International Journal of Pharmaceutics, 89 (1993) 157–167

IJP 02909

Research Papers

Liposome-incorporated dexamethasone palmitate: Chemical and physical properties

H. Benameur [a], G. De Gand [a], R. Brasseur [b], J.P. Van Vooren [c] and F.J. Legros [a]

[a] *Laboratory of Physiopathology, Free University of Brussels, Campus Erasme Bldg E, 808, route de Lennik, 1070 Brussels (Belgium),* [b] *Laboratory of Physical Chemistry of Macromolecules at Interfaces, Free University of Brussels, Brussels (Belgium) and* [c] *Chest Department, Erasme Hospital, Brussels (Belgium)*

(Received 31 March 1992)
(Accepted 8 May 1992)

Key words: Multilamellar vesicle; Small unilamellar vesicle; Dexamethasone; Dexamethasone palmitate; Minicolumn centrifugation; Lipid complex; Conformational analysis

Summary

Dexamethasone palmitate (DMP) has been prepared by esterification. This molecule and its isotopic marker have been encapsulated into 20 mg multilamellar liposomes (MLV) and small unilamellar vesicles (SUV) made of egg phosphatidylcholine (egg-PC) alone or of egg-PC and cholesterol (CH) in molar ratios of 4:3 and 4:6. Amounts of DMP ranging from 0.1 to 3000 μg/ml were added to the lipid film. Liposomes were formed in 1 ml phosphate-buffered saline, pH 7.4, 300 mOsm/kg H_2O. MLV were first isolated by centrifugation. This method proved to be inappropriate, since DMP not only was encapsulated into MLV but also formed micellar structures and aggregates which could not be separated from MLV by centrifugation nor by filtration through a glass microfiber filter. An alternative rapid method was developed, attempting to isolate unencapsulated DMP complexes by minicolumn centrifugation from liposomal suspensions. This method was successfully applied to DMP associated SUV. The encapsulation efficiency was 65% for 4:3 SUV. This fell to 50% for a DMP concentration of 3000 μg/ml. This should be related to a maximal DMP encapsulation of 13 mol% (Fildes and Oliver, *J. Pharm. Pharmacol.*, 30 (1978) 337–342) which corresponds to 2600 μg/ml under our experimental conditions. The 75% optimum efficiency observed for liposomes made of egg-PC alone suggested that DMP could replace CH in these vesicles. Conformational analysis indicated that DMP inserted into monolayers with its carbonyl group oriented towards the aqueous surface while the aliphatic chain became aligned parallel with the acyl chains of phospholipids. The consideration of the incorporation of DMP into an assembly of phospholipids as taking place in a manner similar to that of CH would appear to be reasonable. This reinforces the validity of the hypothesis of CH being replaced by DMP in liposomes composed of egg PC alone.

Introduction

The treatment of inflammatory diseases by systemic administration of glucocorticoids is often accompanied by serious side effects (Goodman and Gilman, 1985) which limit the use, or necessitate the withdrawal of otherwise effective therapeutic agents. The incorporation of a number of drugs in liposomes has been shown to increase their effectiveness and to reduce their toxicity (Fendler and Romero, 1977). The local administration of liposomes into body anatomical cavities containing phagocytic cells, e.g., pulmonary

Correspondence to: F.J. Legros, Laboratory of Physiopathology, Campus Erasme Bldg E, 808, route de Lennik, 1070 Brussels, Belgium.

macrophages, could greatly enhance the probability of cell targeting. It is known that these phospholipid vesicles are readily taken up by and retained in phagocytic cells such as macrophages (Fidler et al., 1989).

Liposome-incorporated corticosteroids inhibit certain types of inflammation in man and animals more intensively than a corresponding dose of free drugs. Dexamethasone palmitate (DMP) was designed for this purpose and has been proven to be 5.6-times as potent as the plain formulation after intra-articular application (Mizushima et al., 1982).

In order to determine the exact mechanism of delivery of the drug, it is important to characterize its chemical and physical properties. Specifically, detailed knowledge of the mode of interaction with membrane lipids will help in the elucidation of the attachment of DMP to liposomes.

In the present report, multilamellar (MLV) and unilamellar (SUV) liposomes were used. The vesicle size was measured by laser-light scattering. The state of DMP in monolayers was determined by conformational analysis. We also achieved the successful separation of the lipid-DMP complexes and insoluble drug precipitate

