International Journal of Pharmaceutics, 14 (1983) 191–208
Elsevier Biomedical Press

The in vitro release of steroids from liposomes

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(Received July 27th, 1982)
(Accepted September 14th, 1982)

Summary

The partitioning of cortisone-21-aliphatic derivatives into dipalmitoylphosphatidylcholine (DPPC) liposomes at 37°C was found to increase with steroid acyl chain length. The free energy of partitioning per methylene addition decreases with increasing chain length, particularly if longer than 16 carbon atoms. The release rate of the cortisone esters from DPPC liposomes decreases with increasing chain length of the 21-substituent and log k (first-order efflux rate constant) is linearly related to n (number of carbon atoms in the steroid ester chain) between n = 6 and 14. Release rates of cortisone esters of acyl chain lengths of 16 to 22 carbon atoms show a faster rate of release than would be expected through extrapolation of the results from n = 6 to 14 chain length esters. The free energy of partitioning of cortisone esters into DPPC liposomes is linearly related to their release rate but short chain esters deviate from this relationship. Release of cortisone hexadecanoate was slower from DPPC liposomes than both dimyristoylphosphatidylcholine (DMPC) and distearoylphosphatidylcholine (DSPC) liposomes.

Introduction

During the elucidation of liposome structure it was realized that selective release of aqueously entrapped material could be achieved. Initial permeation studies used phospholipid 'spherules' as an analogy of the cell membrane (Lundberg, 1979). Following suggestions for the therapeutic use of liposomes, similar efflux experi-

0378-5173/83/0000-0000/$03.00

ments have been used to assess the stability of the drug–liposome complex in vivo (McDougall et al., 1974; Tyrrell et al., 1977); also upon storage (Freise et al., 1979). Indeed it was recommended that the determination of release rates is a necessary prerequisite to the administration of liposome associated drugs (Ryman and Tyrrell, 1979).

Dissolution/release testing has been commonly used in the preliminary testing of implantable sustained release devices, but in vitro evaluation of oily formulations is fraught with problems. Evaluation of release from a liposome preparation may be accomplished by several techniques used to separate bound and free drug. Dialysis has been used to follow drug leakage from liposomes. Unless efflux is much faster through the dialysis membrane than from the liposomes, calculation of true efflux rates is complicated (Bangham et al., 1974). Such rectification of release rates is not required when centrifugation is used to separate non-entrapped material. However, there is the possibility that small unilamellar vesicles may not be completely sedimented (Tyrrell et al., 1976). Centrifugation may also be used to concentrate a liposome suspension and pellets may be redistributed to a well-defined lipid weight per ml. It has been reported that ultrafiltration is not a recommended method for separating entrapped material (Ryman and Tyrrell, 1979). However, it is prospectively useful in concentrating liposome suspensions diluted through gel filtration, or dialysis procedures, and in determining drug efflux rates (Ahmed et al., 1981). Gel filtration is commonly used; the free material remaining on the column while the liposomes are eluted in the void volume. Sharma et al. (1977) have reported some of the problems associated with this method. The influx of hydrophilic materials has been measured, by following spectrophotometrically the osmotic swelling of liposomes (Bangham et al., 1967).

In some of the few instances of the attempted use of liposome encapsulation for sustained systemic therapy the in vitro drug efflux was determined prior to in vivo efflux (Arakawa et al., 1975; Alpar et al., 1981). Other workers, investigating the intramuscular administration of steroids (Shinozawa et al., 1979) and sterols (Fountain et al., 1980) did not perform initial release studies, which were particularly desirable as some hydrophobic materials are known to be released rapidly from liposomes.

Early studies reported the differential permeabilities of liposomes to different materials. Liposomes are freely permeable to water, but cations are released more slowly than anions (Bangham et al., 1974). The speed of efflux of non-electrolytes has been related to their aqueous hydrogen bonding ability (Cohen, 1975). De Gier et al. (1968) have reported effects when varying the alkyl chains of liposomal phosphatidylcholines. Decreases in chain length and increases in unsaturation generally lead to a rise in liposome permeability. However, permeability/temperature studies have led to the conclusion that liposome permeability is more fundamentally related to the degree of disorder of the bilayer. The release of $^{22}Na^{+}$ from DPPC vesicles was very low, below 35°C, but increased to a maximum around the phospholipid transition temperature of 41°C (Papahadjopoulos et al., 1973). The efflux rate fell to a post-37°C minimum at 45°C above which permeability rose steadily with increasing temperature. Similar effects have also been reported for the

release of non-electrolytes from DPPC liposomes, but egg lecithin multilamellar vesicles (MLVs) showed a much less severe temperature dependence (Inoue, 1974). This increase in permeability around the phase transition temperature is attributed to efflux through areas of high disorder and, hence, permeability at this transition.

The rate of release of entrapped material is faster from sonicated preparations than from MLV suspensions.

Also the maximum permeability was found to occur at a temperature a few degrees lower than that recorded for MLV suspensions (Papahadjopoulos et al., 1973). This latter effect reflects constraints due to the smaller radius of curvature of the sonicated liposomes. However, the greater efflux rates of ions from small liposomes may also be attributed to a large surface area to volume ratio and fewer hydrophobic barriers to be traversed before ions can be detected in the external aqueous phase.

The incorporation of 50 mole percent of cholesterol into a liposome formulation decreased the efflux of cations and release rates showed little temperature dependence (de Gier et al., 1968). Such evidence concurs with evidence that cholesterol modulates the fluidity of lecithin bilayers.

The efflux of hydroxocobalamin-HCl from various liposome preparations has recently been studied with a view to use as an alternative sustained release preparation (Alpar et al., 1981). Release rates from the shorter DMPC lecithin was much greater than that following entrapment in DPPC liposomes. Liposomes composed of mixed phospholipids (DPPC/DMPC, 50 mol %) gave intermediate release rates.

The sustained release of drugs from oily vehicles has been achieved by use of highly hydrophobic aliphatic drug esters. The use of such derivatives for the improvement of the stability of the corticosteroid–liposome complex was first suggested by Shaw et al. (1976). Despite the presence of bilayer cholesterol, the amphiphilic steroid, hydrocortisone, its 21-succinate, 21-phosphate and 21-acetate all diffused rapidly from egg lecithin liposomes following aqueous phase incorporation (Shaw, 1978). The incorporation and release of hydrocortisone 21-acyl derivatives from DMPC was studied at 37°C (Shaw et al., 1976). It was found that shortening the alkyl chain of the lecithin increased the rate of release of hydrocortisone-21-octanoate. Use of the 21-palmitate ester significantly lengthened the period of release from DPPC liposomes.

