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The interaction of cortisone esters with liposomes as studied by differential scanning calorimetry

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Summary

The interaction of a series of cortisone esters with dipalmitoylphosphatidylcholine (DPPC) multilamellar vesicles (MLV) has been investigated using differential scanning calorimetry (DSC). The extent of the interaction is dependent on the ester chain-length with increased interaction observed as the chain lengthens. For cortisone hexadecanoate the maximum incorporation is 11.25 mole · %. The presence of cholesterol excludes cortisone hexadecanoate from DPPC liposomes. This effect increases with increasing cholesterol concentrations.

Cortisone hexadecanoate is incorporated into DPPC liposomes in such a manner that it is probable that the steroid moiety interacts strongly with the bilayer.

A correlation exists between the in vitro cortisone ester release rate at 37°C from DPPC liposomes and broadening of DPPC MLV thermograms only over the range of 6–14 carbon chain-length. This indicates that the interaction of the ester chain of the steroid with the acyl chain of the lecithin are not necessarily the sole factors controlling efflux rate.

Introduction

Over the past decade thermal methods have become a popular tool in the investigation of membrane physiology and more recently the mode of action of non-specific (Lee, 1976; Mountcastle et al., 1978; Cater et al., 1974) and other drugs (Cater et al., 1974). Attempts have been made to characterize the changes in bilayer organization, caused by membrane interaction with drugs by study of the phase transition profiles produced using differential scanning calorimetry (DSC) techniques (Jain and Wu, 1977). There is little published material reporting the use of DSC techniques to

evaluate drug–liposome interactions, as an aspect of carrier formulation (Juliano and Stamp, 1978; Chawla, 1980). However, Fildes and Oliver (1978), Shaw (1978) and Knight and Shaw (1979) have shown that if the drug is sufficiently hydrophobic and creates an easily quantifiable thermogram change, DSC may be a very useful tool for liposome formulation. Instruments of moderate sensitivity are ideal for the characterization of the transition profiles of aqueous phospholipid systems. Current, high sensitivity differential scanning calorimeters are yielding information of the actual molecular basis of these changes. Such techniques have recently been reviewed (Maybrey and Sturtevant, 1978).

The transition profile of a pure synthetic phospholipid is very sharp, occurring over a narrow temperature range. The presence of increasing concentration of hydrocortisone palmitate has been shown to broaden the temperature range in which fluid and gel domains may co-exist in the bilayer. Fildes and Oliver (1978) used this property to quantify maximum steroid ester incorporation into DPPC liposomes through use of the half transition peak height, width (HHW) criteria which had been previously initiated by Jain et al. (1975) and Jain and Wu (1977). Shaw (1978) preferred to assess maximum liposomal incorporation through comparison of the reduction in transition temperature found with increasing hydrocortisone palmitate. Similar values were obtained from each of these methods but assessment using proton nuclear magnetic resonance (NMR) yielded a higher value (Fildes and Oliver, 1978).

Steroid esters have been reported to have limited solubilities in phospholipid systems (Smith et al., 1980). It is expected that due to the low aqueous solubilities of steroid derivatives, material present in excess of this limit will be present in a microcrystalline form, internal or external to liposomes. It would therefore be possible to attribute release rates, at least partially, to dissolution of particulate steroid rather than efflux from liposomes.

It has been suggested that a material's effect upon the phase transition profile of phospholipid bilayer is a representation of its interaction with that bilayer (Jain and Wu, 1977). The effect of varying chain-length of cortisone derivatives upon the HHW has therefore been investigated with this objective in mind. Also cortisone hexadecanoate effects upon the DPPC thermogram have been compared with those of its constituent parts to assess which, if either, interacts most strongly with the bilayer.

The inclusion of cholesterol in liposome formulations of hydrophilic materials has become very common due to its properties in increasing in vivo liposome stability (Kirby et al., 1980), and also reducing the permeability of fluid liposomes (Papahadjopoulos and Kimelberg, 1973). However, its properties with respect to hydrophobic materials is less predictable and would appear to depend on the position of solute partitioning with the bilayer. Juliano and Stamp (1978) report that the presence of cholesterol enhanced the incorporation of both hydrophilic and hydrophobic anticancer agents while Wu et al. (1978) found that gramicidin-S was excluded from cholesterol-containing bilayers. It has also been reported that cholesterol increased liposome permeability to hydrophobic ionophores. It would appear that cholesterol has varied effects upon liposomal incorporation of hydro-

phobic materials and therefore its effect upon the incorporation of cortisone hexadecanoate has been undertaken. Cholesterol has a great effect upon the phase transition of hydrated phospholipids and therefore assessment of steroid incorporation in its presence cannot be undertaken by thermal means.

Materials and Methods

The lipid and steroid sources and the synthesis and purification of cortisone-21-esters are detailed in Arrowsmith et al. (1983).