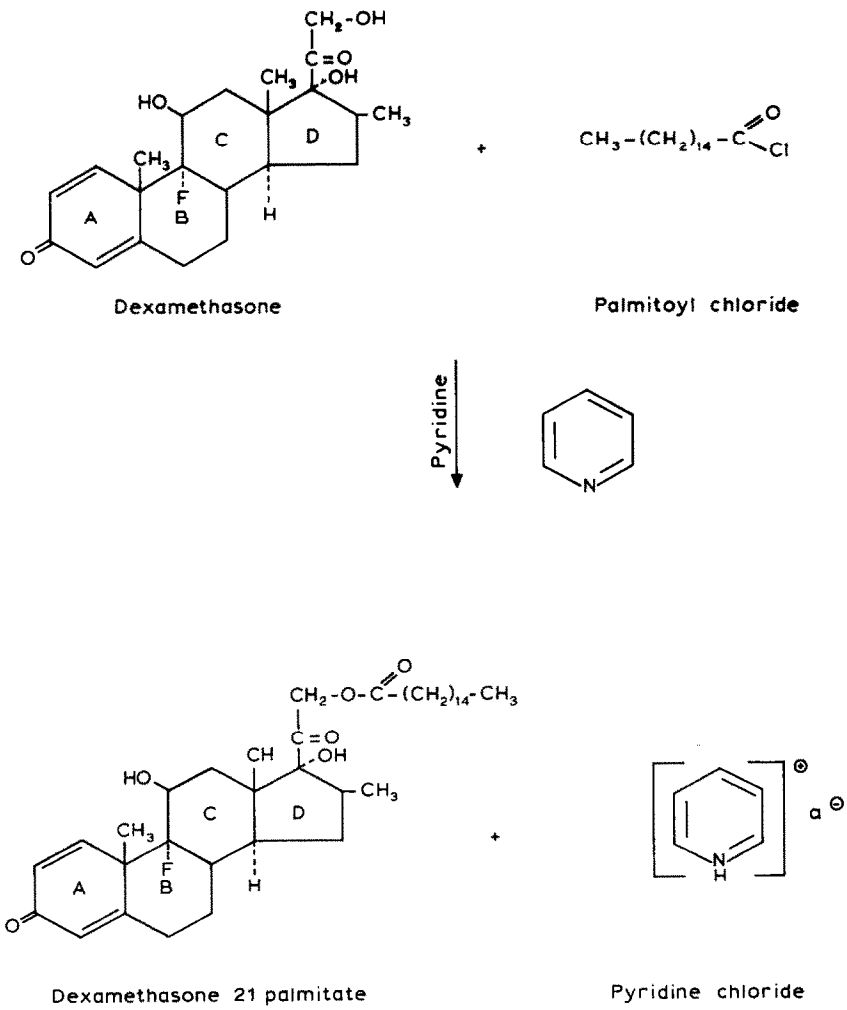

Fig. 1. Chemical structure of DM and DMP in the esterification reaction.

from liposome-associated compound. These complexes are known to be quite difficult to separate by centrifugation, whereas attempts to dialyse out the unencapsulated compound are frustrated by its low solubility in water.

Materials and Methods

Materials

Egg phosphatidylcholine (egg PC type VII-E) 100 mg/ml in a chloroform solution, cholesterol (CH) and dexamethasone (DM) 9α-fluoro-16α-methylprednisolone were purchased from Sigma (St. Louis, MO, U.S.A.). Cholesterol was dissolved at a concentration of 20 mg/ml in chloroform. [1,2,4,6,7-^{3}H]Dexamethasone (37 MBq/ml) and [^{14}C]inulin (1.85 MBq/ml) were purchased from Amersham International (Amersham, Bucks, U.K.). All the above materials were stored at -20°C.

Phosphate buffer was used by mixing precalculated amounts of monobasic and dibasic sodium phosphates. Stock solution A was prepared by dissolving 27.6 g (0.2 mol) of monobasic sodium phosphate (monohydrate) in deionized water to a total volume of 1000 ml. Stock solution B was prepared by dissolving 28.4 g (0.2 mol) of dibasic sodium phosphate in deionized water to a total volume of 1000 ml. By mixing 19 ml of A and 81 ml of B and diluting to a total volume of 200 ml, a 0.1 M phosphate buffer of pH 7.4 was prepared. The osmolality was brought to 300 mOsm/kg H_2O by adding NaCl.

Palmitoyl chloride was obtained from Aldrich Chemical Co (Brussels, Belgium). Sephadex G-50 (100–200 mesh) was purchased from Pharmacia (Uppsala, Sweden) and TLC silica gel 60 WF_{254} plates were obtained from Merck (Darmstadt, Germany). Dexamethasone 21-palmitate (DMP) was kindly provided by Roussel UCLAF (Romainville, France); [1,2,4,6,7-^{3}H]dexamethasone 21-palmitate (D*MP) was prepared in our laboratory. TLC was performed in an Eastman chromogram developing apparatus with chloroform/ethyl acetate (1 : 1, v/v) as a solvent. Liposomes were dissolved in an organic solvent (Soluene 350®, Packard, Meriden, U.S.A.). Radioactivity was counted in a Packard liquid scintillation cocktail (Insta-Fluor®, Packard). A scintillation spectrophotometer (Pharmacia LKB) was used for radioactivity counting.

The sizes of the MLV were measured with Coulter® LS-130 particle size analysers based on laser diffraction. This equipment had a particle size measurement range of 0.1–800 μm on 100 size channels and a PIDS (polarization intensity differential scattering) optical system used as a specially engineered third optical train to measure and analyze the symmetry of the particle sizes in the 0.1–0.5 μm range.

Methods

Synthesis of dexamethasone palmitate

Tritiated dexamethasone palmitate (D*MP) was prepared by esterification (Fig. 1) according to the method of Shaw et al. (1976) with slight modifications. Dexamethasone (24 mg, 6.11×10^{-5} mol) was dissolved in pyridine (1 ml) at room temperature. [1,2,4,6,7-^{3}H]Dexamethasone (50 μl, 50 μCi) in ethanol and palmitoyl chloride (6 μl) was added, and the mixture was stirred at 4°C for 24 h. The solvent was removed under vacuum at 45°C. Distilled water (5 ml) and ethanol (20 ml) were added in that order. The mixture was concentrated under vacuum at 45°C to remove water. This procedure was repeated three times. The residue was dissolved in a minimum amount of ethanol and chromatographed on TLC using chloroform/ethyl acetate (1 : 1, v/v) as solvent; 98% of the radioactivity migrated with the solvent front. This material was presumed to be the ester product as judged by UV detection with DMP as a control, and was used without further purification. The silica was collected from the TLC plate and DMP was eluted by filtration with chloroform in a Pasteur pipete plugged with glass wool.

