preventing efficient DRV formation. Presumably, diluting to 10 ml reduced the concentration of cyclodextrin to levels that were too low for cryoprotection to occur.

As with DHEA/HPβCD, similarly low entrapment values and a high drug to HPβCD entrapment ratio (4.01) were observed when the R/HPβCD complex solution was used for incorporation into PC liposomes (Table 2). In contrast, entrapment of both the R and RA complex solutions was considerable with near unity entrapment ratios being achieved when liposomes were made of DSPC with or without cholesterol (Table 2). It would thus appear that poor entrapment of two different drugs (DHEA and R) (and corresponding entrapment ratios of well above unity) by cholesterol-free DRV made of low melting phospholipids is probably related to the presence of cyclodextrin rather than the type of drug used. It is conceivable for instance that HPβCD interferes with liposome formation or bilayer stability (and hence solute entrapment) to an extent that is directly dependent on the degree of bilayer fluidity. In this respect, it is of interest to note that, generally, entrapment values for both drug and HPβCD become greater and/or entrapment ratios approach unity in the presence of cholesterol in DRV made of low melting phospholipids (including SM) and in its absence in DRV made of high melting phospholipids (Tables 1 and 2). The respective presence and absence of cholesterol in such liposomes is known to promote bilayer rigidity (New, 1990). It is also probable that, because of the high entrapment ratios for

Table 3
Release of drugs and HPβCD from liposomes

Liposomes	Drug	Final drug/HPβCD molar ratio	Medium	% released			
				2 min		60 min	
				^{3}H	^{14}C	^{3}H	^{14}C
DSPC	DHEA	1:1.8	PBS	34.3	3.8	29.4	1.5
	DHEA	1:1.8	plasma	58.3	1.2	62.0	4.6
DSPC	DHEA	1:5.0	PBS	30.2	1.7	35.6	3.6
	DHEA	1:5.0	plasma	49.3	1.4	60.2	6.1
DSPC/CHOL	DHEA	1:5.0	PBS	55.4	1.9	48.4	3.3
	DHEA	1:5.0	plasma	86.0	5.2	88.2	3.4
DSPC	R	1:4.5	PBS	12.6	1.1	15.3	1.8
	R	1:4.5	plasma	12.6	0.7	26.8	2.5
DSPC/CHOL	R	1:4.5	PBS	17.1	5.2	25.9	3.8
	R	1:4.5	plasma	25.0	2.5	35.6	2.0
DSPC	RA	1:5.0	PBS	12.6	4.7	17.5	5.6
	RA	1:5.0	plasma	14.7	4.3	26.6	3.2
DSPC/CHOL	RA	1:5.0	PBS	28.4	8.8	29.2	8.5
	RA	1:5.0	plasma	32.2	5.4	35.1	11.9

DRV liposomes composed of DSPC without or with equimolar cholesterol and containing [^{3}H]DHEA, [^{3}H]R or [^{3}H]RA complex solutions with [^{14}C]HPβCD were incubated in the presence of PBS or rat plasma. Values of released ^{3}H (drug) and ^{14}C (HPβCD) radioactivity are% of total in the incubated DRV preparations. For other details see section 2.

DHEA/HPβCD observed with the least stable, fluid liposomes (e.g., PC, DMPC and SM DRV; Table 1), HPβCD complex interaction with the phospholipids leads to drug loss from the cyclodextrin cavity (perhaps through drug displacement by phospholipid molecules; Uekama and Otagiri, 1987), with the freed drug being preferentially retained by the vesicles (Tables 1 and 2).

3.2. The effect of blood plasma on the stability of liposomes containing cyclodextrin complex solutions

Use of liposomes for the transport of cyclodextrin-drug inclusion complexes to target tissues in vivo requires substantial retention of the complexes by the carrier in the blood circulation. Previous work (Gregoriadis, 1988) has indicated that liposomes made of phospholipids with a high T_c (e.g., DSPC) retain nearly all of their solute content in the presence of blood, both in vitro and in vivo. As already shown (Tables 1 and 2), DSPC liposomes without cholesterol, and to a lesser extent with cholesterol, are the vesicles of choice for optimum entrapment of cyclodextrin drug complex solution. Therefore, such liposomes would be expected to retain much of the entrapped complex solution in the presence of blood plasma.

