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# Effect of dolichyl monophosphate on the permeability properties of lipid membranes

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**The effect of dolichyl monophosphate on the permeability properties of dimyristoylphosphatidylcholine bilayers to alkaline cations, $Ca^{2+}$ and glucose has been determined by stop-flow spectrophotometry. The results show that, in contrast to free dolichol effects, the monophosphate derivative increased the permeability following a decreasing order of the permeating particle size. Phase diagrams indicate that dolichyl monophosphate is fully incorporated into the phosphatidylcholine bilayer around 0.75% weight/weight ratio. For these ratios, the permeation of ions is higher in the gel than in the liquid crystalline state.**

## Introduction

Dolichyl phosphate plays an important role in the biosynthesis of glycoproteins [1–3]; in particular, these polyisoprenoid derivatives may be responsible for the transport of sugar units during glycoprotein biosynthesis.

On this regard, several laboratories have reported the leakage of molecules such as TEMPO-choline from liposomes composed of dolichyl monophosphate, phosphatidylcholine and phosphatidylethanolamine [4–6]. Part of this effect has been ascribed to the influence of dolichol and dolichyl monophosphates on the fluidity, polymorphism and transbilayer movement of the phosphatidylethanolamine molecules [5].

In a previous paper [6], we reported results obtained with stop-flow spectrophotometry in regard to the permeability to alkaline cations of egg and dimyristoylphosphatidylcholine bilayers dopped with dolichol. In that investigation, we found that the ion permeability followed the order $Li^+ > Na^+ > K^+ > Cs^+$ which corresponds to the radius of the dehydrated ions [6]. The rate of permeation was higher when the bilayer was in the fluid state. Glucose and $Ca^{2+}$ did not permeate the bilayer at the temperatures and dolichol/PC ratios assayed.

In this paper we studied with the same methodology, the effect of dolichyl monophosphate on the permeability of multilamellar liposomes composed of dimyristoylphosphatidylcholine to alkaline cations, $Ca^{2+}$ and glucose below and above the phase transition temperature.

The kinetic results and the phase diagrams show that the effect of temperature and dolichyl

Abbreviations: Dol-*P*, dolichyl monophosphate; DMPC, dimyristoylphosphatidylcholine.

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phosphate ratio are different from that found previously with dolichol.

## Materials and Methods

Dimyristoylphosphatidylcholine was purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. A simple spot was obtained by thin-layer chromatography using a mixture chloroform/methanol/water (65 : 35 : 8, v/v) and developed under iodine vapours.

Dolichyl monophosphate was from Sigma Grade III (Cat. number D 7014). The cations used as chloride salts were from Merck p.a. quality.

All other chemicals were analytical grade and water twice distilled and deionized in a MilliQ standard equipment.

Liposomes were prepared according to standard procedures [8].

A dry film was obtained by evaporating the solvent of a chloroform solution of DMPC and the dolichyl monophosphate in the required ratios in a round bottom flask under vacuum. The dry film was subsequently dispersed in an aqueous solution of the chosen composition.

All the solutions were prepared in 10 mM Tris-HCl buffer (pH 7.2).

The incorporation of Dol-*P* to DMPC liposomes was tested measuring the absorbance at 450 nm. A phase diagram was done mixing a DMPC dispersion with aliquots of buffer solutions containing Dol-*P*. The absorbance values were taken after incubating the mixture during 30 min and the molar fraction in each mixture was calculated from the final Dol-*P* and DMPC concentrations in the cuvette.

The gel-liquid crystalline transition temperature for different Dol-*P*/DMPC ratios was determined by the changes at 450 nm as a function of temperature in a double beam Hitachi 100-60 spectrophotometer. These results were comparable to those reported in literature and were taken as an indication of the purity of the Dol-*P* samples [5].

The permeability experiments were carried out in a rapid reaction stop-flow spectrophotometer Durrum D110.