Fractions (2 ml) were collected and 10 μl of each fraction were taken for scintillation counting. Fractions representing the radioactivity were pooled (20 ml). The specific radioactivity was determined as 2.09 μCi/ml.

Unlabelled dexamethasone palmitate was synthesized in the same way on a large scale and was purified on a Sephadex column (2.5 cm internal diameter, 30 cm height; Sephadex G-50, Pharmacia LKB) using chloroform/ethyl acetate as solvent (1:1, v/v). Fractions of 10 ml were collected; a sample (10 μl) of each was taken for TLC using toluene/ethyl acetate (7:3, v/v) as solvent. Fractions containing DMP were evaporated to dryness. The product was shown to be dexamethasone 21-palmitate by IR and NMR spectroscopy (250 MHz).

Encapsulation of DMP into MLV

Egg-PC/CH molar ratios of 4:0, 4:3 and 4:6, DMP and D*MP as a tracer (0.1, 1, 10, 100, 1000 and 3000 μg/ml) were dissolved in chloroform. The total weight of PC and CH in each preparation was 20 mg. The chloroform was removed from the lipid mixture by rotary evaporation under N_2, at a temperature of 20°C, i.e., above the phase-transition of egg PC. After drying under vacuum for 24 h, three glass beads were added. The dried lipid film was dispersed in 0.1 M phosphate buffer, pH 7.4 (300 mOsm), by vigorous shaking at room temperature. Liposomes formed spontaneously when the dried film was hydrated and consisted of multilamellar structures with concentric phospholipid bilayers separated by water.

These MLV suspensions were washed three times in the same buffer (1 ml) by centrifugation at 4000 rpm for 20 min at 4°C, in order to separate associated from nonassociated drug. The mixture was resuspended in buffer (1 ml) and passed through a GF/C filter.

The percentage of DMP encapsulation efficiency was calculated following Eqn 1 where cpm_1 denotes the radioactivity in 0.1 ml of liposome suspension and cpm_2 represents that in 0.1 ml of washed and resuspended pellet. All samples were dissolved in 0.5 ml Soluene 350 and incorporated into 10 ml of scintillation cocktail prior to counting. All samples were counted for 10 min.

$$\% \text{ encapsulation} = \left[\frac{cpm_2}{cpm_1}\right] \times 100 \qquad (1)$$

Results were expressed as means ± SD. Statistical significance between experimental groups was determined by Student's unpaired *t*-test.

Encapsulation of [^{14}C]inulin

Lipid films (egg PC/CH molar ratio 4:3) and three concentrations of DMP (100, 500 and 3000 μg/ml), total weight of lipids 20 mg, were prepared as described above. Inulin encapsulation into DMP-MLV was performed in 0.98 ml phosphate buffer in which 0.02 ml [^{14}C]inulin was dissolved as a marker (Basworth and Hunt, 1982). Filtration of these liposomes was performed on Millipore apparatus (Brussels, Belgium) with a GF/C filter.

Particle size distribution of MLV

Empty and DMP-loaded MLV (4:3, 4:0, 20 mg lipids, 0.5 mg/ml DMP) were prepared in phosphate buffer, pH 7.4. The equipment was a Coulter® LS-130 particle size analyser, based on measuring the patterns of light scattered from particles exposed to a collimated light beam. Differently sized particles produced a characteristic pattern of light scattering. This pattern was analyzed in order to produce an accurate size distribution (Hoff, 1990).

Auto-rinsing of the particle size analyser with buffer was realized and the run cycle was performed with both MLV preparations. All operations were computerized including statistical calculations.

Encapsulation of DMP into SUV

Small unilamellar vesicles (SUV) were prepared by ultrasonic disintegration for 20 min at 60 W under a nitrogen atmosphere (Soniprep, MSE Scientific Instruments, Crawley, U.K.).

Minicolumn centrifugation of SUV

This procedure is based on the method of Fry et al. (1978). Sephadex G-50 was allowed to swell in the phosphate buffer described above. A GF/C Wathman filter (Batra, 1975) was placed in the barrel of a 5 ml plastic syringe to support the gel and to retain complexes and aggregates. The syringe was filled with Sephadex.

The column was inserted into a test tube so that it was supported at the top of the tube by the finger grips of the syringe. Each column was weighed before being spun at 2000 rpm for 5 min in a centrifuge with swinging buckets to remove excess buffer from the Sephadex beads.

The syringe was transferred to another test tube and up to 1 ml of SUV preparation containing entrapped and free solute was applied to the Sephadex bed. The column was weighed and spun under the conditions employed for expelling the liposomal material from the column and into the test tube. Solute retained by the Sephadex beads was recovered by washing the column with buffer and eluting by centrifugation at 2000 rpm.

Radioactivity was measured using a Pharmacia LKB Rackβ scintillation spectrophotometer using Soluene 350 and Instafluor scintillation cocktail.

Conformational analysis

A theoretical approach was undertaken which allowed the elucidation of the conformation of the entire DMP molecule and its mode of insertion into the lipid layer. This approach was based on the technique developed by Brasseur et al. (1981). The total conformational energy of the molecule at the air-water interface was empirically calculated as the sum of all contributions resulting from local interactions. The values used for the valence angles, bond lengths, atomic charges, and the X-ray crystalline form of DMP were those reported by Mitsunobu et al. (1989). In the calculation procedure, the hypermatrix method was used to surround one drug with lipid molecules (Brasseur et al., 1981). This approach was limited to the number of molecules that sufficed to surround one central molecule.