The thermal analysis of DPPC liposome systems

(a) Preparation of samples for DSC

15 mg of DPPC and the required quantity of steroid or additive were weighed (with [^{3}H]tracer if used) into 25 ml round-bottom quickfit flasks and dissolved in 5 ml ethanol (cortisone) or chloroform (cortisone-21-esters and methylpalmitate). The solvent was evaporated by prolonged (30 min) rotary evaporation at 40°C and the final traces of solvent removed at room temperature, under vacuum, overnight. 3 ml of sterile saline, 0.9% w/v, was added to each flask, which were then sealed and gently shaken at 50°C, immersed in a shaking water bath for 1 h. A few glass beads were added and a suspension formed during 30 s periods of mechanical shaking, alternated with suspension rewarming. The liposome suspension was transferred to dry tared polycarbonate centrifuge tubes with the aid of 2×1 ml washings of sterile saline, and centrifuged at $50{,}000 \times g$ for 1 h. The bulk of the supernatant was removed and the final weight of the suspension adjusted to 0.15 g. Steroid loss was assessed by UV assay of the supernatant at 245.5 nm or scintillation counting. The centrifuge tubes were sealed and spun briefly at 3000 rpm to collect all material in the tip of the tube. Following the addition of two glass beads, the pellets were resuspended to make a final DPPC content of 10% w/w. All samples were left at room temperature for 3 days prior to DSC analysis.

(b) Differential scanning calorimetry procedure

Approximately 6 mg samples were weighed accurately (to 10^{-5} g tolerance) and hermetically sealed in aluminium pans (Perkin-Elmer) and analyzed in a differential scanning calorimeter (Perkin-Elmer, DSC-2), following calibration of both temperature and quantitative heat changes with pure indium (Perkin-Elmer). The reference pan contained a similar amount of sterile saline. A freezing mixture of water, salt and solid carbon dioxide was used to cool the calorimeter head and nitrogen was constantly passed over the samples. Thermograms over the temperature range 292.2–330.2 K were recorded at a scanning rate of $5°C \cdot min^{-1}$ and a range setting of 1 $mCal \cdot s^{-1}$. Each system was scanned twice using different preparations.

(c) Experiments performed

(1) Assessment of the maximum incorporation possible of cortisone hexadecanoate into DPPC MLVs.

(2) The effect of the incorporation of 5 mole · % of cortisone-21-esters on the phase transition profiles of DPPC MLVs.

(3) Comparison of the effects of cortisone palmitate, methyl palmitate and cortisone on the thermograms of DPPC MLVs.

The effect of cholesterol upon cortisone palmitate incorporation into DPPC liposomes

Since cholesterol fundamentally affects the phase transitions of phospholipids, it is impossible to assess the effect of cholesterol incorporation into DPPC liposomes upon its retention of cortisone palmitate by thermal methods. Hence an equilibrium technique has been employed as follows.

DPPC liposome suspensions were prepared containing 9 mole · % of [^{3}H]cortisone palmitate (1 μCi) and varying mole · % of cholesterol. Following equilibration at 37°C for 7 days, the distribution of cortisone palmitate was assessed by scintillation counting of the whole suspension and supernatant following centrifugation of 50,000 × g for 1 h. The amount retained was described as a percentage of the incorporation found for the formulation containing 0% cholesterol (i.e. 59% incorporated).

Calculations

The half-height width of the DSC peaks were measured using a travelling microscope and converted to °C. The enthalpy of transition (ΔH_t) was calculated using Eqn. 1 where the constant 27.53 was predetermined through calibration with indium. The area under the peak was calculated through cutting out and weighing of traces of the transition profile. Reproducibility of this procedure was found to be ± 1%.

$$\Delta H_t = \frac{27.53\ A \cdot R}{W \cdot S} \tag{1}$$

where A = area under transition peak cm^2; R = range control setting, mcal · s^{-1}; S = chart speed, mm · min^{-1}; W = weight of sample, i.e. weight of lecithin, mg; ΔH_t = enthalpy of transition, cal · g^{-1}.

Conversion of ΔH_t values of J · mol^{-1} was performed by multiplication of ΔH_t calculated in Eqn. 1 by a factor of 4.18 times the molecular weight.

The following were determined: T_c (onset temperature of transition), T_m (the temperature of the maximum amplitude of the transition peak) and HHW (half peak height, width).

As mentioned above, steroid loss from the sample due to dissolution of material in the excess supernatant was assessed. Gross amounts (80%) of steroid were lost when cortisone was the incorporated steroid but the loss of cortisone-21-derivatives was generally negligible due to their poor aqueous solubility.

Results and Discussion

The incorporation of cortisone hexadecanoate into DPPC liposomes

The concentration-dependent changes in the phase transition profile of increasing cortisone hexadecanoate concentration is shown in Fig. 1. The transition of pure DPPC MLVs was found to commence at 41.6°C (T_c) with a T_m of 42.6°C; however, this is preceded by a small transition at 35.5°C. These figures are in agreement with those previously reviewed (Kimelberg and Mayhew, 1978). However, the enthalpy of transition was higher than literature values at 43.5 kJ · mol^{-1} although the pretransition value of 7.2 kJ · mol^{-1} confirmed previous results.

The introduction of 2.5 mole · % of cortisone hexadecanoate completely removed the pretransition and lowered, with broadening the phase transition profile. This effect continued with increasing concentration of cortisone palmitate until, at 12.5 mole · % steroid, there is some evidence of multiple peaks. Further increases in steroidal content led to broad low multiple transition peaks over a range of temperature of 35–55°C.

