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Interaction of cortisol-21-palmitate with liposomes examined by differential scanning calorimetry

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Liposomes have been suggested as carriers for corticosteroids in the local treatment of arthritis by intra-articular injection. The long chain 21-esters of cortisol such as the palmitate or octanoate are taken up and retained by liposomes in higher concentration than cortisol itself. Differential scanning calorimetry has been used to show that the cortisol ester is anchored in the liposome phospholipid bilayer by the acyl side chain. In addition, the limiting concentration of cortisol-21-palmitate which can be incorporated into dipalmitoyl phosphatidylcholine liposomes has been measured by observing changes in the DSC spectrum at different steroid concentrations. Steroid in excess of this concentration limit forms a separate phase which can be identified by nuclear magnetic resonance. For optimum effect, the treatment of arthritis with liposomes must be carried out with liposomes containing steroid below the limiting concentration.

Liposomes, or multilayer phospholipid vesicles consist of concentric structured bilayers of phospholipid separating discrete aqueous compartments. They are biodegradable, non-antigenic and biocompatible and in addition, a wide variety of both lipid soluble and water soluble compounds can be incorporated into the liposomes. These properties have led to their study as vehicles for carrying and controlling the action of drugs. Interest has centred mainly on the possibility of using liposomes to direct drugs to specific target cells or organs and to minimize uptake by non-target tissue.

Shaw, Knight & Dingle (1976) introduced the possibility of using liposomes to improve the local therapy of rheumatoid arthritis with corticosteroids. By injecting unsonicated liposomes containing the drug directly into the articular cavity, drug should be localized in the inflamed joint and the liposomes should reduce the escape of steroid into the systemic circulation leading to undesirable side effects in remote, non-target tissue. In addition, the proven ability of liposomes to enter cells through endocytosis or membrane fusion might increase the uptake of encapsulated corticosteroid by the target synovial cells lining the joint cavity. The combined effect should reduce the effective therapeutic dose of steroid and also eliminate undesirable side effects.

Cortisol itself is incorporated into liposomes only in low concentration and is rapidly lost from the carrier (Shaw & others, 1976). However, steroid retention is increased with the more lipid soluble esters of cortisol such as the octanoate and palmitate. Maximum drug incorporation and retention was found by Shaw & others (1976) with cortisol-21-palmitate in dipalmitoyl phosphatidylcholine liposomes. We have confirmed this and have also shown the palmitate to have a greater affinity for liposomes than the octanoate which, in turn, is bound more than the 21-butyrate. The non linear pivalate ester is less bound than the linear ester derivatives suggesting the ester side chain anchors the corticosteroid in the liposome bilayers and that the palmitate is most suitable for this function.

In the experiments of Shaw & others (1976) radiolabelled steroid uptake and retention in liposomes was measured from its association with the liposome phospholipid and its loss into an external aqueous medium. Since both lipid and steroid have low solubility in water these observations might simply reflect the further reduction in aqueous solubility of the steroid esters with increasing chain length. For example, Snart & Wilson (1967) have shown that the distribution coefficients of various steroids between lipid suspensions and water are determined mainly by the aqueous solubilities of the steroids. It is evident that when liposomes containing cortisol esters are formed by dispersing a mixed film, a given amount of phospholipid will have a limited affinity for a particular ester. Steroid in excess of this limit might separate as a discrete phase or as insoluble particles stabilized in the dispersion by adsorbed phospholipid. These would be undistinguishable from liposomes containing steroid by the radiotracer method.

The use of liposomes in arthritis therapy requires that the total dose of corticosteroid is incorporated into the liposome. Therefore, there is a need to

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characterize more clearly the nature of the association between phospholipid and drug and, in particular, to determine the concentration limit for steroid uptake by the chosen phospholipid.