Aliquots of the liposome dispersion and of the buffer solution containing the ion to test were injected simultaneously in a mixing chamber in a mixing time of less than 4 ms. The final lipid concentration in the cuvette was about 0.3 $\mu$mol/ml. Repetitive injections were made until reproducible results with a given liposome dispersion were obtained.

In control experiments, DMPC liposomes without Dol-*P* were dispersed in hypertonic ion solutions. The osmotic shrinkage was followed by the decrease in transmittance. From the slope immediately after the mixing time, the water permeation rate can be calculated (see Fig. 1A) [10].

When liposomes contained Dol-*P*, the entrance of permeating ions was noticed by a transmittance increase after the water outflux. From the slope after the minimum (see Fig. 1B) the ion permeation rate was calculated [10].

After the mixing the absorbance changes were amplified and displayed in a Tektronik 546B storage oscilloscope. The trace was then photographed or plotted in an $x/y$ plotter.

The time decay constant ($\tau$) was calculated from the slopes of the logarithm of the transmittance ($T$) versus time ($t$) according to the expression

$$T = T_0 e^{-t/\tau}$$

for a first-order process. $T_0$ is the transmittance at time zero [9]. The ion permeation rate was calculated according to Ref. 7

$$\frac{dT}{dt}\% = \frac{1}{\tau} \times \frac{100}{T_0}$$

## Results

Typical curves obtained when liposomes compared with different Dol-*P*/DMPC ratios are mixed with an hypertonic KCl solution above the liquid crystalline transition temperature are shown in Fig. 1.

The water and the ion permeabilities can be calculated from the slopes before and after the minimum, respectively (see Fig. 1).

Control experiments with pure DMPC (part A), show a decrease in the transmittance due to water outflux. The curves corresponding to liposomes containing 0.50% or 0.75% Dol-*P* show an in-