The configuration of the final mixed monolayer was projected onto the interface plane (X,Y) and the areas occupied per molecule were estimated. The mean interaction energy between one drug molecule surrounded by lipids was equal to the sum of lipid-drug and lipid-lipid interaction energies divided by the number of surrounding lipids (Brasseur, 1991).

The theoretical analysis supposes a procedure comprising two steps, namely, determination of (a) the conformation of the isolated DMP molecule and (b) the conformation of the DMP assembled in the monolayer.

The hydrophobic/hydrophilic balance (ϕ) was calculated according to Eqn 2 where E_{pho} represents the hydrophobic transfer energy and E_{phi} the hydrophilic transfer energy.

$$\phi = \log \frac{E_{pho}}{E_{phi}} \tag{2}$$

The molecular hydrophobicity potential showing hydrophobic and hydrophilic regions was calculated taking into account the transfer energy of each atom employing a recently reported methodology (Brasseur, 1991).

All calculations were performed using an Olivetti CP486 with an 80486 processor. The software was PC-TAMMO + (Theoretical Analysis of Molecular Membrane Organization) and PC-MSA + (Molecular Structure Analysis) procedures. Graphs were drawn with the PC-MGM + (Molecular Graphics Manipulation) program.

Results

Conformational analysis

The mean energies of interaction, computed by the hypermatrix method between DMP and DPPC, are reported in Table 1. This value was quite different from the interaction energy between DPPC-DPPC molecules. The molecular area of the polar DMP group was within the range of DPPC. The hydrophobic/hydrophilic

TABLE 1

Conformational analysis

	DPPC-DPPC	DPPC-DMP	DPPC-CH
Energy (kJ/mol)	−54.3	−33.4	−58.9
Molecular area (nm^2/mol)	7	6	5

Mean interaction energies and molecular area between DPPC, DMP and CH incorporated in DPPC monolayer have been calculated and compared.

balance (ϕ) was 0.73 indicating that DMP is a hydrophobic molecule.

The calculation procedure (hypermatrix methods) showed that, in order to surround one DMP molecule, six lipid molecules are required. Comparison between the molecular packing of phospholipids and DMP showed a common characteristic: hydrophobic interactions led to parallel stacking of hydrocarbon chains in a nonpolar matrix, and thus to the formation of a polar matrix consisting of head groups which establish the link between the hydrocarbon chains. In contrast, the polar matrix forms a relatively large space and often contained polar solvent molecules such as water.

Figs 2 and 3 show a proposed mechanism for the insertion of DMP into the DPPC monolayer. In Fig. 2, the oxygen group of DMP ring A is oriented in the same direction as DPPC which stabilizes the molecular arrangement. Fig. 4 shows the molecular hydrophobicity potential of DMP. The hydrophobic envelopes surround the DMP molecule. Only the carboxyl and ester functions induce very small hydrophilic envelopes.

DMP encapsulation into MLV

Multilamellar vesicles were prepared at different molar ratios of lipid and at various DMP concentrations.

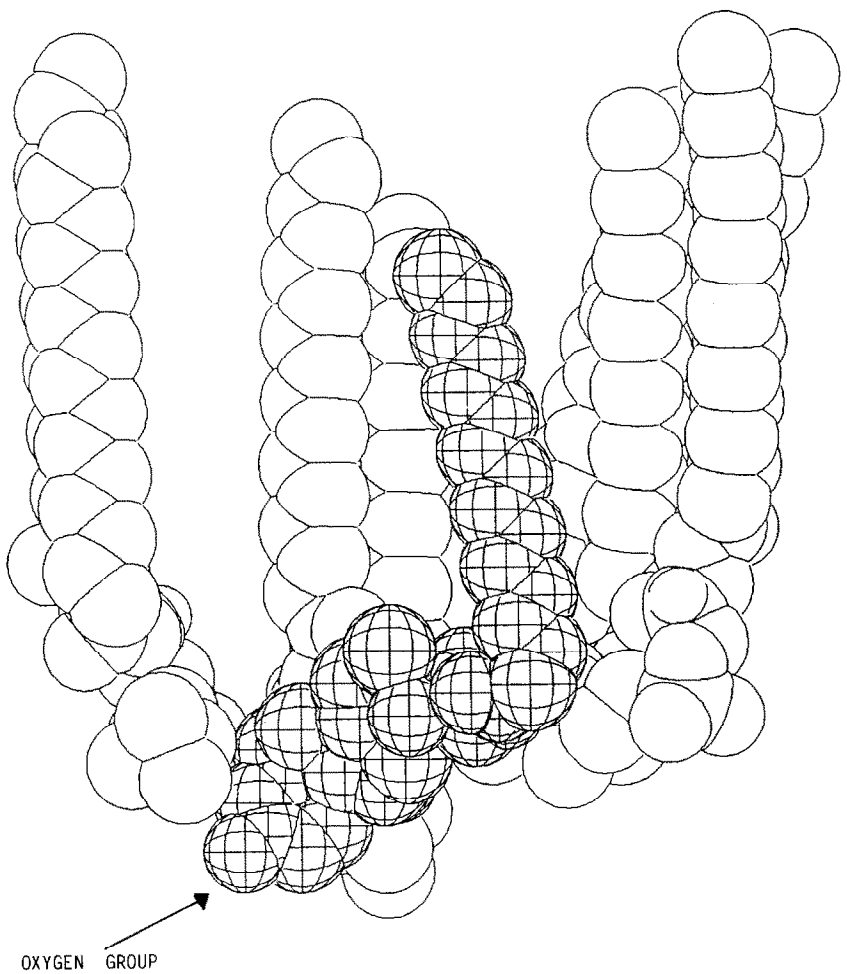

Fig. 2. Space-filling view of the mode of insertion of DMP into DPPC monolayers. DMP is represented by space-filling cross-hatching. The DPPC in front of DMP is not drawn.

