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Effect of insulin on the lytic action of lysophosphatidylcholine in lipid bilayers

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The effect of insulin on the bilayer properties of dimyristoylphosphatidylcholine liposomes at the gel and the liquid crystalline state was measured by differential scanning calorimetry and absorbance at 450 nm. It is found that insulin promotes a decrease in the enthalpy of the gel-liquid crystalline transition without displacing the transition temperature. Under these conditions the lytic action of monomyristoylphospatidylcholine is enhanced, decreasing the critical lytic concentrations to values comparable to the bilayer at the gel state. The effect of the lysoderivate on liposomes in contact with increasing concentrations of insulin promotes a reorganization of the lipids into smaller particles as inferred from fluorescence dequenching, turbidity and exclusion chromatography assay. It is concluded that the action of lysoderivates can be enhanced, at temperatures above the transition temperature, by proteins that without spanning the lipid bilayers can perturb the bilayer interface.

Introduction

The secretion of insulin implies a number of physiological events accompanied by chemical and physical modifications in the lipid bilayer of the granules and plasma cell membranes [1–3]. One of these chemical changes corresponds to the appearance of lysoderivates [4–5]. The physical changes are represented by modifications in the membrane fluidity which favours the fusion of the granule with the cell membrane [6].

The effectiveness of lysoderivates to promote lysis in lipid membranes is closely related to the physical state of the bilayer. The maximum lytic effect is produced around the phase transition temperature or in a phase separation boundary [7–12]. In the later case, insertion of proteins that span lipid membranes such as glycophorin or band 3 protein, enhances the lysoderivate lytic action upon bilayers at the liquid crystalline state [7,8]. On the other hand, liposomes in the gel state are more sensitive to the lytic action when shrunken in hypertonic media [9]. Thus, water-soluble compounds could affect lytic action by changes in the water activity or interacting directly on the membrane surface. Insulin adsorbs on liposome surfaces and it is able to promote changes in the fluidity of membranes containing acidic phospholipids [13,14]. Since different stimulators of insulin secretion induce the production of lysoderivates the lytic effect of these compounds might be enhanced by changes promoted by insulin itself on the granule membrane. Therefore, it appears of interest to test whether this hormone modifies the response of liposomes to lytic agents. With this purpose we have studied the membrane stability induced by lysophosphatidylcholine in relation to the insulin–bilayer interaction below and above the phase transition temperature.

Abbreviations: DMPC, dimiristoylphosphatidylcholine; LPC, monomyristoylphosphatidylcholine; DSC, differential scanning calorimetry; CLC, critical lytic concentration; Rh, octadecylrhodamine.

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Materials and Methods

L-α-Dimyristoyl (DMPC) and L-α-monomyristoyl-phosphatidylcholines (LPC) were obtained from Avanti Polar Lipids Inc. (Birmingham). The purity of these compounds was tested by thin-layer chromatography. All samples gave a single spot when developed under I_2 vapours. Therefore, they were used without further purification. Water was twice distilled in a milli Q standard equipment. All other chemicals used were of analytical grade. Insulin solutions were prepared with crystalline sheep insulin-Zn dissolved in 0.1 M HCl and adjusting the final volume to 1 ml with 10 mM Tris-HCl buffer (pH 7.4) to obtain a final concentration of 3 mg/ml. Care was taken to maintain the pH at 7.4 in all dilutions in order to avoid protein precipitation which may interfere with the measurements.

Multilamellar liposomes were prepared according to Bangham's method [15]. A film obtained by evaporation of a chloroform solution in a round bottom flask was dispersed above the transition temperature in buffer 10 mM Tris-HCl (pH 7.4). Insulin was directly added to the external media of the preformed liposomes or added to the aqueous solution in which the lipid film was dispersed.

The influence of insulin on the phase transitions was measured by absorbance changes at 450 nm according to the method developed by Rowe [17] and by calorimetry. Insulin was present either in the outer and the inner media or only on the outer media in concentrations below and above those corresponding to physiological conditions.