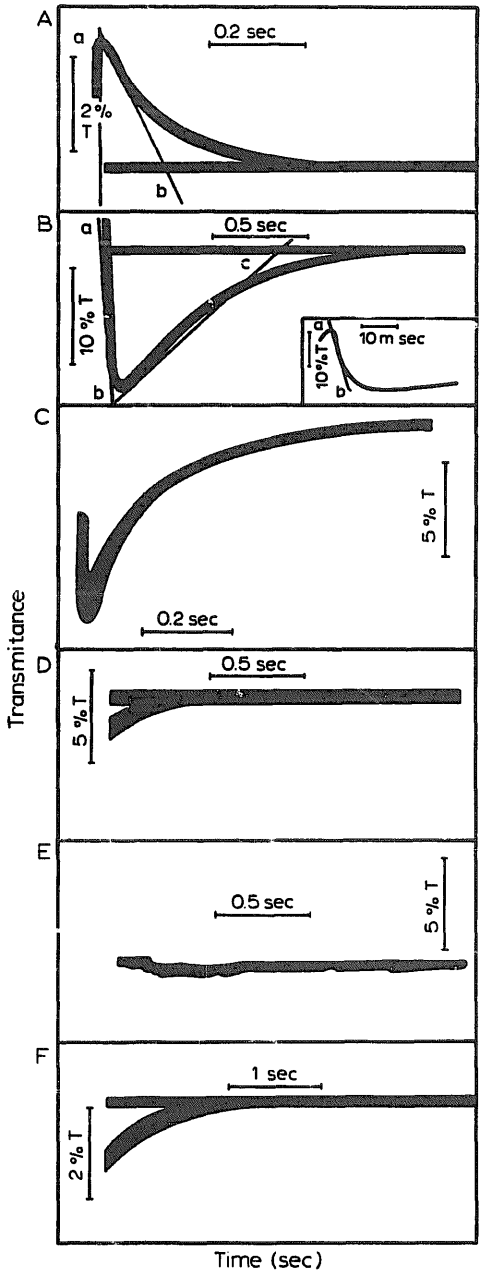


Fig. 1. Stop-flow transmittance versus time traces corresponding to the osmotic response of dimyristoylphosphatidylcholine/dolichyl monophosphate liposomes maintained at 25 °C. Liposomes were prepared as described in Materials and Methods. The different scales for each composition were chosen in order to obtain the maximum definition in the slopes for permeability calculations (see text). (A) Control curve with no Dol-*P* (0% Dol-*P*) against $K^+$ gradient. (B) 0.50% Dol-*P*/DMPC ratio against $K^+$ gradient. (C) 0.75% Dol-*P*/DMPC ratio against $Ca^{2+}$ gradient. (D) 1.50% Dol-*P*/DMPC ratio against $K^+$ gradient. (E) 2.00% Dol-*P*/DMPC ratio against $K^+$ gradient. (F) 2.80% Dol-*P*/DMPC ratio against $K^+$ gradient. a b (———) Initial volume decrease rate (water outflux). b c (———) Initial volume increase rate (solute influx). Inset: transmittance versus time traces for the water outflux of Dol-*P*/DMPC liposomes. Time scale: milliseconds.

crease in transmittance after the minimum which is ascribed to the entrance of $K^+$ into the liposomes driven by its concentration gradient.

The curves of Fig. 1 also indicate that the minimum is not clearly defined at Dol-*P*/DMPC ratios higher than 1% (Fig. 1, D, E, F). Therefore, the ion permeabilities obtained in these cases from the slopes after the minimum, are affected by a large error.

The ion permeabilities for each Dol-*P*/DMPC ratio was calculated as described before taking the mean value of the slope obtained after at least eight assays for a given preparation. The final value for each ion was the average of two different liposome preparations. Under these conditions, the reproducibility was higher than 85%.

The results obtained with the above approach for different Dol-*P*/DMPC ratios are summarized in Fig. 2. It is observed that the maximum $K^+$ permeability is obtained for 0.50%. However, for reasons given later, this maximum in permeability is not coincident with the maximal incorporation of Dol-*P* into DMPC bilayers. It must be noticed, in addition, that the higher amplitude of the curves for 0.75% allows a better osmotic response of the liposomes. This, in principle, can be a consequence of optical properties of the dispersions for the different Dol-*P*/DMPC ratios.

As shown in Fig. 3, the absorbance of the dispersion decreases when the Dol-*P*/DMPC ratio increases from zero until 1.00% (Fig. 3A). Above this ratio little variation is observed. This can be

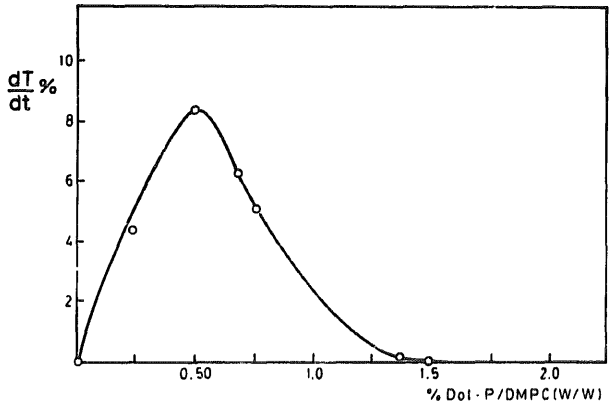


Fig. 2. Permeation rate of $K^+$ entrance as a function of the dolichyl monophosphate/dimyristoylphosphatidylcholine ratio at 25 °C. Each point is an average from at least twenty curves as those shown in Fig. 1.