Table 3 shows results from an experiment in which DSPC or DSPC/CHOL DRV containing doubly radiolabelled complex solutions of DHEA, R or RA with HPβCD were monitored in terms of drug (^{3}H) and HPβCD (^{14}C) release in the presence of plasma or PBS at 37°C. Data indicate that, whereas only 2.0–11.9% of HPβCD is released at 60 min (all drug formulations with DSPC or DSPC/CHOL DRV), release of drugs (^{3}H) is considerable, with values being significantly greater for DHEA (60.2–62.0%) than for R or RA (26.6 and 26.8% respectively) (Table 3; 60 min). Liposomes made of DSPC and cholesterol lose even more of their drug content in the presence of plasma and again, drug loss is greater for DHEA (88.2%) than for R or RA (35.6 and 35.1% respectively) (Table 3; 60 min).

It appears that most of the DHEA, R and RA are released in the media as such since HPβCD presence in the media (both plasma and PBS) is too low (see Table 3) to significantly reflect release of soluble drug-HPβCD complexes. This is especially so with the DRV preparation where the final molar ratio of DHEA and HPβCD in the solution used for entrapment was relatively high (1:1.8). Moreover, molecular sieve chromatography of the PBS and plasma supernatants (60 min incubation) on a Sephadex G10 column, revealed elution patterns for the drugs that were different from the pattern obtained with the original HPβCD/drug complex solutions (e.g., DHEA) used for entrapment. For instance, much (96%; see above) of the [^{3}H]DHEA radioactivity in the latter case eluted together with [^{14}C]-HPβCD whereas, with PBS and plasma supernatants, the drug trailed in later fractions, presumably as a free entity. Taking into account the amount of drugs used for entrapment, average entrapment yield, amount of drug used for incubation and average drug release into the media (see section 2 and Tables 1–3), it is estimated that approx. 0.01–0.02 mg per ml of any of the drugs used is present in the media as free. Although a proportion of these highly insoluble drugs may be solubilized by proteins in the plasma media, their appearance in PBS reflects an estimated solubility in water of at least 0.001–0.002%. The possibility that radiolabelled drugs used were partly degraded, with the labelled moiety escaping from both HPβCD (if included) and liposomes into the media was examined but thought unlikely since ^{3}H radioactivity (drug) release was observed with all three drugs tested. Furthermore, after thin-layer chromatography of radiolabelled DHEA mixed with unlabelled DHEA, over 95% of the radioactivity was recovered in the DHEA spot (results not shown). Another plausible explanation for the appearance of drugs in the media following incubation could be that displaced drug molecules are either somehow associated with phospholipid fragments or micelles from destabilized liposomes to appear in the supernatants, or incorporated into the lipid phase of small liposomes that do not sediment on centrifugation. However, in experiments where ^{14}C-labelled DSPC liposomes containing the complex solution of [^{3}H]DHEA with unlabelled HP-

βCD were incubated with plasma or PBS, whereas DHEA release was similar to that seen in Table 3, practically no DSPC was recovered in the supernatants (less than 1.6% of the total in liposomes) (results not shown).

The mechanism of such release of drugs from liposomes is unclear to us at present. On the other hand, cyclodextrins are known to remove lipid components from cell membranes by forming inclusion complexes with the lipids, especially cholesterol in the case of β-cyclodextrin (Uekama and Otagiri, 1987). It is thus possible that during or after entrapment of the HPβCD/drug complex solutions in liposomes, lipids (e.g., cholesterol, phospholipid or both) enter the cyclodextrin cavity, replacing the drug when the cavity contains it. This could destabilize the bilayers to some extent, rendering them more vulnerable to attack by plasma high density lipoproteins (Gregoriadis, 1988) and to partial loss of drug. Judging from the release paterns of ^{3}H radioactivity (drug) observed in the presence of PBS (Table 3), this (putative) liposomal destabilization must be great enough to allow significant leakage of drugs from the vesicles even in the absence of plasma (PBS).