It appears that increasing concentrations of cortisone palmitate, in common with other materials (Jain and Wu, 1977; Fildes and Oliver, 1978), reduce the cohesion of changes in molecular motion in the phase change. This effect is represented in Fig. 2 where increasing steroid concentration leads to broadening of the phase change up to a maximum at a cortisone palmitate content of 11.25 mole · %. Thermograms produced from liposomes of steroid content greater than this concentration cannot be compared with traces obtained from lower concentrations due to their multiple

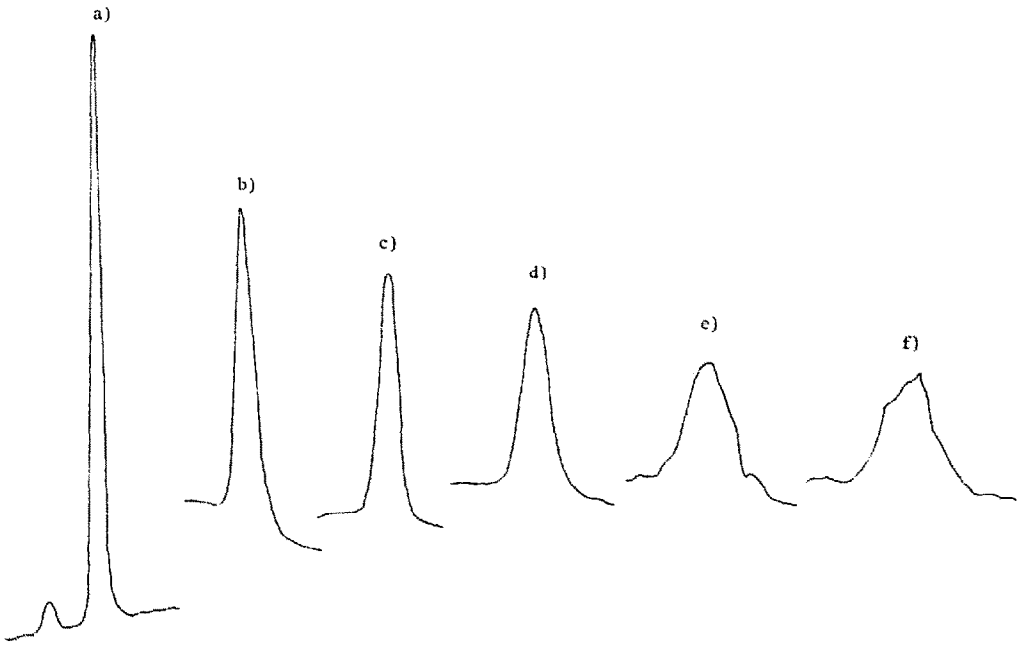

Fig. 1. The effect of increasing incorporation of cortisone hexadecanoate upon the phase transition profile of DPPC liposomes: (a) DPPC alone. Remaining thermograms for equivalent amount of DPPC and the stated mole · % of cortisone hexadecanoate: (b) 2.5; (c) 5; (d) 7.5; (e) 10; and (f) 12.