Absorbance changes in the lipid suspension were used to monitor the phase transition. The absorbance decrease in the main phase transition is primarily due to a change in the refractive index which accompanies the lipid density change [17,26]. No aggregation was detected in the presence or in the absence of insulin when liposomes above the transition temperature were eluted through Sepharose 2B columns. In both cases, liposomes were eluted through the column without adding any dye marker in order to avoid modifications in the insulin adsorption. The lipid content in the fractions eluted from the column were detected adding to each of them an aliquot of merocyanine 540 and measured at 570 nm.

The calorimetric study as a function of the insulin/lipid ratio was performed in a Dupont 910 calorimeter. Batches of 10–15 mg of wet samples were warmed up at a rate of 2 C°/min between 0 and 40°C. In these cases, in order to increase the sensitivity, liposomes were prepared by dispersing the lipids in insulin solutions of different concentrations. Similar results were obtained with scanning rates of 1 C°/min. The enthalpic changes at the phase transition were calculated from the area under the main peak. The calorimeter was calibrated using the isotherm for indium (In) fusion as stated by ASTM-E 698-79 norm. The baseline was obtained previous to each run with two samples of equal calorific capacitance (usually air). The total mass of phospholipids was obtained by phosphorus determination using the Fiske-SubbaRow method after convertion to inorganic phosphate by digestion in perchloric acid during one hour at 200°C [16].

The lytic effect of LPC was determined by measuring the decrease in the absorbance at 450 nm upon successive additions of LPC to a liposome dispersion as shown in previous work [9–11].

A sample of liposomes which represents 0.2 mg/ml of lipids was titrated with LPC in a thermostated cuvette of a spectrophotometer with automated stirring. The absorbances were recorded after equilibration. At a given LPC concentration the absorbance of the liposome sample decreases as a function of the lyso-compound concentration. The intersection of the straight line for absorbance values obtained at low LPC concentrations with that obtained at high LPC concentrations is taken as the critical lytic concentration (CLC) The changes in absorbance were correlated with the lipid redistribution and the changes in the mean size of the liposomes induced by the LPC action by means of fluorescence dequenching and exclusion chromatography, respectively. The fluorescence assays were performed using octadecylrhodamine as a membrane probe. Chromatography was performed in Sepharose CL-2B gel columns using isotonic buffer (pH 7.4) for the elution. Liposomes prepared with 6–7% octadecylrhodamine were seen in the column before and after the incubation with LPC at 30°C in the presence of insulin. According to previous reports, these percentages of octadecylrhodamine do not affect the membrane [9] The volume fractions of 0.5 ml were treated with Triton X-100 to obtained the 100% of dequenching (F_T) and measured in a spectrofluorometer (excitation wavelength: 560 nm; emission wavelength: 590 nm). The percentage of dequenching at each volume fraction was calculated by means of the relation $100 \cdot (F_T - F)/F_T$. The absorbance and fluorescence values were obtained in a continuously stirred cuvette where temperature was controlled within ± 0.5°C. The fluorescence anisotropy determinations were performed using diphenylhexatriene as a probe. Calculations were done by means of the relation

$$r = (I_{\parallel} - I_{\perp} G)/(I_{\parallel} + 2\, I_{\perp} G)$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities of the emission at 444 nm obtained with the analyzer parallel or perpendicular, respectively, to the direction of polarization of the excitation beam. The molar ratio of probe to PC was 1:200. In all experiments, corrections for light scattering blank were made measuring the excitation/

emission ratio in the absence of the fluorophore. $I_{\parallel}$ and $I_{\perp}$ values were corrected with the phototube sensitivity by the geometrical factor (G).