The interaction of steroids with natural cell membranes has been reviewed by Willmer (1961) who concluded that their pharmacological action results, at least in part, from their direct interaction with the phospholipid in the membrane. Willmer proposed that steroids act like cholesterol in associating with the lipid acyl chains, with the molecule buried in the hydrophobic regions of the bilayer. However, recent monolayer studies by Cleary & Zatz (1977) imply that although cortisol is taken up by lecithin monolayers, the association is primarily with the polar head group of the phospholipid. Therefore, the nature of the association between the hydrophobic esters of cortisol and lecithin in liposomes is uncertain. We report here our attempts to clarify the nature of this steroid-lipid interaction by differential scanning calorimetry (DSC).

When phospholipids such as the natural or synthetic lecithins are heated, they undergo an endothermic transition at a temperature which characterizes the melting and increased mobility of the acyl side chains. The temperature at which this occurs depends on the chain length and degree of unsaturation of the phospholipid. In the presence of excess water, the transition temperature is lowered and when it is reached the hydrated phospholipid transforms from a gel phase to a smectic mesophase or liquid crystal. Ladbrooke, Williams & Chapman (1968) and Ladbrooke & Chapman (1969) have shown that thermal analytical techniques such as DSC are suited to studying these phase transitions. DSC is especially useful for examining the interaction of phospholipids with proteins (Pache, Chapman, Hillaby, 1972), cholesterol (Ladbrooke & others, 1968) and metal ions (Houser, Chapman & Dawson, 1969) because these compounds modify the temperature and intensity of the phospholipid transition endotherm, leading to a clearer understanding of their interaction with phospholipids in both liposomes and natural cell membranes. Consequently, we have used DSC to investigate the association between cortisol-21-palmitate and dipalmitoyl phosphatidylcholine in liposomes.

MATERIALS AND METHODS

Materials

Dipalmitoyl phosphatidylcholine was prepared from egg lecithin using the methods of Chadha (1970) and Baer & Buchnea (1959). Cortisol-21-palmitate was synthesized by esterification of cortisol with palmitoyl chloride in pyridine. Both compounds were kindly prepared for us by Dr G. Jones of this Organisation.

Liposome preparation

To simplify interpretation of the DSC studies, neutral liposomes containing only dipalmitoyl phosphatidylcholine and cortisol-21-palmitate were prepared. The incorporation of a charged lipid was deliberately avoided, so that the effect of corticosteroid on the phospholipid transition endotherms could be studied in a simple two component system.

Dipalmitoyl phosphatidylcholine (14·9 mg) and amounts of cortisol-21-palmitate ranging from 0·5 to 3·32 mg were dissolved in chloroform (5 ml) and each solution was evaporated to dryness to form an even film on a flask wall. The final traces of chloroform were removed with a stream of dry nitrogen. Distilled water (5 ml) was then added and the flask and its contents were heated to 60° on a water bath. The mixed film was dispersed at this temperature by agitating the flask contents on a vortex mixer, to give a milky suspension of liposomes.

Sample preparation for differential scanning calorimetry

Ladbrooke & Chapman (1969) have shown the importance of water content on the mesomorphic behaviour of phospholipids and have emphasized the need to maintain constant the amounts of water in samples for thermal analysis. However, when the lipid contains 50 % weight water or more, the phase transitions and thermal spectrum are insensitive to variations in the actual water content of the phospholipid. Consequently, in the present study, all samples were deliberately prepared to contain 50% water.

Two methods of sample preparation were compared. In the first, the aqueous liposome dispersions were completely dehydrated and then rehydrated to 50% water. In the second, the samples were uniformly dehydrated until this figure was reached. This second method was eventually chosen as the more suitable.

Each liposome suspension was transferred to a 12 ml volume ultracentrifuge tube previously dried to constant weight, centrifuged at 40 000 rev min^{-1} for 1 h in a Beckman L5-65 ultracentrifuge, cooled to 4°, and the clear supernatant separated. A weighed glass stirring rod was inserted into each tube and the solid was dispersed round the tube wall. Excess

water was then removed under vacuum, at room temperature (20°) in a vacuum dessicator. To ensure uniform drying, the samples were stirred frequently with the rod. The drying was continued until the weight of the sample indicated a residual water content of 50 weight percent. At this point, duplicate samples weighing approximately 6 mg were hermetically sealed into metal DSC sample holders.