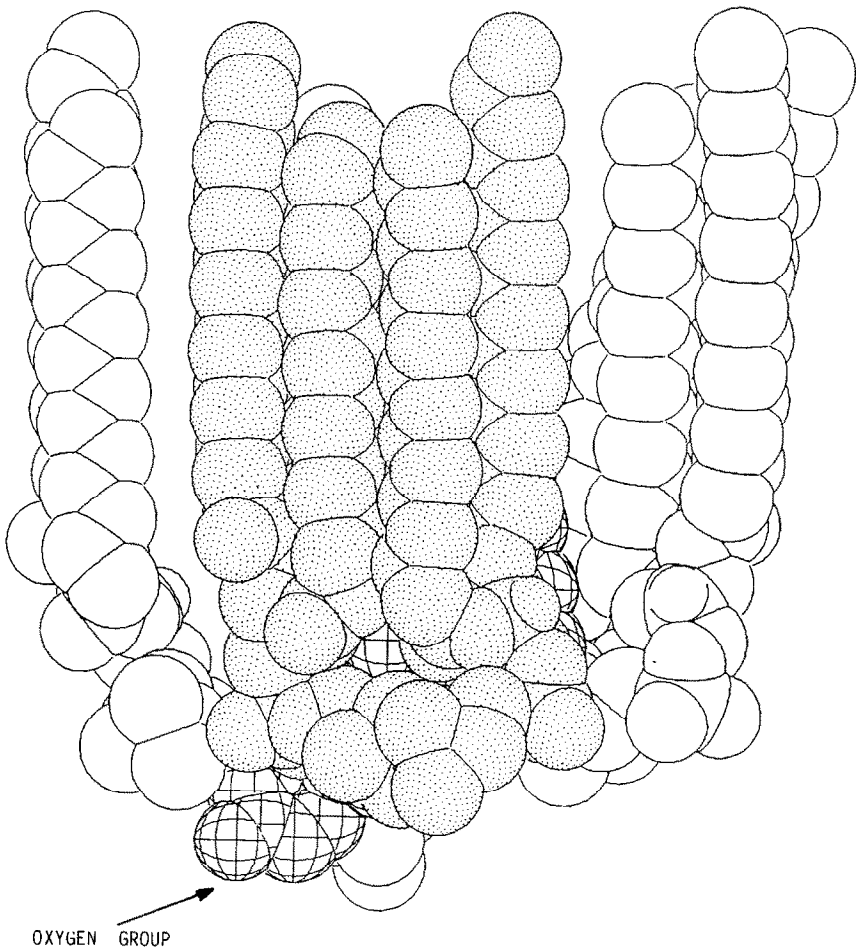

Fig. 3. Space-filling view of the mode of insertion of DMP into DPPC monolayers. DMP is shown in space-filling cross-hatching. The DPPC in front of DMP is represented by dotted filling.

The encapsulation efficiency was statistically identical ($p > 0.05$) at PC/CH molar ratios of 4:0 and 4:3 and at almost all concentrations of DMP used, averaging 85% (Fig. 5). Only the 4:6 MLV at 3000 μg/ml DMP concentration had a statistically significant ($p < 0.05$) lower encapsulation efficiency dropping to 60%.

When vesicle size was measured by laser-light diffraction, the smallest liposomes of the MLV population had a diameter of 0.2 μm while the largest vesicles exhibited a diameter equal to or greater than 8 μm. Diameters of empty and DMP-loaded liposomes were equivalent (Fig. 6). The largest entrapped volume was found for liposome diameters equal to or greater than 8 μm.

When DMP-MLV entrapping [^{14}C]inulin were passed through a GF/C Whatman filter, radioactivity was almost completely retained on the filter (Table 2) indicating that multilayer liposomes were adsorbed on this filter.

Suspensions of DMP with D*MP as a marker and without liposomes were also filtered either immediately after preparation or following sonication. In both cases all of the radioactivity was counted on the GF/C filter.

DMP-SUV

DMP-SUV were prepared by sonication of DMP-MLV as described in Materials and Methods. After minicolumn centrifugation, including