Results

The results shown in Figs. 1, 2, and 3 denote that the response of DMPC liposomes to heating-cooling cycles across the phase transition temperature are markedly different in the presence of insulin (Fig. 1), monomyristoylphosphatidylcholine (Fig. 2) and insulin plus monomyristoylphosphatidylcholine (Fig. 3). The decrease in absorbance observed when liposomes go from the gel to the liquid crystalline state disappears in the presence of insulin. The absorbance values obtained during the heating and the cooling of each type of samples show no hysteresis in all cases. The 100% values for liposomes with and without insulin correspond to the same absorbance. The transition disappears because the absorbance at temperatures higher than the transition increases with insulin to reach those values corresponding to the gel state.

The absorbance values obtained after cooling DMPC liposomes heated in the presence of lysoPC and in the absence of insulin are below those obtained before the heating (Fig. 2). However, the same type of experiments performed in the presence of insulin shows that, after the heating, the decrease of temperature promotes a further decrease in absorbance. When liposomes are heated in the presence of lysoPC the absorbance decreases to the same extent both in the absence or in the presence of insulin (0.65 to 0.4 in the first case and 0.65 to 0.33 in the second). However, a more pronounced absorbance decrease is observed when the samples are cooled in the presence of insulin. This suggests that the action of the lysoderivate takes place when the membrane phase goes from the fluid state to the gel state.

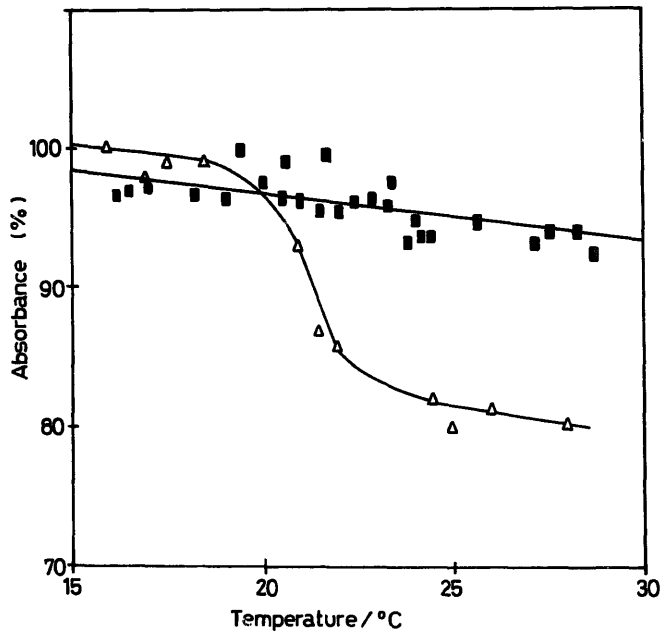

Fig. 1. Absorbance changes as a function of temperature for dimyristoylphosphatidylcholine. Liposomes without insulin (△). Liposomes with 3.63 mol% insulin (■). Data correspond to warming up and cooling down assays in both cases.

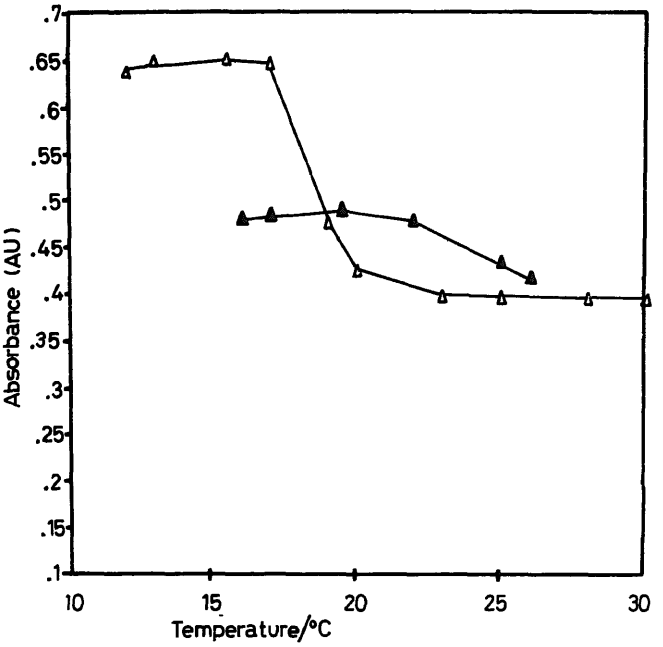

Fig. 2. Absorbance changes as a function of temperature for dimyristoylphosphatidylcholine liposomes in the presence of monomyristoylphosphatidylcholine. Absorbance changes during the warming up (△) and the cooling down (▲) of a dispersion containing 0.057 mM LPC in the absence of insulin.