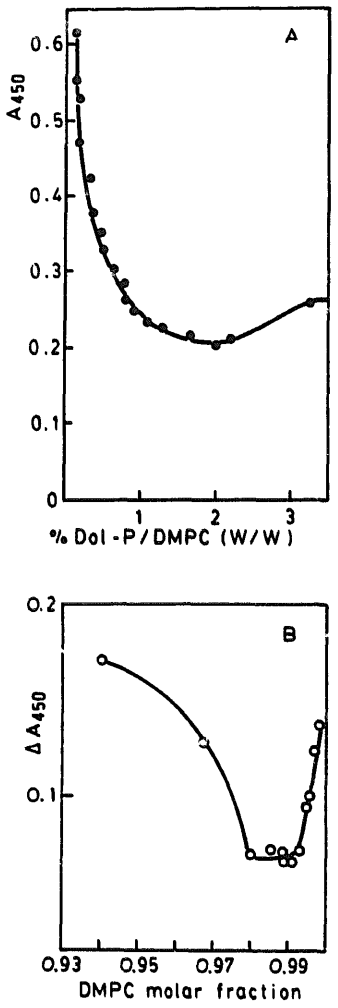


Fig. 3. Effect on the dispersion absorbance of the varying dolichyl monophosphate/dimyristoylphosphatidylcholine ratios. (A) Absorbance decrease obtained when a buffer solution containing 0.021 mg/ml of dolichyl monophosphate is titrated with pure DMPC liposomes at 25°C. (B) Relative absorbance of Dol-*P*/DMPC dispersions with respect to pure DMPC dispersions as a function of the molar fraction of DMPC. The molar fractions 0.99 and 0.98 correspond to 0.75% and 2.50% w/w ratios, respectively. Experimental conditions were as indicated in Materials and Methods.

taken as an indication that the Dol-*P* incorporation into the liposome bilayer, above the gel-liquid crystalline transition temperature is maximal around 1.00% It is seen that the absorbance converges to a minimum for Dol-*P* percentages ranging between 0.75% and 2.50%. Within this range, the Dol-*P*/DMPC dispersion has lower absorbances than those corresponding to liposomes of pure DMPC.

The same data of Fig. 3A were used to build the phase diagram of Fig. 3B.

The curve of Fig. 3B can be analyzed as a phase diagram of three components: pure dolichyl monophosphate, pure dimyristoylphosphatidylcholine liposomes, and liposomes composed with Dol-*P* and DMPC.

The left-hand branch of the curve corresponds to the high absorbance obtained when an excess of Dol-*P* is present in the solution. The right-hand branch corresponds to the increase of the absorbance as a function of the DMPC liposome excess. The flat portion ranging between 0.98 and 0.99 molar fraction corresponds to mixed liposomes Dol-*P*/DMPC. The constancy in the relative absorbance is an indication that within that range Dol-*P* and DMPC are mixed in all the proportions. Below 0.98 and above 0.99 the excess of Dol-*P* or DMPC appears in the solution as segregated phases, respectively.

Interestingly, within those composition ranges the transmittance vs. time curves, as shown in Fig. 1, present a clear osmotic behaviour demonstrating that Dol-*P*/DMPC liposomes are formed. Therefore, the absorbance minimum of curves in Fig. 3B can be used as a criterion for the incorporation of Dol-*P* to lipid bilayers. According to the results shown in Fig. 3A, the 0.75% ratio is the minimum at which all the Dol-*P* is incorporated to the bilayer. Above this percentage Dol-*P* would also completely incorporate to the bilayer but this increase does not induce an increase in permeability (Figs. 1 and 2). However, it should be stressed that the minimum at these Dol-*P*/DMPC ratios is not clearly defined and therefore slopes for ion permeability determinations are affected by a large error.

The gel-liquid crystalline transitions shown in Fig. 4 indicate that at percentages above 0.75% a shift of the pretransition to higher temperatures is occurring. No change is detected for 0.5% liposomes. These results are similar to those obtained with dolichyl 20-phosphate in DMPC model membranes [5].

Therefore, according to these results, the ratio at which permeability measurements can be done with a relatively high degree of confidence that Dol-*P* is completely incorporated in the bilayers and with a minimum error is 0.75%. In conse-

TABLE I

PERMEATION RATES FOR ALKALINE CATIONS, $Ca^{2+}$, WATER AND GLUCOSE IN LIPOSOMES COMPOSED BY 0.75% w/w Dol-*P*/DMPC AT 25°C

$P_X$, $P_W$ are the permeabilities of the cations and water, respectively. $P_X/P_K$ is the relative permeability with respect to $K^+$. Hydration number ($n$) and ionic radii ($r_s$) were calculated from the limiting mobility by Stoke's law [11]. All the ions were added as chloride salts. Note the different time scales for water and ion permeabilities.