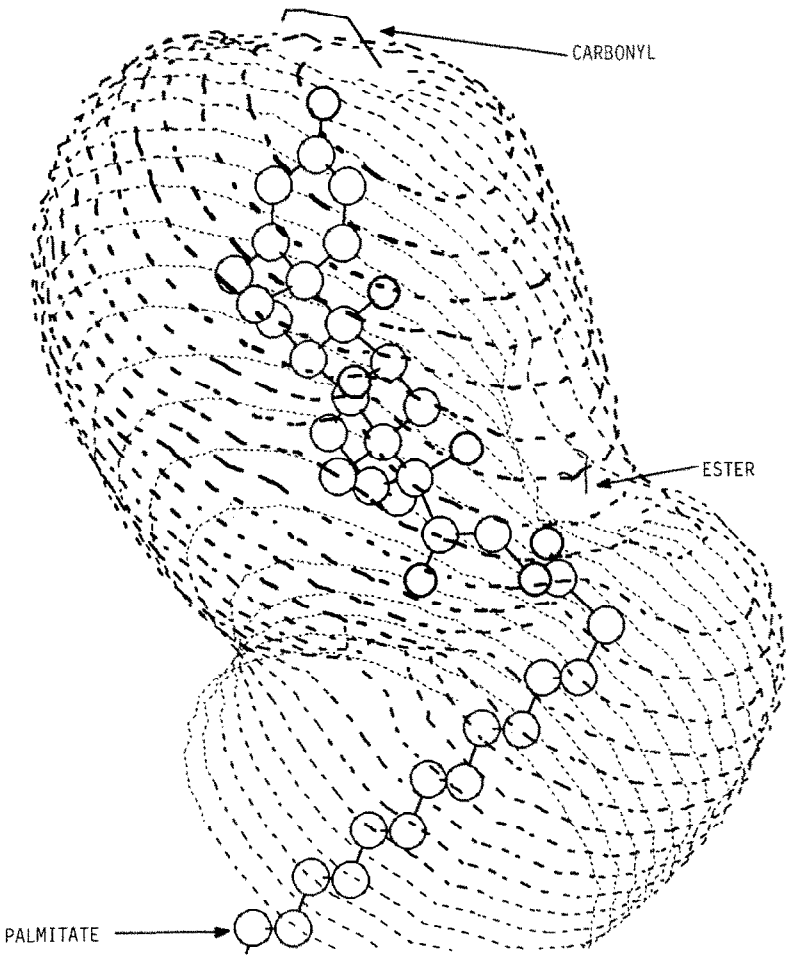

Fig. 4. Perspective view of the molecular hydrophobicity potential of DMP. Hydrophobic envelopes are indicated using continuous lines; hydrophilic envelopes are represented using broken lines. Carbonyl, ester and palmitate are indicated.

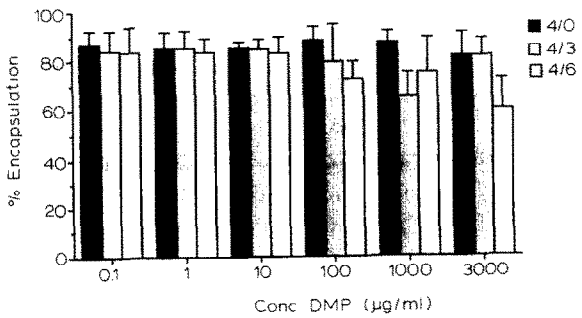

Fig. 5. Encapsulation efficiency of dexamethasone palmitate at concentrations of 0.1, 1, 10, 100, 1000 and 3000 μg/ml ($n = 5$) in MLV of egg PC and of egg PC/CH in molar ratios of 4:3 and 4:6.

TABLE 2

Filtration data of MLV

DMP concentration (μg/ml)	CPM filtrate	CPM filter (GF/C)
100	52.5 ± 1.00	208643 ± 6809
500	53.3 ± 1.00	203745 ± 8404
3000	49.3 ± 1.00	208928 ± 7308

Radioactivities of the filter (GF/C) and of the filtrate are expressed in cpm.