In this paper the relationship between the hydrophobicity of cortisone-21-aliphatic derivatives and their in vitro release from synthetic lecithin formulations will be examined. It is thought that such studies will be of critical importance in the optimization of prospective liposome formulations for in vivo use. The assessment of release from MLVs only, has been undertaken as it is reasonable to assume that the release from these vesicles would be the slowest (Papahadjopoulos et al., 1973).

Materials and methods

Phospholipids

L-α-Dimyristoylphosphatidylcholine (DMPC), L-α-dipalmitoylphosphatidylcho-

line (DPPC) and L-α-distearoylphosphatidylcholine (DSPC), all 98% crystalline, were purchased from Sigma Chemicals, U.K.

Steroids

Δ^4-Pregnen-3,20-dione (progesterone); Δ^4-Pregnen-21-ol,3,20-dione (deoxycorticosterone); Δ^4-Pregnen-11β,17α,21-triol-3,20-dione (hydrocortisone) and Δ^4-Pregnen-17α,21-diol-3,11,20-trione (cortisone) were purchased from Sigma Chemicals, U.K. Cortisone acetate was donated by Boots Co., U.K.

Synthesis of cortisone-21-derivatives

1.4 mmol of cortisone was dissolved in chilled pyridine (4°C) and 2.8 mmol of acid chloride added dropwise whilst stirring constantly. The reaction mixture was stirred at 4°C for 72 h. Acylation was confirmed by TLC analysis using a toluene–ethylacetate (13 : 7) solvent. Pyridine was removed by rotary evaporation at 37°C under vacuum for 20 h. Resulting oil was dissolved in 20 ml chloroform and 5 g of Kieselgel 60 (Merck, F.R.G.) added and the solvents removed. The silica gel was packed into a 1 cm glass column to produce a bed height of 12 cm. This was eluted with petroleum ether (60–80°C boiling fraction) with increasing concentration of ethylacetate and 20 ml fractions collected. The latter were analyzed by TLC and identification was by UV light and a 50% concentrated sulphuric acid spray followed by charring at 150°C for 1 h. Appropriate fractions were pooled and recrystallized twice from hot aqueous ethanol (20% water) before air-drying. Yields varied from 40 to 70%, the higher value being obtained from short chain esters.

Radiolabelled steroids

[4-^{14}C]Progesterone (53 μCi/μmol); [1,2-^{3}H]deoxycorticosterone (44 mCi/μmol); [1,2,6,7-^{3}H]hydrocortisone (100 mCi/μmol); [1,2,6,7-^{3}H]cortisone (35.4 mCi/μmol) were purchased from Amersham International, U.K.

Synthesis of [1,2,6,7-^{3}H]cortisone 21-esters

500 μCi [^{3}H]cortisone (Amersham International, U.K.) was diluted to a specific activity of 36 μCi/μmol in 0.5 ml dry pyridine, 20 μl of the acid chloride added and the flask sealed for 2 (short esters) to 7 days (long esters) at room temperature. TLC analysis ensured that the reaction was complete (see synthesis of cortisone-21-derivatives) by scintillation counting of the ester and cortisone spots. After removal of the pyridine by rotary evaporation at 40°C under vacuum, the residue was dissolved in chloroform–methanol and applied to preparative TLC plates (silica gel GF 254 0.5 mm) and developed as above. The ester band was identified and removed before extracting the ester from a short column of the silica gel upon fine scintre, with 20 ml of ethylacetate. The volume was accurately adjusted to 25 ml and aliquots assayed to find the yield (80–94%). Radiochemical purity was confirmed through TLC of 10 μl portions of this solution and counting of the developed plates, divided into equal areas.

Solvents

Ethanol, absolute (98%). Redistilled 78–79°C boiling fraction collected. Chloroform AR (99%) (Koch-Light, U.K.). Redistilled 61–62°C boiling fraction collected.

Assay of radiolabelled steroids

All aqueous samples were adjusted to 1 ml and incorporated into 10 ml of a scintillation cocktail prior to counting. The cocktail consisted of PPO 15 g, dimethyl POPOP 300 mg, toluene 2 litres and Triton X-100 1 litre. Small amounts of phospholipid did not cause any noticeable decrease in counting efficiency. All samples were counted for 4 min (Intertechnique PG 4000, Kontron, U.K.) using the facilities for computerised background subtract and cpm to dpm conversion using automatic external standardisation. Counting efficiencies were of the order 90% for ^{14}C samples and 40–45% ^{3}H samples.

Liposome preparation

Films of phosphatidylcholine and steroid were prepared in 50 or 100 ml Erlenmeyer quickfit flasks by rotary evaporation at 40°C of a solution in ethanol (parent steroids) or chloroform (cortisone esters). 4 μCi of tritiated steroid (36 μCi/mol) or 2 μCi of ^{14}C-labelled progesterone was included in each film. 25 ml of 0.9% sterile saline was added, the flasks sealed and the films hydrated at a temperature of 15°C in excess of that of the phase transition temperature. (DMPC = 38°C, DPPC = 56°C, DSPC = 69°C). Mechanical shaking for 2 × 1 min in the presence of 4 glass beads led to the formation of an MLV suspension of mean particle size 0.75–2 μm; the smaller mean vesicle size occurring in the more dilute suspensions.

Assessment of steroid efflux rates

Steroid partitioning between phosphatidylcholine and water was predetermined through equilibration at 37°C. Phase ratios were calculated that gave rise to two-thirds liposome associated steroids following equilibrium partitioning. These ratios are listed in Table 1.

Liposomes for assessment of efflux rates were prepared as described above and equilibration of partitioning achieved by shaking at 37°C over a 3–7-day period. Duplicate 5 ml samples were centrifuged to assess steroid distribution at time zero. Efflux of steroid was monitored by periodical centrifugation of samples at 50,000 × g for 1 h (High Speed 25, M.S.E., U.K.) in a temperature pre-equilibrated head, following a 1 in 10 dilution of the initial suspension with sterile 0.9% w/v saline. Duplicate 1 ml samples of suspension and supernatant were taken for scintillation counting.