| Ion | $P_X$ ($s^{-1}$) | $P_W$ ($ms^{-1}$) | $P_X/P_K$ | $r_s$(Å) | $n$ |
|---|---|---|---|---|---|
| $Li^+$ | 2.80 | – | 0.54 | 2.37 | 7.1 |
| $Na^+$ | 2.80 | – | 0.54 | 1.85 | 3.5 |
| $K^+$ | 5.16 | – | 1.00 | – | 1.9 |
| $Cs^+$ | 5.97 | 1.01 | 1.16 | – | – |
| $Ca^{2+}$ | 5.10 | 1.53 | 0.99 | 3.09 | 12.0 |
| Glucose | 1.86 | 0.75 | 0.36 | – | – |
| Control DMPC (without dolichol) | – | 1.25 s | – | – | – |

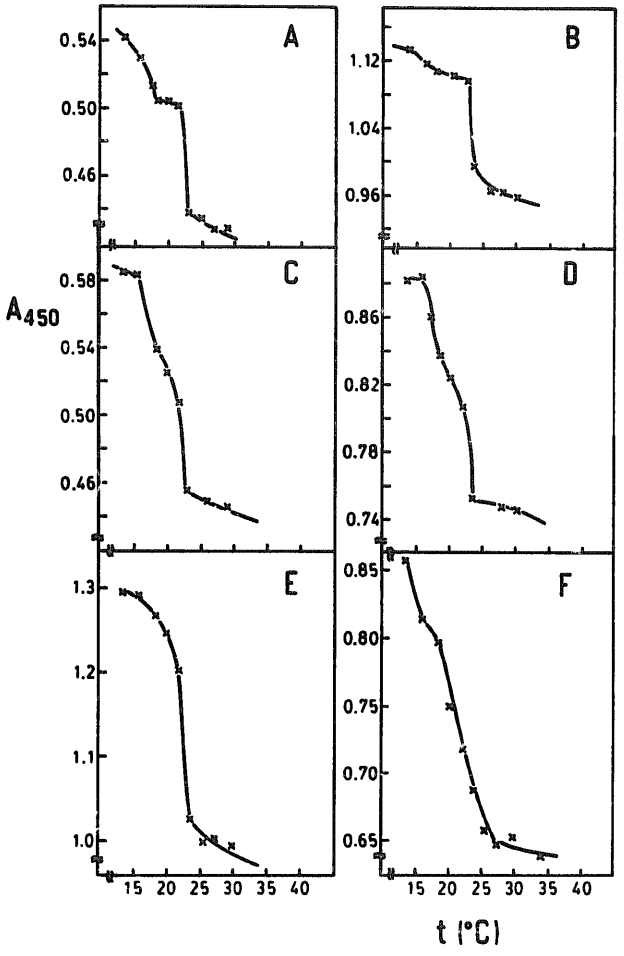


Fig. 4. Gel-liquid crystalline transition of DMPC liposomes containing different percentages of Dol-*P*. Absorbance at 450 nm was measured as a function of temperature for DMPC liposomes with the following percentages of Dol-*P*: (A) 0% or 0.5%; (B) 0.66%; (C) 0.75%; (D) 1.36%; (E) 1.50%; (F) 2.84%.

quence, the selective permeability of alkaline cations, $Ca^{2+}$ and glucose was studied at this composition.

Table I shows the permeability values obtained with liposomes with 0.75% at 25°C for $Li^+$, $Na^+$, $K^+$, $Cs^+$, $Ca^{2+}$ and glucose.

In addition, the water permeability and the relative ion permeabilities with respect to $K^+$ are also given.