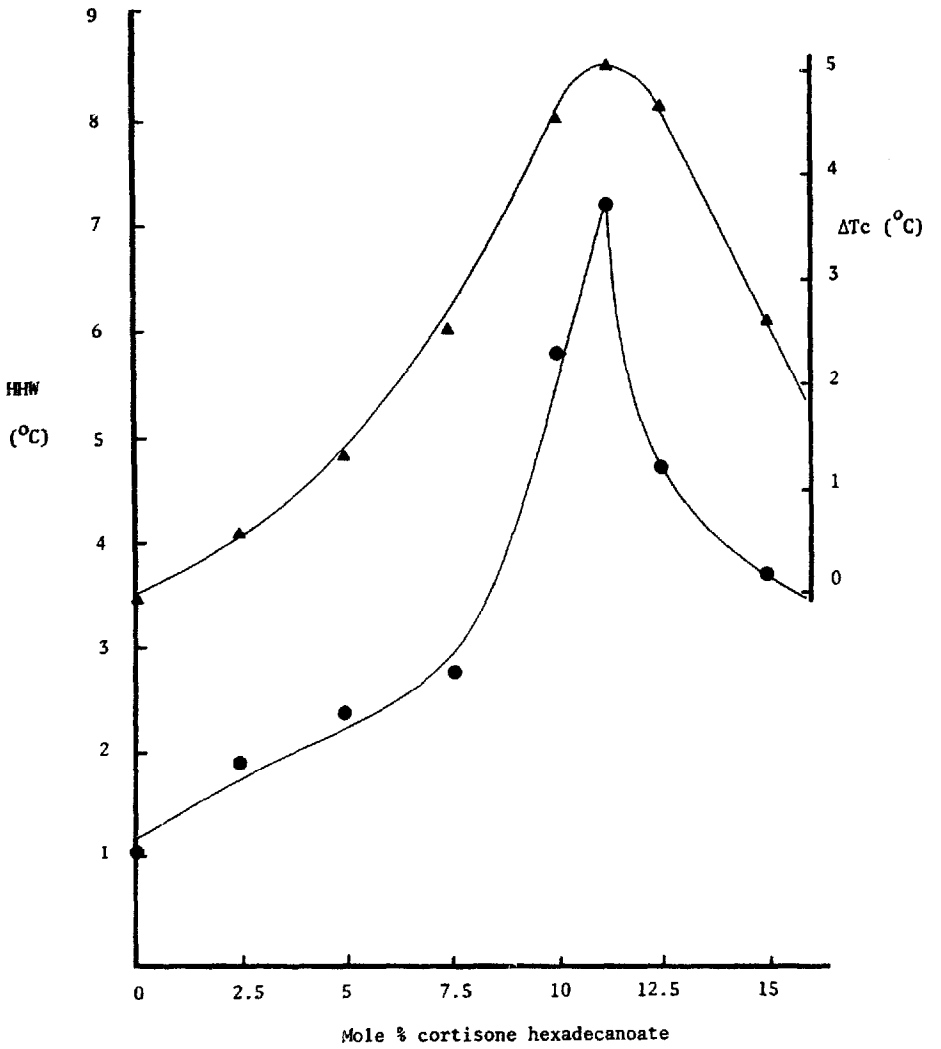

Fig. 2. The relationship between the onset temperature of the main phase transition (T_c), the endotherms half-height width (HHW) and the incorporation of cortisone hexadecanoate into DPPC liposomes. ●, HHW; ▲, ΔT_c.

nature. Maximum half-height widths have been previously used as an indication of DPPC bilayer saturation of hydrocortisone hexadecanoate (Fildes and Oliver, 1978). Steroid in excess of this limit may separate out into crystalline drug or complexes having thermal properties different from the liposomes, resulting in the multiple peaks recorded. The decrease in phase transition onset temperature (ΔT_c) has been also used to assess hydrocortisone ester saturation of the liposomal bilayer (Shaw, 1978; Knight and Shaw, 1979). In contrast, Fildes and Oliver (1978) reported little deviation in transition temperature with increasing content of the same ester. In both cases it is not reported whether T_c or T_m was measured. Since a lowering in T_c is an integral part of the increasing HHW, it follows the same pattern as HHW (in Fig. 2) and is equally viable in estimation of liposome saturation with steroidal esters. T_m,

which is probably the best criterion for quantification of additive-induced shifts in transition temperature, was little altered with increasing cortisone hexadecanoate concentration but was affected by the addition of other cortisone esters.

Fig. 3 shows that the enthalpy of the phase transition is reduced with the increasing cortisone hexadecanoate content of the DPPC liposomes. However, possibly due to the problems associated with defining the areas of the broad transitions found at high additive concentrations it is not possible to define a break in this graph which may have use in assessment of liposome saturation.

The value of 11.25 mole · % as the maximum incorporation of cortisone hexadecanoate into DPPC liposomes may be compared with values for hydrocortisone hexadecanoate of 15 mole · % by DSC techniques (Fildes and Oliver, 1978; Shaw, 1978; Knight and Shaw, 1979) and 19–20 mole · % using NMR.

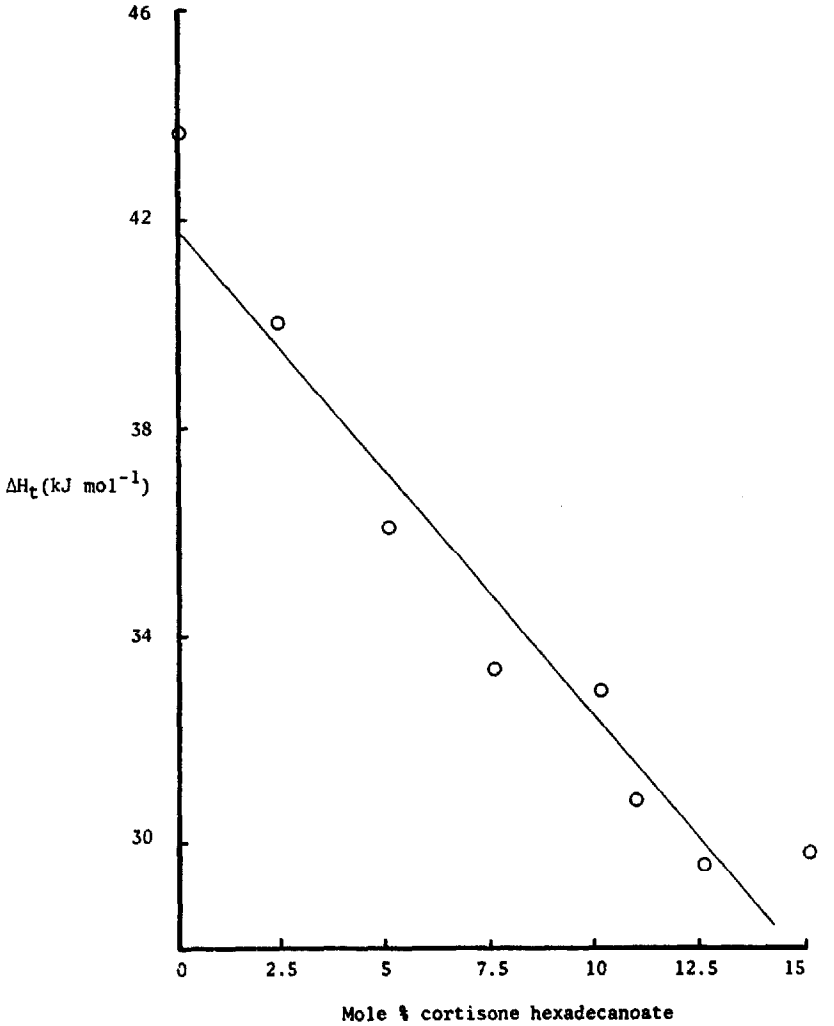

Fig. 3. The effect of increasing incorporation of cortisone hexadecanoate upon the enthalpy of the DPPC phase transition. Each point is the mean of two determinations ($r = 0.973$, $P > 99.9\%$, $n = 8$).