Calculations

The percentage of steroid released at each time point was calculated from the ratio of supernatant to suspension radioactivity. In order to enable comparison of the release data of steroids and derivatives whose initial partitioning (time zero partitioning) differs, the time zero percentage liposome associated was normalized to

TABLE I

INITIAL PHASE RATIOS USED, PRIOR TO 10×DILUTION, TO STUDY EFFLUX RATES

Steroid	H_2O:DPPC phase ratio
Progesterone	1000
Deoxycorticosterone	250
Cortisone	125
Hydrocortisone	125
Cortisone-21-acetate	303
-butyrate	400
-hexanoate	1000
-octanoate	2000
-decanoate	4000
-dodecanoate	5000
-butadecanoate	6667
-hexadecanoate	10000
-octadecanoate	10000
-docosanoate	12500

100% retained. All subsequent release data were multiplied by the same factor as illustrated in Eqn. 1.

$$\underset{(N_t)}{\text{Normalized \% retained}} = \frac{100}{A_0} \times A_t \tag{1}$$

where A_t = % retained at time, t; and A_0 = equilibrium % retained (prior to dilution).

The first-order rate constants of efflux of cortisone esters from DPPC were calculated from least-squares linear regression analysis of plots of ln normalised % retained (N_t) versus time through means of Eqn. 2.

$$\ln N_t = -kt + \ln N_0 \tag{2}$$

where k = the first-order rate constant of efflux; and N_0 = normalized % retained at time zero ($\simeq$ 100%).

Results and discussion

The release of steroid hormones from DPPC liposomes

The release of parent steroid hormones was too fast to be monitored accurately using centrifugation techniques. However, a dialysis procedure proved inadvisable due to steroid adsorption to polymeric membranes. Unfortunately it was not possible to devise any means of assaying steroid efflux without first separating liposomes and aqueous phases.

The time of sampling stated in the results is the interval between dilution of the initially equilibrated suspension and the commencement of centrifugation. Obviously the mean time of liposome separation is a finite interval after the start of centrifugation but this error is constant provided the centrifugability, i.e. size distribution of the suspension, is constant. No change in DPPC liposome size was found over the first 12 h after which such errors are less significant. Due to these inherent errors in estimation of sampling time and 1 h period required to reproduce sedimentation it was impossible to calculate rate constants of efflux of these parent steroids. However, some estimation of relative release rates may be made from the comparison of steroid retention 1 h post-dilution (Table 2).

The polypolar steroids cortisone and hydrocortisone are released at a faster rate than the more hydrophobic deoxycortisone and progesterone. From Overton's rules it is generally expected that increasing hydrophobicity confers a faster membrane permeation rate, due to the increased partitioning of the substance into the lipophilic membrane. However, in Table 2 an opposite effect is described, the efflux of the less hydrophobic material leaching faster from liposomes. Highly hydrophobic materials when in aqueous solution cause structuring of the surrounding water leading to a large negative entropy change. Hence self-association and partitioning into a hydrophobic phase is energetically favoured. Such effects lower partitioning into the aqueous phase, restrict diffusion in this phase and also may promote any interactions between steroid and bilayer. Hence, the discrepancy between rate of permeation and hydrophobicities of the steroids may be explained by steroid retention in the bilayer.

The partitioning of cortisone-21-aliphatic esters into DPPC liposomes at 37°C

The lengthening of acyl 21-substituents is shown to have a great effect upon the partitioning behaviour of cortisone derivatives. It is apparent that the relationship between the DPPC liposome/water partition coefficient (K) and the carbon chain length of the 21-substituent is sigmoidal (Fig. 1). The effect of increasing chain length is greatest between n = 6 to 16 where K increases regularly with increasing chain length. In this range an increase in K of approximately 1300 results from each methylene added.

TABLE 2

THE RETENTION OF STEROIDS IN DPPC LIPOSOMES 1 h POST-DILUTION

	Partition coefficient 37°C (DPPC/H_2O) (± S.D.) (n = 3)	% retained, 1 h
Progesterone	1300 ± 83	35 ± 10
Deoxycortisone	325 ± 24	43 ± 8
Cortisone	175 ± 13	16 ± 5
Hydrocortisone	168 ± 26	18 ± 6

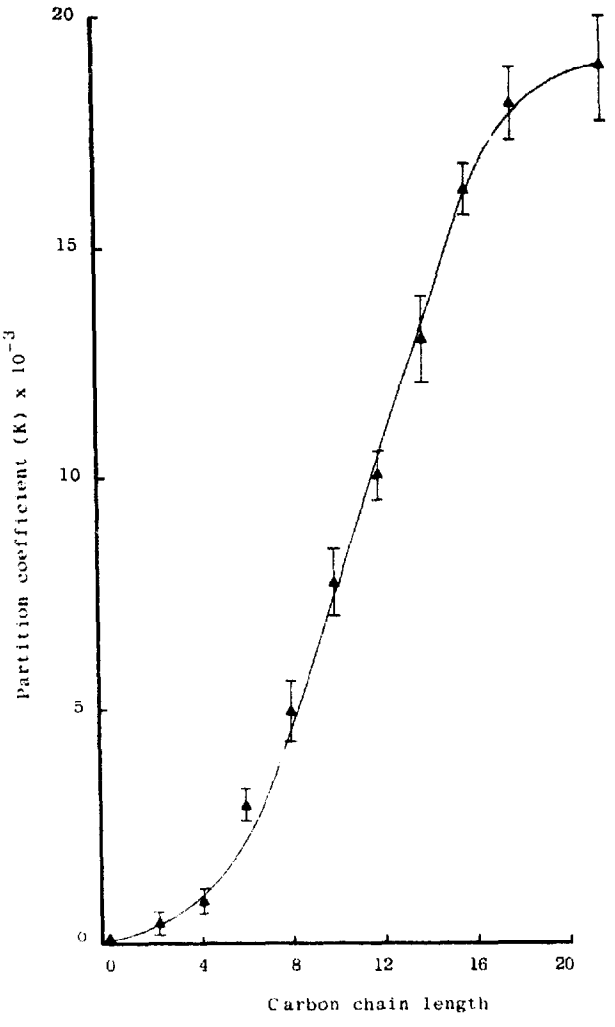

Fig. 1. The variation with chain length of the partitioning of cortisone-21-aliphatic esters into DPPC liposomes at 37°C.