It must be noticed that the sequence found for alkaline cations is

$$Li^+ \simeq Na^+ < K^+ < Cs^+ \simeq Ca^{2+}$$

which is opposed to that found previously for

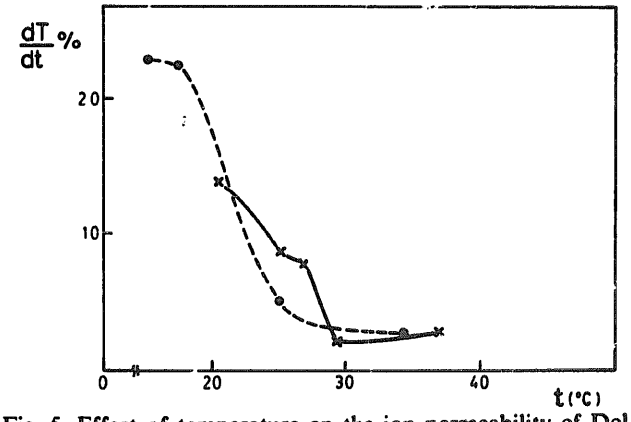


Fig. 5. Effect of temperature on the ion permeability of Dol-*P*/DMPC liposomes. (×) 0.50% w/w; (●) 0.75% w/w. Experimental conditions were as indicated in Fig. 1, except temperature.

dolichol dopped phosphatidylcholine bilayers [6]. In addition, glucose and $Ca^{2+}$ also permeate the Dol-*P*/DMPC membrane above the transition temperature, a phenomenon not observed in Dol-*P*/DMPC membranes.

The observation that 0.50% gives the higher permeability for $K^+$, although it is not the composition at which Dol-*P* is fully incorporated (Fig. 3B) prompted us to study the permeability at 0.50% and 0.75% at different temperatures.

It can be observed in Fig. 4 that at 0.75% the pretransition overlaps with the main transition. Thus, at temperatures between 15 and 24°C the higher permeabilities observed in Fig. 5 correspond to the transition region.

The 0.75% ratio curve shows a drastic increase in $K^+$ permeability around 21°C, which is the DMPC transition temperature (Fig. 5).

In contrast, the 0.50% ratio curve shows a clear separation of the main and the pretransition regions. The permeability is observed at 20°C, i.e. between the pre and the highest main transition temperatures. The permeability in this gel state region is much lower than that found for 0.75%. Coincident with data in Fig. 2, the curve of Fig. 5 shows that for 0.50% the permeability is higher than that for 0.75% at the fluid state.

This behaviour could be attributed to the different accessibility of Dol-*P* to bilayers in the gel or in the fluid state. An insight into this possibility was obtained measuring the changes in absorbances produced when liposomes composed with different Dol-*P*/DMPC ratios undergo a gel-liquid crystalline transition (Fig. 6).

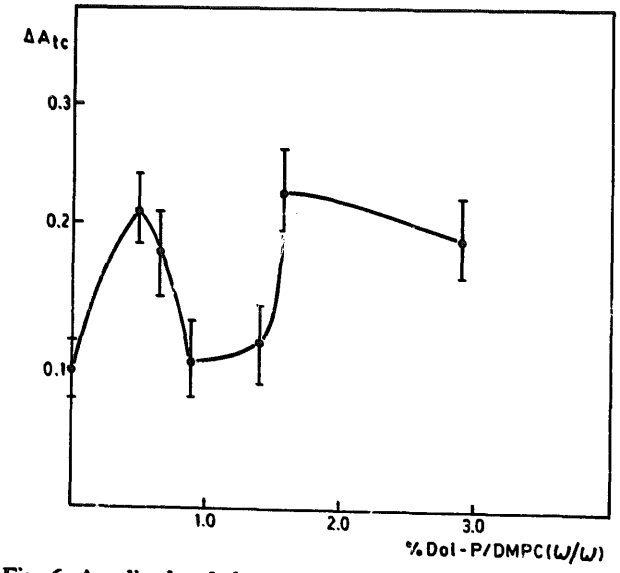


Fig. 6. Amplitude of the absorbance change at the gel-liquid crystalline transition temperature for different Dol-*P*/DMPC ratios. Experimental conditions were as indicated in Materials and Methods.