Cholesterol effects upon liposomal incorporation of cortisone hexadecanoate

The incorporation of cholesterol drastically reduces the capacity of DPPC liposomes to carry cortisone hexadecanoate (Fig. 4). Therefore cholesterol is not a useful addition to this formulation. Similar effects have been reported for the incorporation of cholesterol and hydrocortisone octanoate into DPPC liposomes (Shaw, 1978). Also cholesterol has been previously found to reduce the partitioning of parent steroids into egg lecithin liposomes (Tipping et al., 1979; Heap et al., 1970; Snart and Wilson, 1967). It may be concluded that either cholesterol occupies the same bilayer sites as steroids and corticosteroid esters or its effects upon the bilayer exclude the incorporation of these molecules.

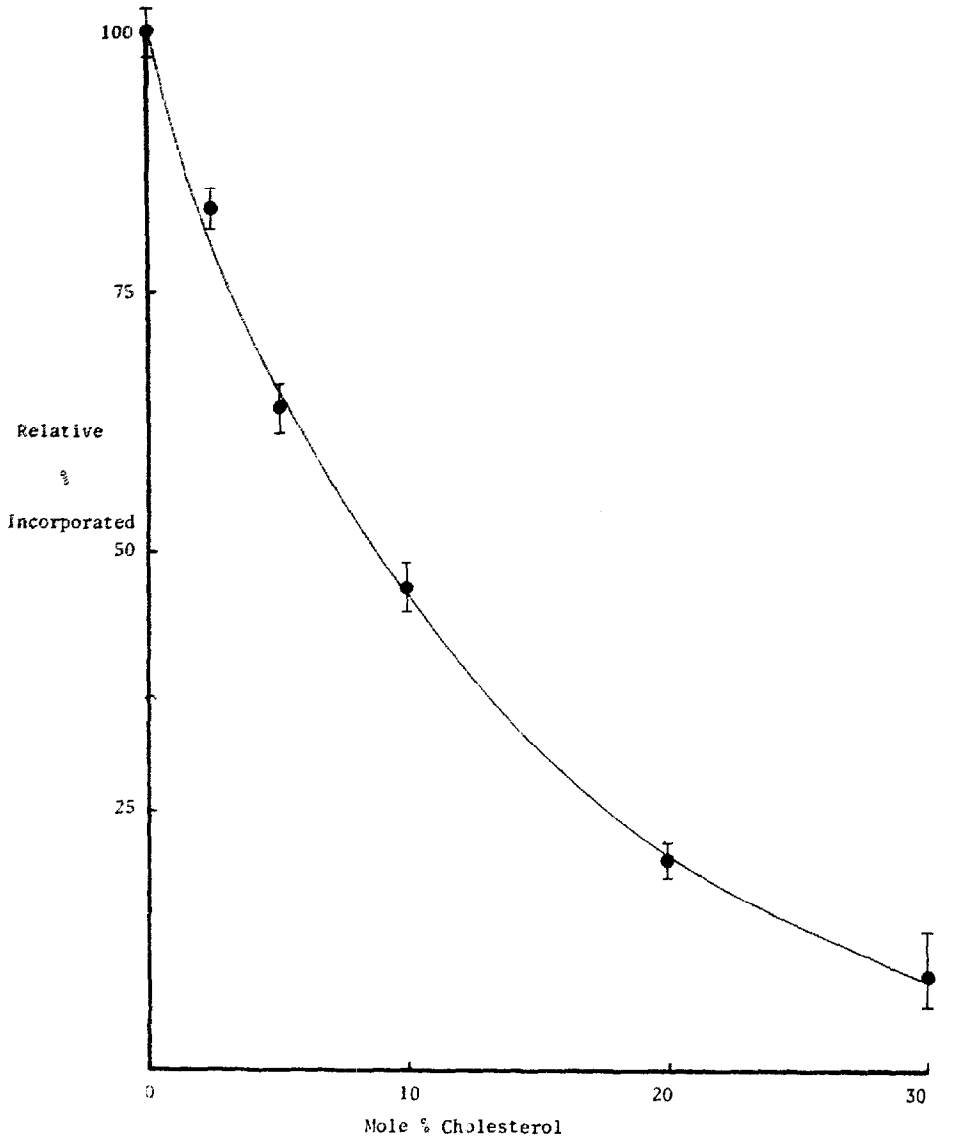

Fig. 4. The reduction of 9 mole·% cortisone hexadecanoate incorporation into DPPC liposomes at 37°C incurred following the incorporation of cholesterol (mean of 3 determinations ± S.D.).