However, a different effect is shown when the methylene group contribution to the free energy of partitioning is considered. These increments have been plotted to facilitate comparison of the free energy of partitioning changes ($\Delta G_{n \rightarrow 1}$) following the addition of a methylene group to cortisone esters of varying chain length (Fig. 2). It is evident that the greatest free energy changes occur upon methylene addition to the short esters, cortisone acetate and butyrate. These effects are similar to those shown by short cortisone esters for partitioning in the DMPC/water system (Arrowsmith et al., 1982). It was concluded that such great effects were unlikely to occur through hydrophobic effects alone and the effect of lengthening short esters was a re-orientation or change of partitioning site of the ester chain within the bilayer facilitating greater hydrogen bonding. However, in DPPC liposomes the results are lower than those found for DMPC liposomes, more similar to the methylene addition effects seen for small alcohols (Diamond and Katz, 1974) and also phenothiazine side chains (Ahmed et al., 1981) in liposome systems.

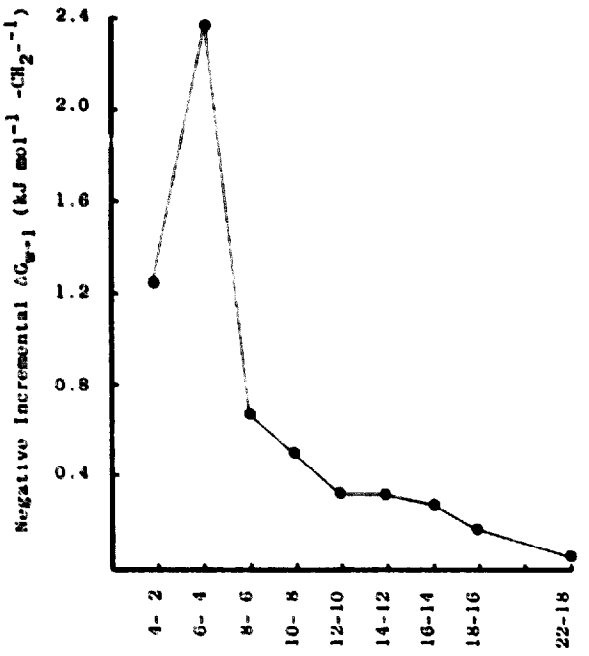

Fig. 2. The variation with chain length of the incremental free energy of partitioning ($\Delta G_{w\to l}$) for the addition of a methylene grouping to the side chain of cortisone-21-aliphatic derivatives, for partitioning into DPPC liposomes at 37°C. Abscissa figures represent the carbon chain length of the esters used to calculate methylene group contribution.

As the series is ascended methylene free energy of partitioning group contribution falls, sharply at first, and then more gradually until the addition to cortisone octadecanoate has little effect. Such results are in conflict with those found for the partitioning of fatty acids into bulk solvents (Smith and Tanford, 1973). A linear increase in $\Delta G_{w\to l}$ per extra methylene grouping is reported for the complete series of acids up to 22 carbon chain length. Discrepancies between the partitioning behaviour of a series of solutes into liposomes and bulk solvents has not been previously reported. This is the first time the partitioning of an aliphatic series into liposomes has been investigated although a homologous series of carbamates has been examined in relation to $^{22}Na^+$ leakage from liposomes (Wood et al., 1979).

The partitioning of cortisone esters is controlled by hydrophobic effects. That is the high negative entropy resulting from water structuring around these esters leads to their great affiliation with the lecithin phase. It is the extra-entropic effect caused by the additional methylenes which raises the partitioning as the ester series is ascended.

The efflux of cortisone-21-aliphatic esters from DPPC liposomes

The first-order efflux profiles of representative cortisone-21-aliphatic esters, from DPPC MLVs is shown in Fig. 3. The linearity of the plots indicate that release proceeds by first-order kinetics once an initial phase of rapid loss is terminated. The importance of this phase decreases with the increasing chain length of the ester, being virtually non-existent during the release of cortisone docosanoate. Similar high

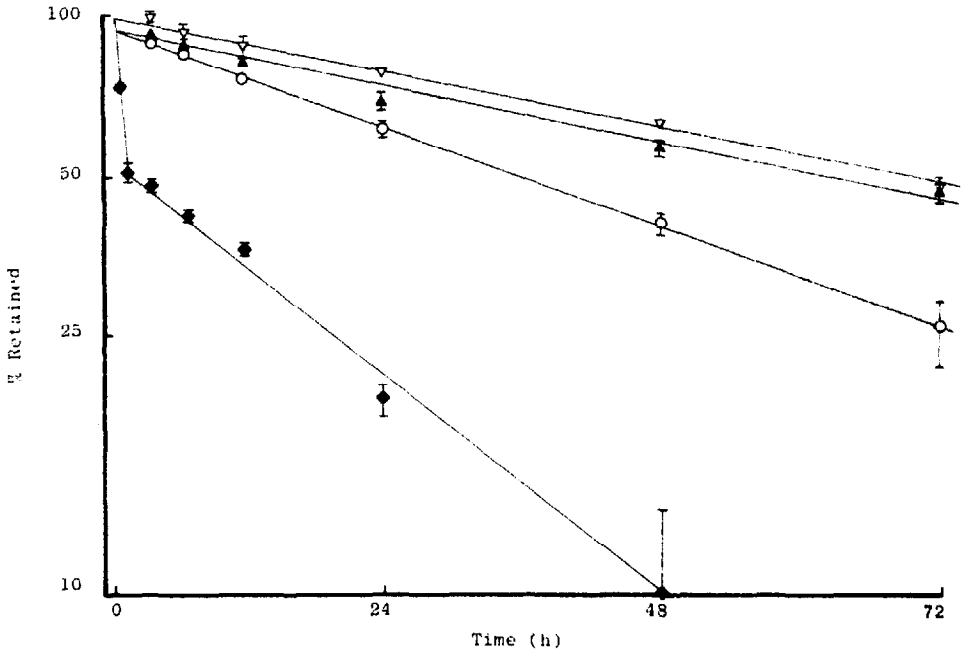

Fig. 3. The release of cortisone esters from DPPC liposomes at 37°C into pH 7 saline. ◆, cortisone butyrate; ○, cortisone decanoate; ▲, cortisone hexadecanoate; ▽, cortisone docosanoate.

initial in vitro release rates of liposome encapsulated hydrophobic drug, vinblastine sulphate, has been ascribed to the desorption of loosely bound drug (Layton et al., 1979). However, similar results have been reported for the release of the hydrophilic drug, hydroxocobalamin hydrochloride, from DPPC and DMPC liposomes (Alpar et al., 1981) and this effect may represent some inherent property of the bilayer or liposome structure.