It is interesting to note that the maximum change in absorbance is obtained with 0.50%, whereas that corresponding to 0.75% shows a similar absorbance change to that found with pure DMPC bilayers.

For a given absorbance value at the gel state, the increase in temperature promotes a decrease in the absorbance at the gel-liquid crystalline transition temperature. The amplitude of the change depends on the Dol-*P*/DMPC ratio.

This is another indication that the physicochemical properties of 0.50% liposomes are different from those composed with 0.75%, in addition to the indications observed in permeability.

## Discussion

The absorbance changes with time have been frequently used to measure water and solute permeabilities in multilamellar liposomes [7]. The osmotic response is reflected by the linear relation between the volume and the inverse of the absorption coefficient.

The inclusion of dolichyl monophosphate in the bilayer may affect the optical properties of the dispersion. There are at least three reasons. In the first place, the incorporation of the dolichyl phosphate molecules may alter the bilayer permeability, allowing the entrance of compounds which are usually impermeable in pure phosphatidylcholine membranes (ions and glucose). In this case, the optical changes with time would be a consequence of the volume variations of the liposomes. Secondly, the resulting absorbances of the mixed liposomes (see Figs. 3A and 3B) may be a consequence of the differences in size and the refraction indices for each composition without implying permeability changes. Thirdly, the absorbance may result from a mixture of liposomes of different composition; one of pure DMPC, another with Dol-*P*/DMPC and a third consisting of aggregates of free Dol-*P* segregated in the water phase.

Each of these dispersed phases would have a different absorbance in the equilibrium which may

respond differently to the osmotic stress in comparison to pure DMPC liposomes.

All these possibilities should be taken into account to interpret the transmittance changes with time obtained in Fig. 1.

Although liposomes used in those experiments are obtained by dispersing in KCl solution a dry film evaporated from a chloroform solution containing Dol-*P* and dimyristoylphosphatidylcholine in the desired ratios, this does not guarantee that the dispersion is composed only by Dol-*P*/DMPC liposomes. In other words, part of the Dol-*P* could remain in the aqueous phase.

This is particularly important when the Dol-*P*/DMPC ratio is increased.

As shown in Fig. 1, the transmittance vs. time curves become diffuse for Dol-*P*/DMPC ratios higher than 1.00%. It may be possible that at high concentrations, only part of the Dol-*P* is incorporated into the liposomes and hence the osmotic changes observed are difficult to detect.

An insight on the incorporation of Dol-*P* to DMPC bilayers can be obtained from the absorbance changes produced when a Dol-*P* buffer suspension is mixed with liposomes of pure DMPC.

The Fig. 3A shows that the absorbance at 450 nm decreases when the Dol-*P*/DMPC ratio in the solution increases. This may indicate that Dol-*P* is incorporated into the bilayer. When the dolichyl monophosphate is included in the bilayer the presence of charges induces larger liposomes as a result of the interbilayer repulsion. Thus, the refraction index difference between the inner and the outer media decreases and the absorbance decreases. A similar behaviour has been found when phosphatidic acid is introduced into phosphatidylcholine bilayers [10].

However, De Kruijff et al. [5] were not able to ascribe to Dol-*P*/DMPC bilayer a net charge measuring the interbilayer distance by means of small X-ray diffraction [5] at much higher concentrations than those employed here.

Thus, if this is the case the absorbance decrease should be ascribed to changes in the bilayer properties instead of to the increase in the interbilayer space. This is reflected in the response of the absorbance to temperature in Fig. 6, which may be related to the differences found in permeability (Fig. 1). The decrease in the absorbance difference is coincident with the shift of the pretransition towards the main transition region (Fig. 4).

It is intriguing to observe that the maximum permeability is found at 0.50%. At this ratio Dol-*P* is not completely incorporated into the bilayer in the light of the results of Fig. 3A and those of Fig. 4. In Fig. 3A it can be observed also that the absorbance becomes constant above 1.00% with some fluctuations around 0.75% and 3.00%.