To eliminate the possibility that the effect of cortisol-21-palmitate on the thermal spectrum of the phospholipid could result from coarse mixing of the two compounds, a series of control experiments was run with hydrated mixtures of steroid and lipid. Weights of solid cortisol-21-palmitate and dipalmitoyl phosphatidylcholine identical to those used in the liposome preparations were mixed by stirring the solids together in weighed sample holders. The amount of water necessary to give 50% in the final mixture was added and the sample was homogenized by stirring. Weighed samples were then hermetically sealed in DSC sample holders as before.

Differential scanning calorimetry

DSC spectra between 0° and 80° were recorded on a Perkin-Elmer F11 differential scanning calorimeter run at a heating rate of 8° min^{-1}. The curves from both the first heating and cooling cycles were recorded and then a second heating curve was run to see if heating the samples above the phospholipid transition temperature had any effect on the subsequent mesomorphic behaviour of the samples. The temperature scale of the calorimeter was calibrated before use with a sample of pure phenol which exhibits a distinct melting transition at 40·9°.

Nmr experiments

Nmr spectra of samples dissolved in deuterochloroform, were recorded on a Bruker HX-90E nmr spectrometer operating in the Fourier transform mode.

RESULTS AND DISCUSSION

The DSC curves for the first heating with pure hydrated dipalmitoyl phosphatidylcholine and for liposomes containing increasing concentrations of cortisol-21-palmitate are shown in Fig. 1 (a). The thermal spectrum of the hydrated lipid shows a major transition endotherm centred at 41° and a less intense transition or pre-transition at about 35°. The incorporation of steroid in the liposomes produces a number of changes in this spectrum. First, the incorporation of only 3·8 mol % cortisol-21-palmitate in the liposome abolishes the pre-transition and this peak is absent with all liposome samples containing steroid. The temperature of the main transition remains approximately constant in all the samples. However, this transition broadens and its intensity diminishes as the concentration of steroid in the preparations increases. This is evident from Fig. 2 which shows the variation of the half-peak width of the first heating transition with increasing cortisol-21-palmitate concentration (half-peak width is defined as the width of the transition, in degrees centigrade, at a point equal to one half the maximum peak intensity). The graph shows that this width increases from an average of 3·3° with pure hydrated lipid, to a maximum of 9·0° when the steroid concentration is 13·2 mol %. Further increase of the cortisol ester concentration to 20·9 mol % causes no further increase in line width and, in fact, a slight narrowing of the transition is observed at this concentration.

In contrast to these effects, the DSC spectra of the control mixtures of phospholipid and corticosteroid show only marginal changes with the same increase in cortisol-21-palmitate concentration. The pre-transition at 35° is maintained with all samples and Fig. 2 shows that the transition width in the heating curve increases from 3·7° with pure lipid to only 4·7° at 20·9 mol % steroid.

These differences between liposomes and simple mixtures support the proposal that the original liposomes from which the DSC samples are prepared, do not behave as mixtures containing discrete phases of phospholipid and steroid. Mixing appears to be at the molecular level, in which state the corticosteroid can interact with and modify the mesomorphic behaviour of the hydrated lipid molecules. This is consistent with the incorporation of cortisol-21-palmitate into the phospholipid bilayer in the liposome suspensions.