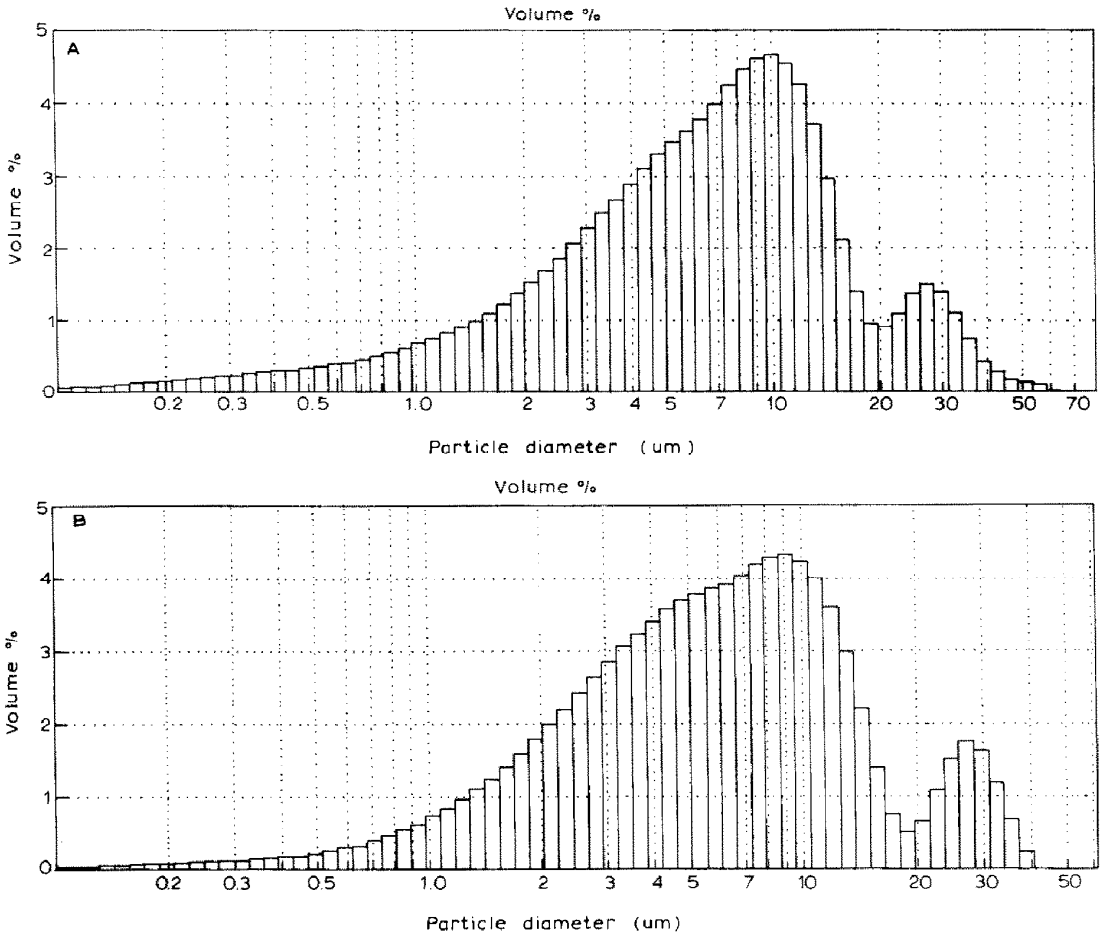

Fig. 6. Particle diameter of empty MLV and of dexamethasone-loaded MLV as a function of the percentage of total liposomal volume in subpopulations ranging from 0.2 to 40 μm. (a) Empty MLV; (b) DMP-loaded MLV.

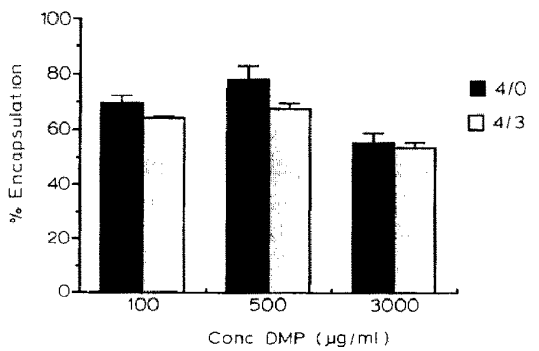

Fig. 7. Encapsulation efficiency of dexamethasone palmitate at concentrations of 100, 500 and 3000 μg/ml ($n = 5$) in SUV of egg PC and of egg PC/CH in the molar ratio of 4:3.

TABLE 3

Filtration data of SUV

DMP concentration (μg/ml)	CPM filtrate	CPM filter (GF/C)
100	227809 ± 9608	43193 ± 5050
500	207504 ± 10508	37146 ± 7908
3000	232441 ± 7568	48240 ± 7608

Radioactivities of the filter (GF/C) and of the filtrate are expressed in cpm.

filtration through GF/C filter, the encapsulation efficiency was found to be 65% for egg PC/CH (4:3, SUV formulation) and 75% for liposomes without CH (Fig. 7). The encapsulation efficiency significantly ($p < 0.05$) fell to 50% for a DMP concentration of 3000 μg/ml.

When DMP-SUV entrapping [^{14}C]inulin were passed through GF/C filter, all of the radioactivity counts were found to be in the test tube, almost no radioactivity being adsorbed on the filter (Table 3).

Discussion

Dexamethasone palmitate has been encapsulated into readily prepared liposomes, namely, MLV and SUV. The phospholipid vesicles were initially centrifuged at 2500 rpm in order to separate encapsulated from nonencapsulated drug. A pellet of liposomes was harvested.

The purity of this pellet had to be questioned taking into account that molecules of DMP are not soluble in water in the accepted sense. In aqueous media, they align themselves in micellar structures in order to minimize the unfavorable interactions between the bulk aqueous phase and the C_{16} hydrocarbon fatty acid chain. These micellar structures cannot be centrifuged at 2500 rpm. In addition, DMP forms aggregates (insoluble drug precipitate) and lipid complexes in water (Szoka and Papahadjopoulos, 1980).

Filtration on 1 μm pore size GF/C filter indicated that DMP complexes formed at DMP concentrations from 0.1 μg/ml. Since they were also retained by the filter, their diameter was at least 1 μm. MLV encapsulating [^{14}C]inulin as a marker of the intraliposomal aqueous phase were retained by this filter. The diameter of the vesicles, DMP-loaded or unloaded, which encapsulated the largest aqueous volume was 8 μm and greater. Thus, it appears that DMP-MLV and DMP complexes were both retained on the filter.

It has been reported that the classical methods of separation, dialysis, chromatography on a silica gel column and ultrafiltration, are not suitable for use in the purification of DMP liposomes (Szoka and Papahadjopoulos, 1980).

An alternative approach has been examined. Attempts were made to separate unencapsulated DMP complexes by performing minicolumn centrifugation according to the procedure of Fry et al. (1978). This method involves centrifugation of a liposomal suspension while being separated from unencapsulated molecules through molecular sieving.

Separation was completed by filtering through GF/C filter during centrifugation. Unfortunately, DMP complexes were adsorbed onto the filter, while DMP-MLV encapsulating [^{14}C]inulin were similarly retained. Thus, the minicolumn centrifugation method could not be used to purify DMP-MLV.