The invariance in the absorbance observed in Fig. 3B in the range 0.98 and 0.99 molar fraction would suggest that in this interval the Dol-*P* and the DMPC liposomes mix in all the proportions. That is, all the Dol-*P* added to the aqueous solution is incorporated into the DMPC bilayer.

The increase in absorbance below 0.98 and above 0.99 (see legend of Fig. 3B) is due to an excess of DMPC liposomes or an excess in Dol-*P*, respectively. In these regions, Dol-*P*/DMPC liposomes will coexist with free Dol-*P* (left branch) and pure DMPC liposomes (right-hand branch).

The permeability measurements done with compositions corresponding to the right-hand branch would be affected by the absorbance corresponding to pure DMPC liposomes coexisting in the dispersion.

Measurements with dispersions whose composition fall in the left-hand branch of Figure 3B would correspond to DMPC liposomes in an excess of Dol-*P*. In this case, the Dol-*P*/DMPC liposomes will be formed and would coexist with free Dol-*P*. The compositions within the flat portion of the curve in Fig. 3A corresponds to the decrease in permeability observed in Fig. 2 after the maximum. However, between 0.75% and 2.50% the permeability values would correspond to a population composed mainly of Dol-*P*/DMPC liposomes. It must be noticed that in this composition range the pretransition disappears gradually superposing with the main transition (Fig. 4). In addition, the mean of the pretransition falls at 18°C which is similar to that found by De Kruijff et al. [5].

The $K^+$ permeability at 25°C is higher for 0.50% in comparison to that found with 0.75%. However, at temperatures below 21°C, 0.75% liposomes show a higher permeability than that found with 0.50%. This indicates that the effect of

Dol-*P* on the bilayer permeability properties is different when it is in the gel or in the fluid state.

The data in Fig. 3 show a decrease in absorbance which is interpreted as a consequence of the Dol-*P* incorporation into the DMPC bilayer in the fluid state. Thus, if the gel state absorbance is similar for 0.50% and 0.75% the decrease in absorbance when going from the gel to the fluid state would be greater with 0.75% than with 0.50%.

However, as shown in Figs. 4 and 6, this is not the case. The changes observed for 0.50% are larger than those for 0.75%. Moreover, this is similar to those obtained with pure DMPC liposomes.

Therefore, in order to explain the smaller changes of absorbance at 0.75% the gel state absorbance should be lower than that corresponding to 0.50%. This suggests a higher incorporation of Dol-*P* at 0.75% when the bilayer is in the gel state. It is clearly observed in Fig. 4 that a displacement of the pretransition towards the main transition temperature is found for 0.75% but not for 0.50%. This would explain the fact that the permeability at 0.75% is higher at the gel state than that corresponding to 0.50%.

The increase of Dol-*P* in the bilayer would give a large area available for permeability. This would explain the selectivity by size found with liposomes with 0.75% shown in Table I. The cation permeability follows the order of the hydrated ionic radii [11].

The selectivity sequence for these ions is opposed to that found for free dolichols [6]. In the case of dolichol the ions with the smaller dehydrated radius are more permeable than those of high ionic radii.

If the phosphate charges are hidden or screened in the bilayer interface [5], the selectivity would be determined by the permeant size. This seems to be confirmed by the fact that glucose also permeates Dol-*P*/DMPC bilayers, a fact not observed with free dolichols.

However, the permeation of $Ca^{2+}$ would indicate that charges are in some way involved in the permeation process at least for divalent cations. In this case, the high affinity of $Ca^{2+}$ by the phosphate groups would allow this ion to enter into the Stern electrical double layer region promoting, as observed with other phosphate lipids, distortions enhancing permeability [12].

In conclusion, Dol-*P* induces the permeability to alkaline cations in a different sequence to that found for free dolichol. Moreover, the presence of the phosphate group in the isoprenoid molecule promotes the permeation of $Ca^{2+}$ and glucose. In addition, there seems to be an optimum range of Dol-*P*/DMPC ratios for which a complete incorporation of Dol-*P* into the bilayer can be assured.

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