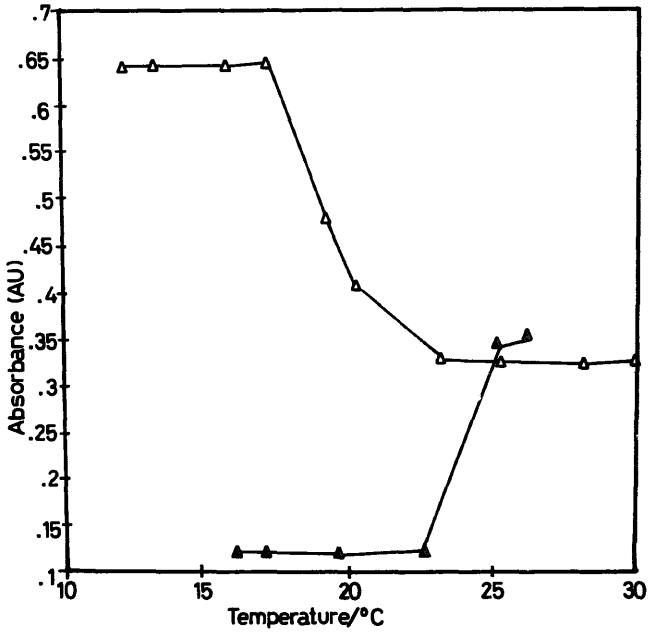

Fig. 3. Absorbance changes as a function of temperature for dimyristoylphosphatidylcholine liposomes in the presence of monomyristoylphosphatidylcholine and insulin. Absorbance changes during the warming up (△) and the cooling down (▲) of a dispersion containing 0.057 mM LPC in the presence of $1.42 \cdot 10^{-2}$ mM insulin.