In an attempt to examine whether or not vesicle destabilization is indeed induced by entrapped cyclodextrins or cyclodextrin complexes, an experiment was carried out in which quenched CF, an appropriate marker of liposomal stability in media of neutral pH (Gregoriadis, 1988), was entrapped in DSPC liposomes alone or together with HPβCD-DHEA or β-CD polymer-DHEA complex solutions. Table 4 shows that, on incubation of CF-containing liposomes with plasma or PBS, release patterns for co-entrapped DHEA and HPβCD (HPβCD/DHEA complex solutions) are similar to those observed in Table 3. Interestingly, release of the drug from liposomes containing the DHEA-βCD polymer complex solutions also occurred (presumably following DHEA displacement by lipids) and was comparable to that seen with DHEA-HPβCD (Table 4). Inspection of the values for released CF, however, shows that these are low (4.7–10.8% at 70 min) for both complex solution-free or complex solution-containing preparations (Table 4). Thus, drug release under the present conditions cannot be explained on the basis of a cyclodextrin-induced bilayer destabilization. A further indication that liposomes with co-entrapped CF and

Table 4
Comparative studies of CF, DHEA and cyclodextrin release from liposomes

DRV content	Medium	% released					
		2 min			70 min		
		^{3}H	^{14}C	CF	^{3}H	^{14}C	CF
DHEA/HPβCD + CF	PBS	30.2	1.6	2.6	27.5	1.6	4.3
	plasma	43.6	1.4	2.2	64.1	2.9	4.7
	water	37.8	13.5	27.4	59.0	40.1	48.4
DHEA/βCD 2009 + CF	PBS	31.1		5.4	28.4		5.9
	plasma	52.0		4.8	52.6		10.8
	water	28.8		14.8	34.0		36.1
DHEA/βCD 2010 + CF	PBS	45.0		6.6	41.4		13.3
	plasma	58.3		8.9	61.4		10.5
	water	42.5		11.3	39.4		31.9
CF	PBS			1.7			5.9
	plasma			4.5			8.3
	water			11.9			30.0

DRV liposomes composed of DSPC and containing CF with or without co-entrapped [^{3}H]DHEA complex solutions with [^{14}C]HPβCD or βCD polymers 2009 and 2010 were incubated in the presence of PBS, plasma or deionized water. Values of released [^{3}H]DHEA and [^{14}C]HPβCD radioactivity or CF are % of total in the incubated DRV preparations. For other details see section 2.

complex solutions are relatively stable is provided by data obtained on their exposure to distilled water (Table 4): release values (e.g., at 70 min) are greatly augmented for both cyclodextrin (HPβCD) (40.1%) and CF (30–48.4%), probably as a result of osmotic shock. As shown previously (Kirby and Gregoriadis, 1984), osmotic shock occurs with loaded stable DRV when exposed to water.

Assuming that drugs used in the present study are indeed displaced from the cyclodextrin cavity by lipids during entrapment of the inclusion complex solutions into liposomes, the question as to the mechanism of their release from stable vesicles into the media still remains. Since all three drugs are highly lipophilic, it is likely that their displacement from the cyclodextrin cavity is followed by accommodation in the lipid bilayers which could, as a result, be rendered more fluid (Castelli et al., 1984) and hence more permeable (Gregoriadis, 1988) to the drugs. Because considerable loss of all three drugs occurs even after 2 min of exposure to PBS (Tables 3 and 4), it is possible that, following their displacement, drugs are localized (to an extent that is dependent on the type of drug used) at or near the lipid-water interface of the vesicles and are thus available to desorption. This is supported by work (Castelli et al., 1984, 1992) showing that interaction between certain amphipathic molecules and phospholipid polar heads occurs only at the surface of lipid bilayers without significant involvement of the acyl chains and by previous findings (Kirby and Gregoriadis, 1983) that lipophilic drugs entrapped in stable liposomes are prone to rapid partial loss on exposure to plasma.

4. Conclusions

Water-soluble cyclodextrin complex solutions of insoluble drugs can be entrapped into the aqueous phase of stable multilamellar liposomes made by the dehydration-rehydration procedure. Optimal entrapment of complexes together with void cyclodextrins is achieved either when liposomes are composed of high melting phospholipids or, when equimolar cholesterol is also present, regardless of the phospholipid used. It appears that during complex solution entrapment, included drugs are partially displaced from the cyclodextrin cavity by liposomal lipids to a degree that is dependent on the drug used, i.e., probably the stability constant of the complex. Displaced drugs are rapidly released from liposomes in the presence of plasma or even PBS. Work is in progress both to establish conditions of entrapment under which drug displacement is minimal and to identify water insoluble drugs that resist significant displacement by liposomal lipids. It is recognized that drugs employed here may not necessarily meet the requirements for a preference of the present approach over that of direct accommodation into the lipid bilayers. It is anticipated however, that entrapment of a wide range of water-insoluble or soluble drugs as cyclodextrin inclusion complexes into the aqueous phase of liposomes will circumvent problems associated with their use as such.

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