The observations also give some indication of the nature of the interaction between steroid and phospholipid in the liposome. The pre-transition in dipalmitoyl lecithin has been identified by Ladbrooke & Chapman (1969) with the motion of the phospholipid polar head groups. Alternatively, Hinz & Sturtevant (1972) suggest that this transition may be associated with co-operative movement of the rigid acyl side chains in a transition between crystal forms below their melting temperature. A third possibility is that the pre-transition is associated with tilting of the hydrocarbon chains before melting (Chapman, Urbina & Keough, 1974). It is evident from the present results that the inclusion of cortisol-21-palmitate sterically interferes with the motion responsible for this transition and eliminates the

endotherm from the DSC spectrum. This could, of course, be consistent with inhibition of the head group motion or motion of the acyl chains and taken alone does not help identify the nature of the steroid-phospholipid interaction. However, the fact that the transition temperature of the main endotherm remains effectively constant in the presence of steroid indicates that there is little effect on the mobility of individual phospholipid acyl side chains. Such an effect would be manifest as a change in fluidity of the lipid core and a reduction in the main transition temperature. The incorporation of cholesterol into hydrated phospholipids has been shown to produce this kind of behaviour (Ladbrooke & others, 1968). Therefore, it seems unlikely that the bulky cortisol nucleus is associated with the lipid core of the bilayer. However, the insertion of the palmitate side chain of the steroid ester into the ordered array of identical phospholipid palmitate chains should lead to minimum disorganization of the bilayer core and should not significantly inhibit the freedom of motion of the phospholipid acyl side chains. This kind of association would not lead to a perturbation of the melting temperature of the chains and would maintain the main transition endotherm at approximately the same temperature. Therefore, these observations are consistent with the incorporation of the corticosteroid molecule 'tail-first', in such a way that the palmitate ester chains pack easily into the bilayer hydrocarbon core, while the corticosteroid nucleus remains 'on the surface'. The loss of the pre-transition lends support to the belief that this transition is associated with motion of the phospholipid polar head groups and that insertion of the cortisol nucleus into the head group array interferes with this motion.

When the steroid concentration is increased, broadening of the main transition without a systematic reduction in its temperature might indicate the existence of more than one phospholipid-steroid mesophase in the liposomes. This implies that the steroid is not uniformly dispersed in the phospholipid matrix but that some segregation of the two molecules leads to domains containing different amounts of steroid or less likely, different amounts of bound water. The evidence for multiple phases in the samples is re-enforced by the DSC cooling curves. Fig. 1 (b) shows that on cooling from above the main transition temperature, samples containing the higher concentrations of cortisol-21-palmitate show a distinct assymetry in the main peak, and at 20·9 mol % steroid, a second transition appears. This transition does not result from free corticosteroid,

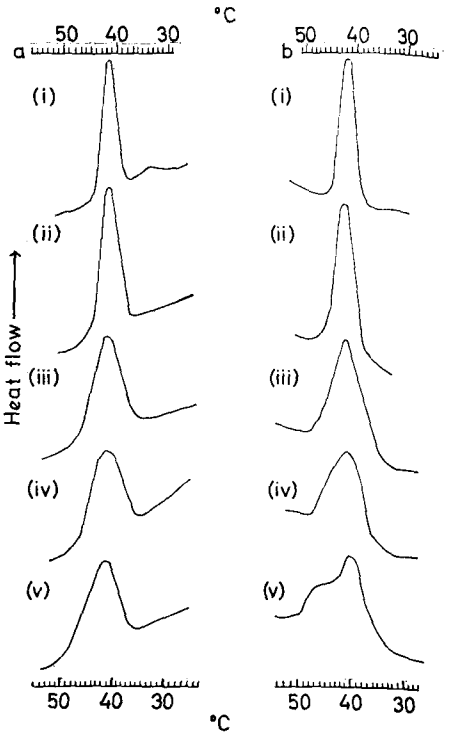

FIG. 1. DSC spectra for dipalmitoyl phosphatidylcholine liposomes containing cortisol palmitate. (a) first heating (b) first cooling. Cortisol palmitate concentration (i) 0 (ii) 3·8 (iii) 7·4 (iv) 13·2 (v) 20·9 mol %.

because the melting temperature of the ester is not in this temperature range. The assymetry and the new peak probably result from the formation of intermediate mesophases, rich in cortisol-21-palmitate. Segregation of these domains in the liposome is facilitated when the samples are maintained in a fluid state above the main transition temperature and the overlapping transitions are, therefore, more evident in the cooling curve. This assymetry was observed in all subsequent heating and cooling cycles, after the sample had been above the transition temperature. Other experiments showed that liposome samples which were maintained above 41° overnight, showed complex thermal spectra containing multiple transitions, the number and intensity of which depend on the steroid concentration and the thermal history of the sample.