Furthermore, the DMP complexes could not be filtered after sonication under the conditions developed in the preparation of SUV from MLV. Using [^{14}C]inulin loaded SUV, it has been shown that these vesicles were readily filtered. It has been proved that identical empty SUV prepared according to the same conditions of sonication had a diameter of 20–40 nm (Szoka and Papahadjopoulos, 1980); the residual fraction of MLV was at least about 5%. Under our experimental conditions, 6.6% of [^{14}C]inulin-DMP-SUV were retained on the filter. These data indicate that DMP-SUV prepared from DMP-MLV by sonication could be purified by minicolumn centrifugation using GF/C filter.

The encapsulation efficiency of DMP was measured for MLV and SUV. For MLV, the efficiency was identical when egg PC alone or egg PC/CH in a molar ratio 4:3 were used and when different DMP concentrations were encapsulated, averaging 85%. This efficiency was not real, since DMP complexes were included in the harvested liposomal fraction. Only the 4:6 MLV with DMP at 3000 μg/ml encapsulated 60%.

For SUV, the encapsulation efficiency amounted to 65% for egg PC/CH 4:3, and 75% for liposomes without cholesterol. It can be suggested that DMP molecules were partly inserted inside the bilayers, replacing cholesterol. The efficiency fell to 50% for a DMP concentration of 3000 μg/ml. This decrease in DMP encapsulation at 3000 μg/ml for both MLV and SUV could be attributed to a limiting concentration of

DMP in the liposomes. This concentration has been shown to be 13 mol% DMP (Fidles and Oliver, 1978).

Under our experimental conditions, this limiting concentration was calculated as being 2600 μg per ml buffer for 14.42 mg egg PC. A lipid film containing this amount of phospholipid and 3000 μg DMP dissolved in 1 ml buffer was beyond the limit of DMP concentration. These observations suggest that if the liposomes must be used as a DMP carrier, the corticosteroid concentration should be maintained below the saturation value of 13 mol%. Although the absence of cholesterol could improve the encapsulation of DMP, it should be borne in mind that cholesterol stabilizes the liposome structure in biological fluids.

Conformational analysis has demonstrated that, being an amphiphatic molecule, DMP inserts into a DPPC monolayer membrane with its carbonyl group oriented towards the aqueous surface, and that the aliphatic chain aligns itself parallel to the acyl chains of phospholipid. The calculation procedure (hypermatrix methods) indicates that DMP preferentially interacts with lipids rather than with itself. It seems reasonable to consider a lamellar structure wherein DMP is incorporated into an assembly of phospholipids similarly to cholesterol. This reinforces the hypothesis of cholesterol being replaced by DMP in liposomes made of phospholipids alone.

Acknowledgements

We wish to thank Professors E. Brachet and A. Moes for careful reading of the manuscript.

References

Basworth, M.E. and Hunt, C.A., Liposome disposition in vivo. II: Dose dependency. *J. Pharm. Sci.*, 71 (1982) 100–104.

Batra, S., Aqueous solubility of steroid hormones: an explanation for the discrepancy in the published data. *J. Pharm. Pharmacol.*, 27 (1975) 777.

Brasseur, R., Goormaghtigh, E. and Ruysschaert, J.M., Theoretical conformational analysis of phospholipids bilayers. *Biochem. Biophys. Res. Commun.*, 103 (1981) 301–310.

Brasseur, R., Differentiation of lipid-associating helix by use of three dimensional molecular hydrophobicity potential calculations. *J. Biol. Chem.*, 266 (1991) 16120–16127.

Chatelain, P. and Brasseur, R., A conformational analysis study of the interaction of amiodarone and cholesterol with lysophosphatidylcholine. *Biochem. Pharmacol*, 41 (1991) 1639–1647.

Fendler, J.H. and Romero, A., Liposomes as drug carriers. *Life Sci.*, 20 (1977) 1109–1120.

Fidler, I.J., Raz, A., Fogler, W.E., Kirsh, R., Bugelski, K. and Poste, G., Design of liposomes to improve delivery of macrophage-augmenting agents to alveolar macrophages. *Cancer Res.*, 49 (1989) 3976–3984.

Fildes, F.J.T. and Oliver, J.E., Interaction of cortisol-21-palmitate with liposomes examined by differential scanning calorimetry. *J. Pharm. Pharmacol.*, 30 (1978) 337–342.

Fry, D.W., White, J.C. and Goldman, I.D., Rapid separation of low molecular weight solutes from liposomes without dilution. *Anal. Biochem.*, 90 (1978) 809–815.

Goodman, A. and Gilman, L.S., Adrenocortical steroids. In Goodman, A., Gilman, L.S., Rall, T.W. and Murad, F. (Eds), *The Pharmacological Basis of Therapeutics*, Macmillan, New York, 1985, pp. 1474–1485.

Hoff, E.V., Is your particle size analyser up to the task? Technical Bulletin, Coulter Electronics, Scientific Instruments, 1990.

Mitsunobu, D., Toshimasa, I., Shigethoshi, S., Takashi, I., and Masatoshi, I., Physicochemical properties of dexamethasone palmitate, a high fatty acid ester of an anti-inflammatory drug: polymorphism and crystal structure. *J. Pharm. Sci.*, 78 (1989) 417–422.

Mizushima, Y., Hamano, T. and Yokoyama, K., Tissue distribution and anti-inflammatory activity of corticosteroids incorporated in lipid emulsion. *Ann. Rheum. Dis.*, 41 (1982) 263–267.

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

Szoka, F. and Papahadjopoulos, D., Comparative properties and methods of preparation of lipid vesicles (liposomes). *Annu. Rev. Bioeng.*, 9 (1980) 467–508.