The proportions of lecithin and steroid (listed in Table 1) have been carefully manipulated to create an initial suspension in which 65–75% of the steroid is liposome associated and the aqueous solubility of the steroid is not exceeded. Ten-fold dilution leads to the efflux of steroid which continues until equilibrium partitioning is re-established with 10–20% of steroid remaining associated with the liposome. Such changes may be predicted from the application of le Chatelier's principle to a partitioning system. The driving force of the efflux of steroid is a discrepancy in the concentration gradient between lipid and water compared to that predicted by the partition coefficient. This situation arises through alteration of the phase ratio by dilution of the aqueous phase. As efflux progresses this driving force is reduced as the concentration ratio becomes similar to the equilibrium partition coefficient. Such a situation predicts that in vitro efflux of hydrophobic materials from liposomes will be of a first-order nature, which is confirmed in Fig. 3.

The rate of release of hydrocortisone-21-octanoate and 21-hexadecanoate reported by Shaw et al. (1976) is considerably slower than the profile presented in Fig. 3. It might be expected that release profiles would be fairly similar, due to their analogous structures. The explanation for these discrepancies lies in the differing

experimental protocol employed. Shaw incorporated into liposome formulation a much greater proportion of steroidal ester than in the experiments presented in this paper, where virtual trace quantities of ester were employed (1 nmol/ml). The severe limitations of the solubilities of steroidal octanoate and particularly hexadecanoate may have retarded measured release rates. Release of steroid may be curtailed due to the approach of the aqueous cortisone ester solution to saturation conditions. Should precipitation of steroid material released in excess of this limit occur such material would also be undetected as Shaw et al. (1976) also used a centrifugation technique for separation of liposomal and free drug. Assuming a hydrocortisone hexadecanoate lecithin/water partition coefficient of 16×10^3 the phase ratio employed of 10^3 (water/lipid) would allow release of only one-sixteenth of the encapsulated palmitate before equilibrium partitioning was re-established. The combination of these two factors leads to data which is non-representative of release from liposomes into a biological sink.

The release rate of esters decreases with increasing chain length of the aliphatic 21-esters (Fig. 3). This effect, for the whole series investigated, is represented in Fig. 4, a plot of first-order efflux rate constant (k) from DPPC MLVs versus the carbon chain length of the aliphatic ester (n). The addition of two methylenes to the acyl chain of cortisone acetate to produce the butyrate and similarly the hexanoate produces large reductions in efflux rate simultaneous with the large negative changes

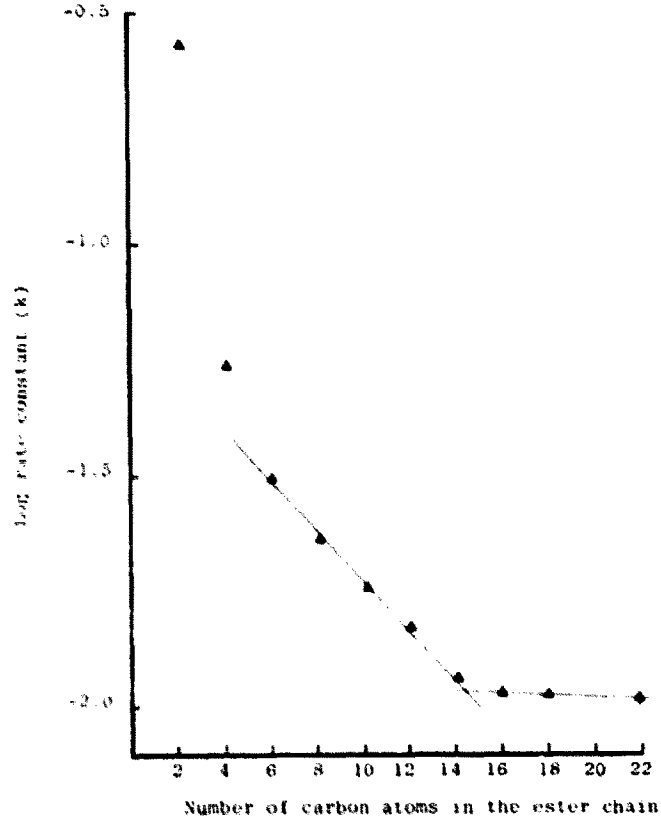

Fig. 4. The relationship between rate constant (k) for release from DPPC liposomes at 37°C and the carbon chain length of cortisone esters.

in free energy of partitioning shown in Fig. 2. The addition of further acyl chain methylenes leads to successively slower reductions in efflux from DPPC liposomes. Between the hexanoate (n = 6) and butadecanoate (n = 14) esters, log k and n are linearly related but an inflexion occurs between 14 and 16 acyl chain length, the release rate of longer esters showing only a slight dependence on carbon chain length (n = 16–22).

These two types of release dependence are represented by the following relationships:

$$\log k = -5.19 \times 10^{-2} n - 1.21 (n = 6 \text{ to } 14) \quad (3)$$

($r = 0.9974$; significant at 0.001 probability level).

$$\log k = -1.59 \times 10^{-3} n - 1.94 (n = 16, 18 \text{ and } 22) \quad (4)$$

($r = 0.7876$).

Fig. 5 illustrates a relationship between log first-order rate constant of efflux (k)