It is clear from Fig. 2 that the increase in half-peak line width with increasing steroid concentration is not continuous, but reaches a maximum at about 13 mol %. We interpret this to mean that the original liposome is 'saturated' with steroid at this concentration and that further addition of steroid

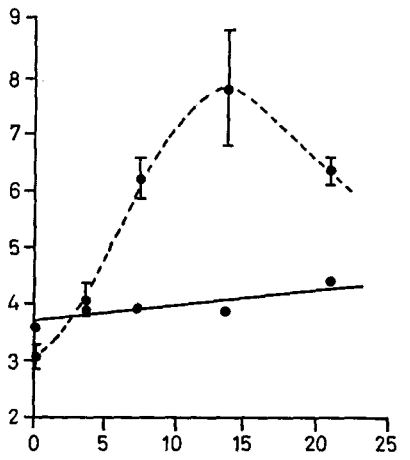

FIG. 2. Half-peak line width of the main DSC peak for (●- -●) dipalmitoyl phosphatidylcholine liposomes containing cortisol palmitate and (●—●) dipalmitoyl phosphatidylcholine–cortisol palmitate mixtures. Ordinate: Half-peak line width (°C). Abscissa: Cortisol palmitate (mol %).

leads to the separation of a separate phase of cortisol-21-palmitate. The nmr experiments described below support this view. The packing of the phospholipid and steroid palmitate side chains is unlikely to limit steroid incorporation in the bilayer, and this 'solubility' limit is probably determined by accommodation of the steroid nucleus in the array of phospholipid head groups.

These observations suggest that if liposomes are to be used as a carrier for cortisol-21-palmitate, the steroid concentration should be maintained below this saturation concentration so that all the drug remains associated with the carrier.

To check this conclusion, liposomes were prepared from mixtures containing steroid in excess of this limiting concentration and the samples were investigated by nmr. The chosen formulations contained dipalmitoyl phosphatidylcholine, 34 mol %, cortisol-21-palmitate and 9·7 mol % egg phosphatidic acid. The charged lipid was added to impart a negative charge to the liposomes which is known to stabilize liposome suspensions against flocculation and sedimentation. For example, negatively charged liposomes containing 13 mol % steroid do not sediment for several days after preparation. However, the liposomes containing 34 mol % steroid, in phosphate buffered isotonic saline, formed a sediment composed of coarse particles within hours of preparation. This sediment was removed by decantation and the suspended liposomes were isolated by ultracentrifugation. The samples were then dried under vacuum and the nmr spectra of the samples were recorded. The molar ratios of cortisol-21-palmitate and dipalmitoyl phosphatidylcholine in the sediment and the liposomes were then computed from the relative intensities of the steroid C_4 proton peak and the phospholipid choline methyl proton peaks.

In two separate experiments, the molar concentration of steroid in the suspended liposomes was computed to be 19 and 20 mol %. This is close to the 13 mol % saturation concentration indicated by DSC with the neutral liposomes. However, the concentration of corticosteroid in the sediments from the two experiments was calculated to be 75 and 50 mol %, showing them to be rich in steroid compared with the suspended liposomes. This supports the proposition that above the saturation concentration, excess steroid separates from the liposomes as a separate phase. The sediment probably contains a mixture of steroid particles which may be associated with adsorbed phospholipid and some true liposomes saturated with steroid.

These experiments confirm the proposition by Shaw & others (1976) that dipalmitoyl phosphatidylcholine can act as a carrier for corticosteroids such as cortisol-21-palmitate. However, they demonstrate that a limited concentration of drug can be accomodated in the liposome and suggest that biological evaluation must be carried out with formulations containing less than this steroid concentration limit.

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