The mode of incorporation of cortisone esters into liposomes

The effect of various mole·% of cortisone, methylhexadecanoate, cortisone hexadecanoate and cortisone octanoate upon the phase transition thermogram of DPPC MLVs is listed in Table 1. It is clear that the interactions of the cortisone hexadecanoate with the DPPC bilayer, as represented by alteration of the thermograms, is much greater than that encountered with either of its constituent parts; the alkyl chain (represented by methylhexadecanoate) and the cortisone nucleus. Neither of these latter additives eliminates the pretransition but it should be realized that due to loss of 80% cortisone in the supernatant this effect is due to the incorporation of only 1 mol·%. Comparison of the HHW or 2% cortisone incorporation (1.25°C) with that of 2.5 mole·% of cortisone hexadecanoate (1.95°C) shows that the ester effect is greater than that of the parent steroid. However, cortisone does appear to have more effect than methylhexadecanoate on the phase change profile when comparing equivalent concentrations. The steroid portion of the molecule may be a considerable contributor to the cortisone hexadecanoate alteration of the thermograms illustrated in Fig. 2. Fildes and Oliver (1978) postulated a model of membrane inclusion of hydrocortisone hexadecanoate in which the steroid portion was excluded from the lipid core of the bilayer, in the polar surface of the bilayer whilst the ester acyl chain "dipped" into the lipid interior. The results of Cleary and Zatz (1977) gave this model credibility, but the thermogram effects mentioned above demonstrate that the corticosteroid nucleus interacts with the lecithin. Mabrey and Sturtevant (1976) have shown that, in low concentrations, the addition of palmitic acid does not disturb the hexagonal packing of DPPC alkyl chains in the liposomal bilayer. This is further evidence that the effects seen in Fig. 2 are due to steroid interactions.

Knight and Shaw (1979) suggest an alternative mode of hydrocortisone ester incorporation in which the steroid moiety penetrates the bilayer in addition to the hydrophobic ester side-chain. As they point out, a 21-esterified hydrocortisone

TABLE 1

THE EFFECT OF DIFFERENT ADDITIVES UPON THE HALF PEAK HEIGHT WIDTH (HHW) OF THE DPPC PHASE TRANSITION PROFILE

Solute	Mole·%	HHW (°C)
Pure DPPC		1.0
Cortisone	1	1.1
	2	1.25
Cortisone octanoate	5	2.0
	10	2.3
Cortisone hexadecanoate	2.5	1.95
	5	2.9
	10	5.8
Methyl hexadecanoate	5	1.4
	10	1.85

(Reproducibility = ±0.2°C).

moiety is a hydrophobic entity and is unlikely to associate with solely polar portions of the bilayer. Such arguments obviously apply to cortisone-21-derivatives and it may be concluded that the true model of corticosteroid partitioning in the lecithin bilayer is not as simple as that suggested by Fildes and Oliver (1978).

The presence of aliphathic additives of chain-length shorter than 11-carbon atoms leads to a reduction in the phase transition temperature of DPPC liposomes. In contrast, the inclusion of longer aliphathic additives increases T_m. Such effects have been reported for alkanes (McIntosh et al., 1980), alcohols (Eliasz et al., 1976; Lee, 1976) and fatty acids (Eliasz et al., 1976; Usher et al., 1978) and are well characterized. If the interaction of cortisone esters with DPPC liposomes is primarily via the side-chain, then upon the inclusion of different esters similar effects would be expected. Fig. 5 shows a stepped descent in peak temperature to a minimum at cortisone butadecanoate; not decanoate as predicted above. Lengthening of the ester chain to 16 and 18 carbons produces an increase in transition temperature to that expected when no additive is present. Such differences indicate that cortisone esters are not liposomally incorporated in an exactly analogous manner to simple aliphatic substances and the steroid nucleus has some interaction with the bilayer. It is probable that the degree of this interaction is influenced by the ester chain-length.

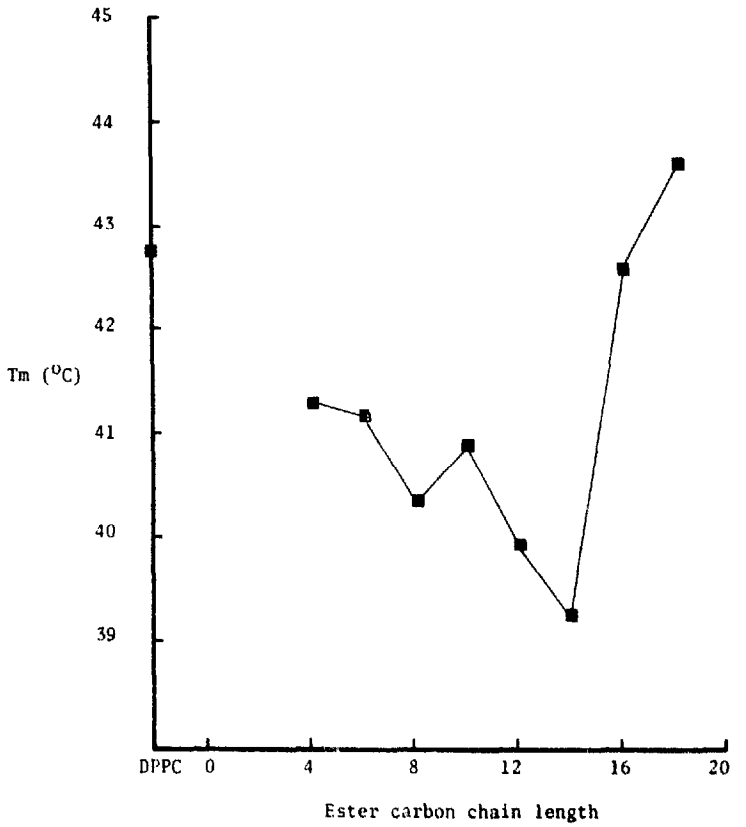

Fig. 5. The relationship between the DPPC transition peak temperature (T_m) and the carbon chain-length of the cortisone-21-derivative at 5 mole·%. Each point is the mean of 2 samples (reproducibility = ±0.1°C).