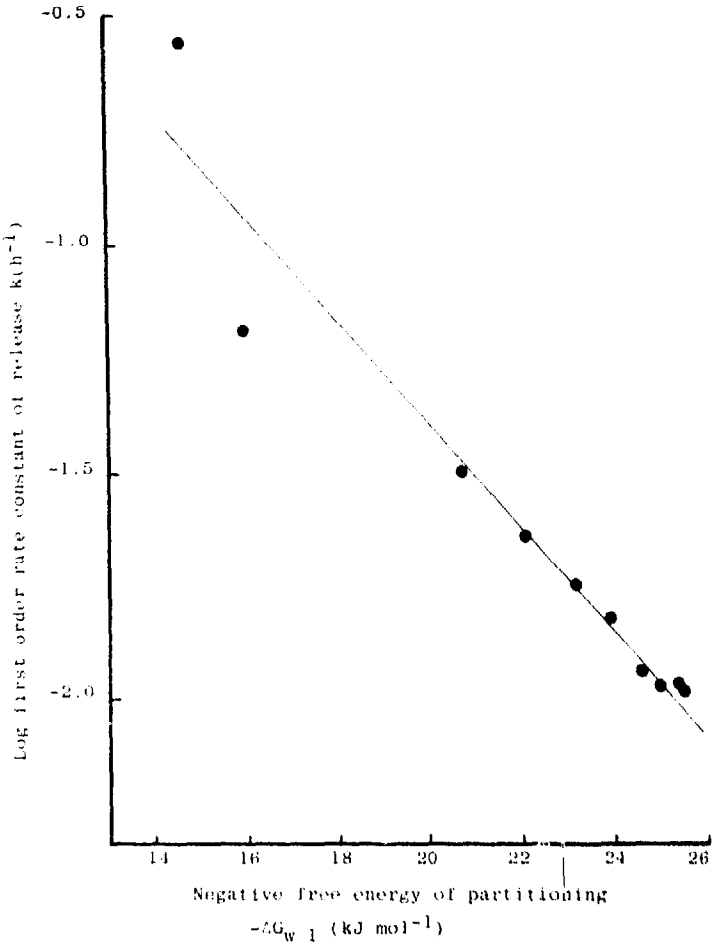

Fig. 5. Log first-order efflux rate constant (k) of cortisone esters from DPPC liposomes at 37°C versus the free energy of partitioning ($\Delta G_{w \to l}$).

and the free energy of partitioning of the esters. A logarithmic decrease in k with increasing negative free energy has been drawn for the full range of acyl chain length (n). Although the values for cortisone acetate and butyrate diverge considerably from this relationship, even including these points the correlation coefficient is significant at the 1% probability level.

$$\log k = 1.10 \times 10^{-1}(-\Delta G_{w \to l}) + 7.95 \times 10^{-1} (n = 2 \text{ to } 22) \quad (5)$$

($r = 0.9535$, significant at 0.001 probability level).

The correlation between log k and the free energy of partitioning is based on the relationship between partition coefficient and the rate constants of transfer between lipid and aqueous phases (Eqn. 6).

$$\Delta G_{w \to l} = -RT \cdot \ln K = -RT \cdot \ln(k_{w \to l}/k_{l \to w}) \quad (6)$$

The release of cortisone-21-hexadecanoate and -octadecanoate from 3 synthetic phosphatidylcholines

The first-order retention profiles of cortisone-21-hexanoate and -octadecanoate from DMPC, DPPC and DSPC MLVs are compared in Fig. 6 and half-lives ($t_{1/2}$) of efflux listed in Table 3. As expected from Eqn. 4, the efflux rates of the two esters from DPPC are very similar, and this is also true for efflux from DMPC liposomes. However, the longer chain ester, octadecanoate, was released significantly slower

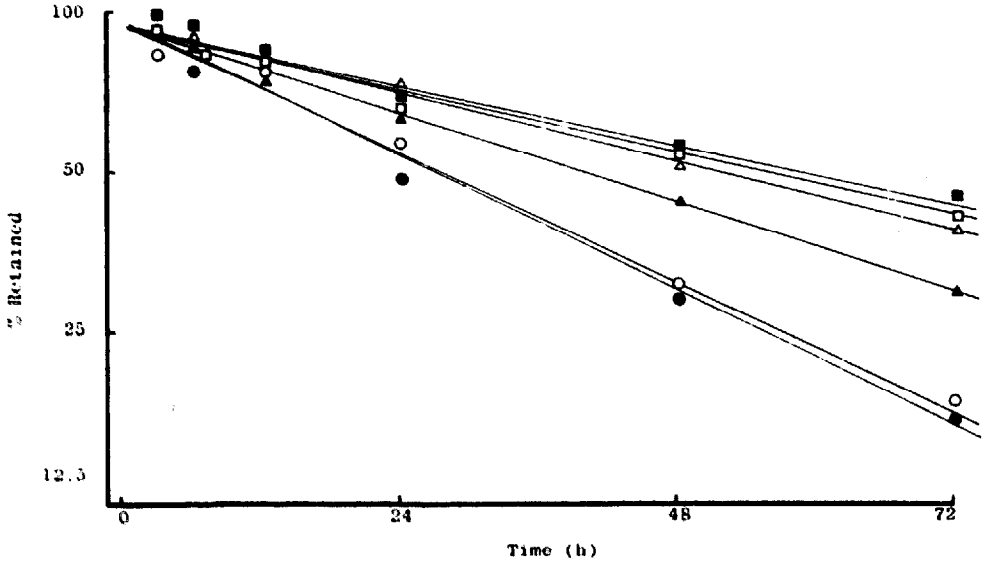

Fig. 6. The release of cortisone hexadecanoate and octadecanoate from liposomes composed of different lecithins, at 37°C.

▲, *hexadecanoate* } DSPC □, *hexadecanoate* } DPPC
△, *octadecanoate* ■, *octadecanoate*
●, *hexadecanoate* } DMPC
○, *octadecanoate*

TABLE 3

THE EFFLUX HALF-LIVES (h) OF CORTISONE HEXADECANOATE AND OCTADECANOATE FROM DMPC, DPPC AND DSPC LIPOSOMES

	$t_{1/2}$ hexadecanoate	$t_{1/2}$ octadecanoate
DMPC	28.8	29.9
DPPC	64.3	63.8
DSPC	43.8	61.9

than cortisone-hexadecanoate release from DSPC liposomes. Efflux of both esters from DMPC liposomes was much faster than efflux from DPPC and DSPC liposomes. Release rates of cortisone-hexadecanoate and -octadecanoate from DPPC liposomes were similar to release of octadecanoate from DSPC liposomes. However, the efflux of hexadecanoate was faster than the release of similar esters from DPPC liposomes.

Factors controlling the efflux of cortisone esters from liposomes

Aqueous phase entropy effects largely govern the partitioning of cortisone aliphatic esters (Arrowsmith et al., 1982). However, their release rate may be modified by other lipid phase and interfacial factors. Diamond and Katz (1974) suggested a model of hydrophobic non-electrolyte permeation across lipid bilayer membranes in which resistance was great at each lipid/water interface. Between these peaks of resistance lies a trough representing dissolution and partitioning in the alkyl chains of the lipid bilayer. The efflux rate of cortisone esters was shown to decrease as hydrophobicity and therefore negative free energy of partitioning increases (Fig. 5). The latter effect may be interpreted as an increase in the energy a molecule requires to diffuse from solution in the core of the bilayer through the lipid/water interface.