Cortisone-21-ester–lecithin interactions as studied by DSC

Cortisone ester–lecithin bilayer interactions thus involve both the steroid nucleus and the acyl chain. The presence of the cortisone moiety has a modifying influence on the ester chain bilayer interactions as compared with simple aliphatic series.

The evidence presented in Fig. 6 shows that increasing steroid ester acyl chain-length successively broadens the DPPC liposome phase transition in the range of n = 6–14 carbon atoms. The interaction of lecithin bilayers and cortisone ester chains of this length increases therefore in an analogous manner. Short- (n = 2 and 4) and long-chain cortisone esters (n = 16 and 18) were exceptions to this series as they were in previously reported plots relating ester chain-length and efflux from DPPC liposomes (Arrowsmith et al., 1983). Fig. 7 attempts to relate liposomal interactions of cortisone derivatives as measured by low HHW with efflux rates from liposomes (log k efflux from DPPC liposomes). However, this analogy is not complete. A good correlation of these two parameters is found only over the range n = 6–14. If HHW was a true measure of effects controlling release from liposomes then such a correlation should be seen over the entire ester chain-length range.

It is possible that cortisone acetate and butyrate deviate from this relationship due to a different mode of interaction with the bilayer compared with the longer esters. The addition of a lengthening acyl chain to cortisone may lead to its

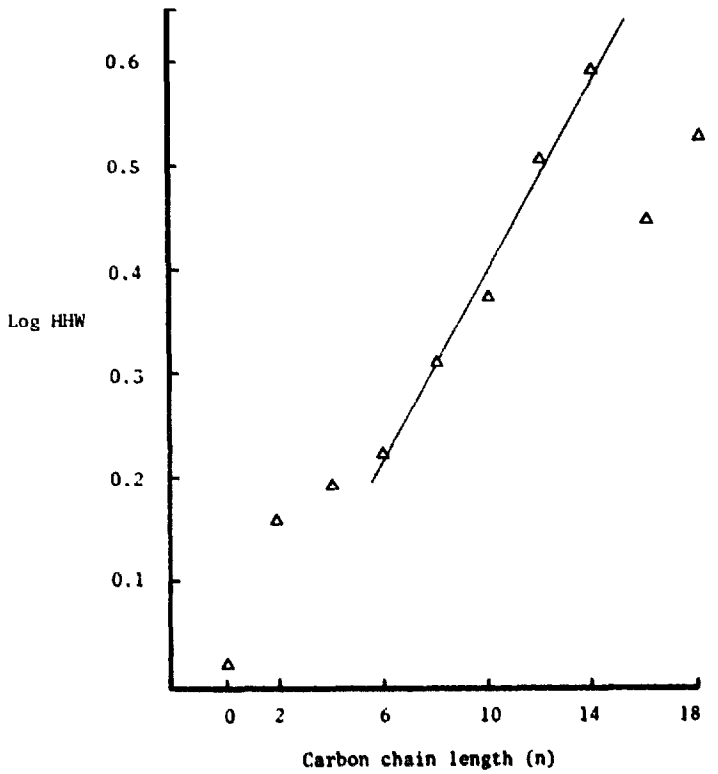

Fig. 6. The peak broadening effect (log HHW) of the incorporation of cortisone derivatives as a function of ester acyl chain-length. Each point is the mean of two independent samples ($r = 0.997$, (n = 6–14), $P > 99.9\%$).