The energy barriers related to crossing from water into lipid are partially enthalpic effects related to the hydrogen bonding capability and molal volume of the material (Cohen, 1975). The hydrogen bonding capability is constant for the series of esters but obviously molecular volume increases as the acyl chain lengthens. The diffusivity of a material is very important in considerations of bilayer permeation, as membranes show great size selectivity (Wolosin and Ginsburg, 1975) and liposome permeation has been compared to diffusion in polymers. Therefore, it is likely that the incremental increases in molecular size due to methylene additions to the acyl chain result in part to the decrease in permeation rate observed in ascending the cortisone ester series. However, if size factors were of overriding importance then no differences in effect would be shown for the addition of a methylene group to a chain of less than 14 carbon atoms compared with additions to esters of greater chain length.

The increase in hydrophobicity shown in a homologous series of low lipophilicity non-electrolytes has led to an increase in permeation rate across various membranes (Diamond and Wright, 1969; Sha'afi et al., 1971). These results, in conflict with

those of Fig. 4 for a series of more hydrophobic materials, are due to an increase in the ability of the material to partition into the lipid core of the membrane, as hydrophobicity is increased (Fig. 1). It is evident that partitioning into and diffusion across the membrane are the rate-determining steps for membrane permeation of low lipophilicity non-electrolytes. However, the rate-limiting step in the transfer of cortisone esters is the passage from the membrane to the water phase. Evidence that this is generally true, for other highly hydrophobic materials, is the efflux of phenothiazines from egg lecithin liposomes, which is inversely related to liposome/water partitioning (Ahmed et al., 1980). Such results may occur through the energetically unfavourable formation of structural water around these molecules as they enter the water phase, or the breaking of solute–lipid hydrophobic interactions which may be of extraordinary strength due to the ordered nature of the bilayer. Hydrophobic interactions essentially involve van der Waal's forces which are known to increase in proportion to the molecular area available for the interaction (Diamond and Wright, 1969).

It has been found that the incremental hydrophobicity following methylene addition to slightly lipophilic solutes decreases as an homologous series is ascended (Sha'afi et al., 1971). As illustrated in Fig. 2, the methylene group free energy additive effect for cortisone esters decreases in a similar manner. This addition results in a linear increase in partition coefficient over the middle of this sigmoidal range. However, at the top of the ester size range, the methylene group contribution to partitioning is very small indeed. An analogous effect is seen when considering the rate of release of steroid esters from DPPC liposomes. The release rate of the longer esters is faster than predicted through extrapolation of the relationship between carbon chain length and rate of release of esters of a shorter chain length.

If a model of cortisone ester partitioning is envisaged with the acyl chains of steroid and lecithin lying in parallel, esters of acyl chain length longer than those of the lecithin, assuming trans-monolayer penetration is not possible, may be viewed as incompletely penetrating the bilayer. This is due to the protrusion of the methylene ester and terminal methyl group, in excess of the length of the alkyl chain of the lecithin. Results from the release of cortisone 16 and 18 carbon esters from different chain length lecithins support such a model.

Table 3 illustrates that no chain length dependence of hexadecanoate and octadecanoate release rate was found from DMPC or DPPC liposomes. Such a model would predict this effect as there is no opportunity for increased penetration of the bilayer of the longer ester acyl chain in both these cases. However, cortisone hexadecanoate and octadecanoate do show differential release behaviour from DSPC liposomes. If the above model is true, the 18 carbon alkyl chains of the lecithin do allow for greater penetration of the octadecanoate in relation to the palmitate ester causing the different release rates. It would appear that the maximum length of steroidal alkyl chain incorporated by a lecithin bilayer, is similar to the alkyl chain length of the lecithin itself.

The theory that acyl chain penetration of the liposomal bilayer is a particularly important factor in determining the liposomal release rate of cortisone esters is substantiated by the above data obtained from different phosphatidylcholines.

However, it is not possible to determine, at this stage, whether retarded release is due to an increased hydrophobic interaction between longer chain esters and the hydrocarbon chain of the lecithin or whether aqueous phase effects are solely responsible. If the former case is true, increased stability of the interaction between lecithin and ester acyl chains, due to the greater area available for van der Waal's interactions would be expected. If bilayer penetration is length-limited, only that portion of the side chain which is incorporated will be available for mutual alkyl chain interaction and in consequence retardation of release.

Aqueous phase entropic effects are proportional to the length of alkyl chain exposed to the water and the resultant degree of water structuring. The longer the acyl chain of solute exposed to water, the greater is the structuring and therefore the greater the barrier to interfacial transfer. This energy barrier increases with the lengthening in alkyl chain of the solute, and release simultaneously decreases, provided the bilayer 'shelters' all the acyl chain. However, if the solute protrudes into the aqueous phase it may cause an entropic effect equivalent to the length protruding, despite being liposomally incorporated. Hence the interfacial energy barriers may become a maximum for cortisone esters of similar length to the bilayer.

It has been known for several years that release rates of solutes from liposomes increase with increasing temperature. Such effects arise from a combination of two effects—the effect of increased temperature on the ordering of the bilayer and the increased energy of solute molecules allowing a faster diffusion across the bilayers. These effects may be separated by consideration of diffusion from liposomes of differing alkyl chain length and therefore phase transition temperature. Traüble (1971) has postulated the theory that non-electrolyte diffusion across lipid membranes is facilitated by 'kinks' in the regular arrangement of lecithin acyl chains. It is reasonable to assume that the incidence of these 'kinks' increases with temperature and so the passage of solute is faster at higher temperatures.

The results presented in Fig. 6 indicate that this effect appears greatest when comparing liposomes in the fluid state with gel state liposomes. The release rate from DMPC ($T_c = 23°C$) liposomes at 37°C was far greater than that from both DPPC ($T_c = 41°C$) or DSPC ($T_c = 54°C$) liposomes. It is perhaps surprising that efflux of cortisone hexadecanoate is slower from DPPC liposomes than DSPC, but this may be due to its lower partitioning into the liposomes of the longer chain lecithin ($K \approx 6000$). The restricted environment of the very ordered gel state of these liposomes leads to a poor affinity for partitioning solutes in comparison with more fluid liposomes such as those of DPPC and DMPC ($K \approx 16{,}000$). This difference in thermodynamic activity of DSPC compared with DPPC at 37°C is analogous to the very much smaller partition coefficients for steroids into DMPC liposomes at 5°C (Arrowsmith et al., 1982).