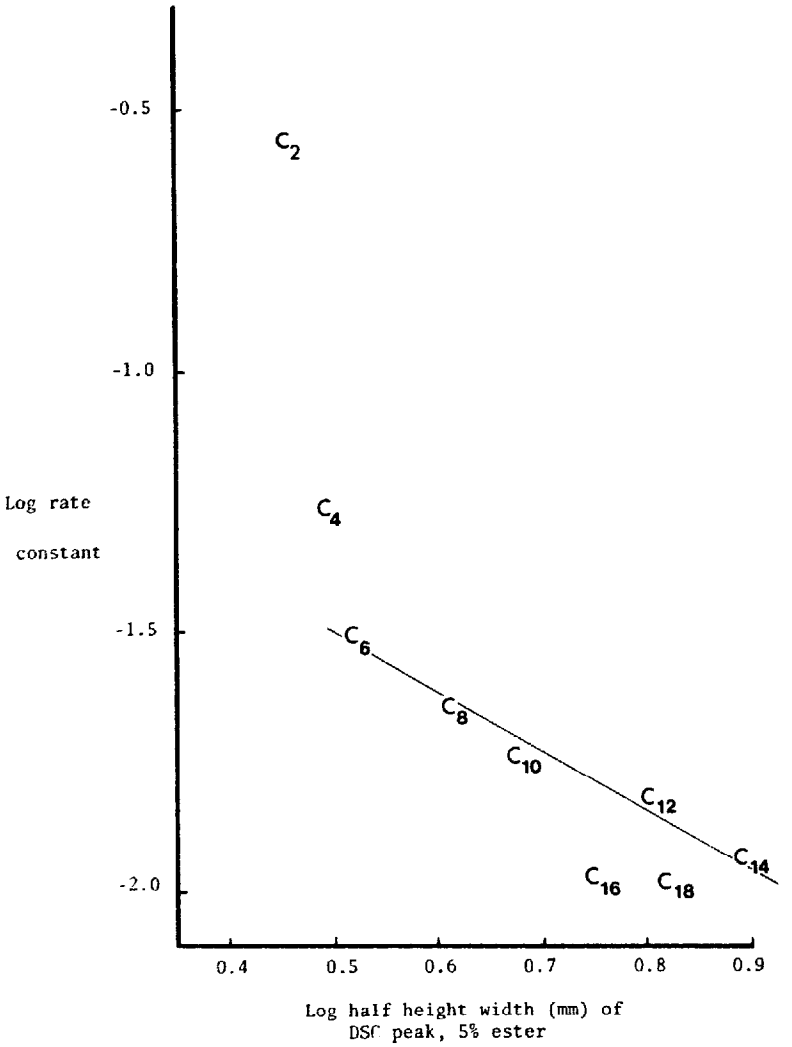

Fig. 7. A plot relating the peak broadening effect of 5 mole·% of cortisone esters (log HHW) with their efflux rate from DPPC liposomes at 37°C (log k). Efflux rate data reported in Arrowsmith et al. (1983). ($r = 0.990$, ($n = 6$–14), $P > 99.9\%$).

re-orientation or re-positioning within the bilayer. This may explain the parameter deviations consistently seen for the short esters in relation to the other cortisone esters.

It has been suggested (Arrowsmith et al., 1983) that the relatively small difference in release rates between cortisone hexadecanoate and docosanoate (22 carbon) was possibly due to their protrusion from the bilayer failing to cause incremental increases in the hydrophobic effect. If this was true, it would be expected that the peak broadening effects of cortisone hexadecanoate and octadecanoate would be similar but slightly greater than that of cortisone butadecanoate since the same hydrocarbon chain-length is available for interaction with lipid hydrocarbon chains. However, the HHWs of both hexa- and octadecanoates are smaller than those of

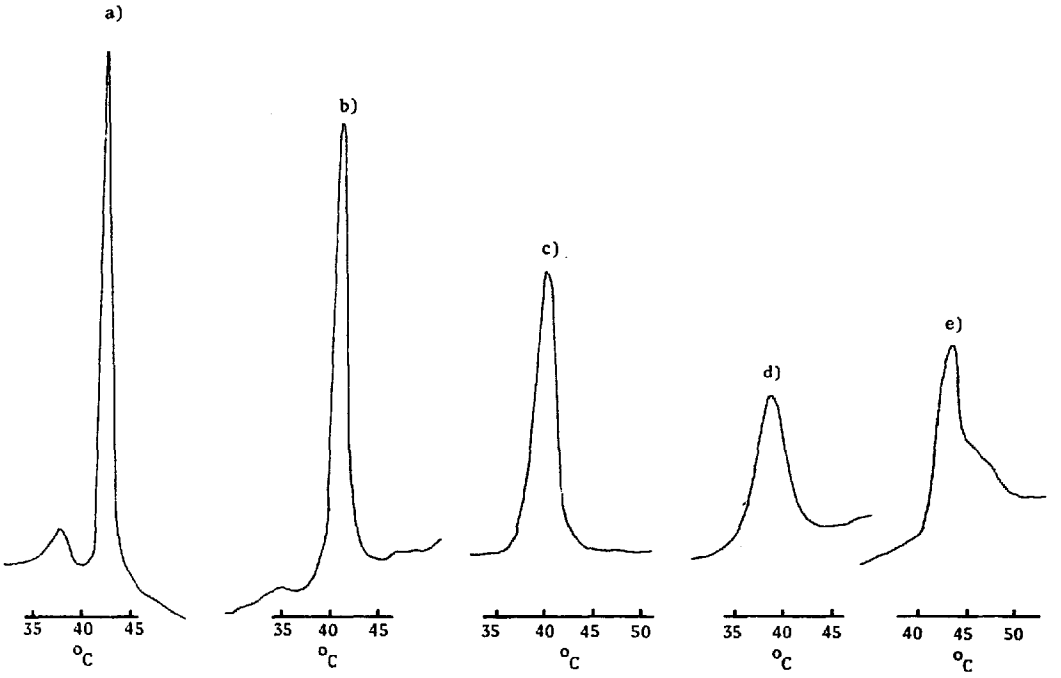

Fig. 8. The effect of incorporation of 5 mole·% of different cortisone derivatives upon the phase transition profile of DPPC liposomes. (a) DPPC alone; (b) cortisone butyrate; (c) cortisone decanoate; (d) cortisone butadecanoate; and (e) cortisone octadecanoate.

cortisone butadecanoate although a fundamental change in transition shape was observed in the former cases. This shape change arises from a disproportionate increase in the base of the former esters transition profiles in comparison with peak height (see Fig. 8). The fundamental cause behind this change is unknown but may be due to irregular packing of these esters into the bilayer.

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