References

Ahmed, M., Burton, J.S., Hadgraft, J. and Kellaway, I.W., Partitioning and efflux of pheothiazines from liposomes. Biochem. Pharmacol., 29 (1980) 2361–2365.

Ahmed, M., Burton, J.S., Hadgraft, J. and Kellaway, I.W., Thermodynamics of partitioning and efflux of phenothiazines from liposomes. J. Membr. Biol., 58 (1981) 181–189.

Alpar, O.H., Bamford, J.B. and Walters, V., The in vitro incorporation and release of hydroxocobalamin by liposomes. Int. J. Pharm., 7 (1981) 349–351.

Arakawa, E., Imai, Y., Kobayashi, H., Okumura, K. and Sezaki, H., Application of drug-containing liposomes to the duration of the intramuscular absorption of water-soluble drugs in rats. Chem. Pharm. Bull., 23 (1975) 2218–2222.

Arrowsmith, M., Hadgraft, J. and Kellaway, I.W., Thermodynamics of steroid partitioning in dimyristoylphosphatidylcholine liposomes. Biochem. Biophys. Acta, 750 (1983) 149–156.

Bangham, A.D., de Gier, J. and Greville, G.D., Osmotic properties and water permeability of phospholipid liquid crystals. Chem. Phys. Lipids, 1 (1967) 225–246.

Bangham, A.D., Hill, M.W. and Miller, N.G.A., Preparation and use of liposomes as models of biological membranes. In Korn, E.D. (Ed.), Methods in Membrane Biology, Vol. 1. Plenum Press, London, 1974, pp. 1–68.

Cohen, B.E., The permeability of liposomes to non-electrolytes: I. Activation energies for permeation. J. Membr. Biol., 20 (1975) 205–234.

De Gier, J., Mandersloot, J.G. and Van Deenen, L.L.M., Lipid composition and permeability of liposomes. Biochem. Biophys. Acta, 150 (1968) 666–675.

Diamond, J.M. and Katz, Y., Interpretation of non-electrolyte partition coefficients between dimyristoyl lecithin and water. J. Membr. Biol., 17 (1974) 121–154.

Diamond, J.M. and Wright, E.M., Biological membranes: physical basis of ion and non-electrolyte selectivity. Ann. Rev. Physiol., 31 (1969) 581–646.

Fountain, M.W., Pedersoli, W.M., Ganjan, V.K. and Ravis, W.R., Phosphatidylcholine-digoxin liposome drug delivery vehicle: an evaluation of the intramuscular route of administration in dogs. Curr. Ther. Res., 28 (1980) 558–561.

Freise, J., Magerstedt, P. and Schmidt, F.W., Die in vitro und in vivo Stabilität des Einschlusses von Methotrexatin negativ geladenen Liposomen nach Sterilfiltration. Zeitschrift. Nat., 34C (1979) 114–119.

Inoue, K., Permeability properties of liposomes prepared from dipalmitoyllecithin, dimyristoyllecithin, egg lecithin, rat liver lecithin and beef brain sphingomyelin. Biochim. Biophys. Acta, 339 (1974) 380–402.

Layton, D., De Meyere, J., Collard, M.P. and Trouet, A., The accumulation by fibroblasts of liposomally encapsulated vinblastine. Eur. J. Cancer, 15 (1979) 1475–1483.

Lundberg, B., Temperature effect on the water solubility and water–octanol partition of some steroids. Acta Pharm. Suecica, 16 (1979) 151–159.

McDougall, I.R., Dunnick, J.K., McNamee, M.G. and Kriss, J.P. Distribution and fate of synthetic lipid vesicles in the mouse. Combined radionuclide and spin label study. Proc. Natl. Acad. Sci. U.S.A., 71 (1974) 3487–3491.

Papahadjopoulos, D., Jacobson, K., Nir, S. and Isac, T., Phase transition in phospholipid vesicles: fluorescence polarization and permeability measurements concerning the effect of temperature and cholesterol. Biochim. Biophys. Acta, 311 (1973) 330–348.

Ryman, B.E. and Tyrrell, D.A., Liposomes—methodology and applications. In Dingle, J.T., Jacques, J.P. and Shaw, I.H. (Eds.), Lysosomes in Applied Biology and Therapeutics, Frontiers in Biology, Vol. 6, North-Holland, Amsterdam, 1979, pp. 549–574.

Sha'afi, R.I., Gary-Bobo, C.M. and Solomon, A.K., Permeability of red cell membranes to small hydrophilic and lipophilic solutes. J. Gen. Physiol., 58 (1971) 238–258.

Sharma, P., Tyrrell, D.A. and Ryman, B.E., Some properties of liposomes of different sizes. Biochem. Soc. Trans., 5 (1977) 1146–1149.

Shinozawa, S., Araki, Y. and Oda, T., Distribution of [^{3}H]-prednisolone entrapped in lipid layer of liposome after intramuscular administration in rats. Res. Commun. Chem. Path. Pharmacol., 24 (1979) 223–232.

Shaw, I.H., Knight, C.G. and Dingle, J.T., Liposomal retention of a modified anti-inflammatory steroid. Biochem. J., 158 (1976) 473–476.

Shaw, I.H., Ph.D. Thesis, University of Cambridge, 1978.

Smith, R. and Tanford, C., Hydrophobicity of long-chain *n*-alkyl carboxylic acids, as measured by their distribution between heptane and aqueous solutions. Proc. Natl. Acad. Sci. U.S.A., 70 (1973) 289–293.

Traüble, H., The movement of molecules across lipid membranes: a molecular theory. J. Membr. Biol., 4 (1971) 193–198.

Tyrrell, D.A., Heath, T.D., Colley, C.M. and Ryman, B.E., New aspects of liposomes. Biochim. Biophys. Acta, 457 (1976) 259–302.

Tyrrell, D.A., Richardson, V.J. and Ryman, B.E., The effect of serum fractions on liposome–cell interactions in cultured cells and the perfused rat liver. Biochim. Biophys. Acta, 497 (1977) 469–480.

Wood, S.G., Symons, A.M. and Bridges, J.W., Interactions of a homologous series of carbamates with phosphatidylcholine liposomes. Biochem. Pharmacol., 28 (1979) 393–395.

Wolosin, J.M. and Ginsburg, H., The permeation of organic acids through lecithin bilayers resemblance to diffusion in polymers. Biochim. Biophys. Acta, 389 (1975) 20–33.