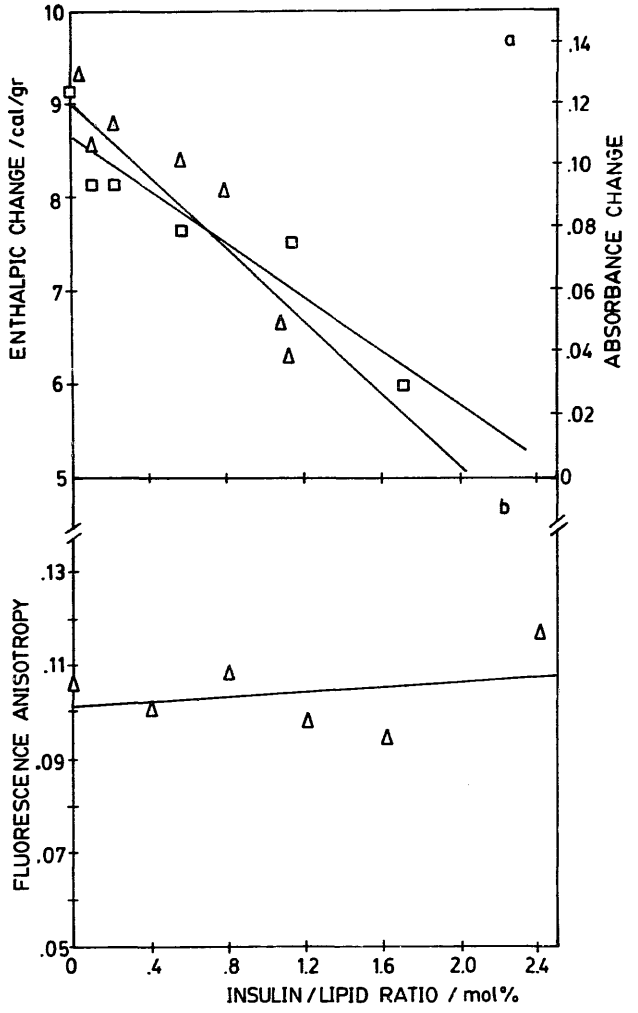

Fig. 4. Effect of insulin on the enthalpy, the absorbance change at the phase transition and the anisotropy of dimyristoylphosphatidylcholine liposomes. (a) Enthalpic changes measured by differential scanning calorimetry (□). Absorbance decrease at the phase transition measured at 450 nm (△). (b) Fluorescence anisotropy of liposomes in the fluid state as a function of insulin concentration.

The contribution of insulin to the formation of a gel phase above the transition temperature is inferred from the absence of the change of absorbance at the phase transition in Fig. 1. The difference in the absorbance between the gel and the fluid state disappears with the insulin/lipid ratio and it is related with the enthalpic change measured by calorimetry (Fig. 4a). While the absorbance change at the phase transition decreases parallel to the enthalpy, no changes in the fluorescence anisotropy were observed in the same range of insulin/lipid ratio (Fig. 4b). The mean standard deviation of the DSC determinations was ±0.4. No changes in the transition and pre-transition temperature were observed.

The observation that insulin promotes a decrease in the enthalpic change would be in agreement with the finding that insulin adsorbs on lipid membranes in the fluid state but not in the gel state [21]. However, the invariance of the membrane anisotropy suggests that the effect of insulin would be constrained to the membrane interface.

Concordantly, the CLC obtained with liposomes in the fluid state decreases with the increase in the insulin/lipid ratio (Fig. 5). It is observed that the values obtained with absorbance and fluorescence approach those found for liposomes in the gel state when insulin is increased (Fig. 6).

The LPC action on liposomes in the fluid state treated with insulin promotes a lipid redistribution and a decrease in the mean size of the particles, most probably micelles as described in previous reports [7]. Liposomes without treatment of LPC incubated with insulin elute in the front of a Sepharose column (Fig. 7). After incubation of liposomes in the presence of 5–6 mol% insulin at 30 °C with LPC concentrations above 0.1 mM according to Fig. 5, the elution gives the major peak at 8–9 ml. In concordance, the amount of liposomes of the original size at 3–4 ml is significantly decreased. Thus, the decrease in turbidity observed above the CLC in Fig. 5 corresponds to a decrease in the mean particle size of the sample. The percentage of dequenching obtained by adding Triton is nearly the same in both peaks denoting that the lipid/octadecylrhodamine ratio is maintained in both types of particles.

However, when LPC is added to a sample containing liposomes with octadecylrhodamine and liposomes without the dye an increase in fluorescence is obtained as a function of the LPC concentration above the CLC (Fig. 5). Thus, the disruption of the liposomes to smaller particles is accompanied by a redistribution of lipids between the membranes.

The CLC is clearly sensitive to the insulin concentration when it is determined by absorbance changes or with the octadecylrhodamine assay. The differences between the CLC values between both methods are due to the fact that absorbance detects the larger particles which contribute in a greater extent to the turbidity. In the fluorescence assay small and large particles are equally affected by the LPC action giving lower values of CLC. When the CLC is determined by fluorescence dequenching, the slope taken near the CLC gives lower values than those obtained with points far from it. These values are within the limits denoted by the error bars in Fig. 6.

Discussion

The changes in the light scattering with temperature have been mainly attributed to the change in the refractive index occurring at the phase transition. This is a reversible change. An irreversible increase of absorbance (or turbidity) is attributed to the fusion of particles [26,27].

The results from Sepharose columns indicate that the changes in absorbance in the absence of LPC corre-

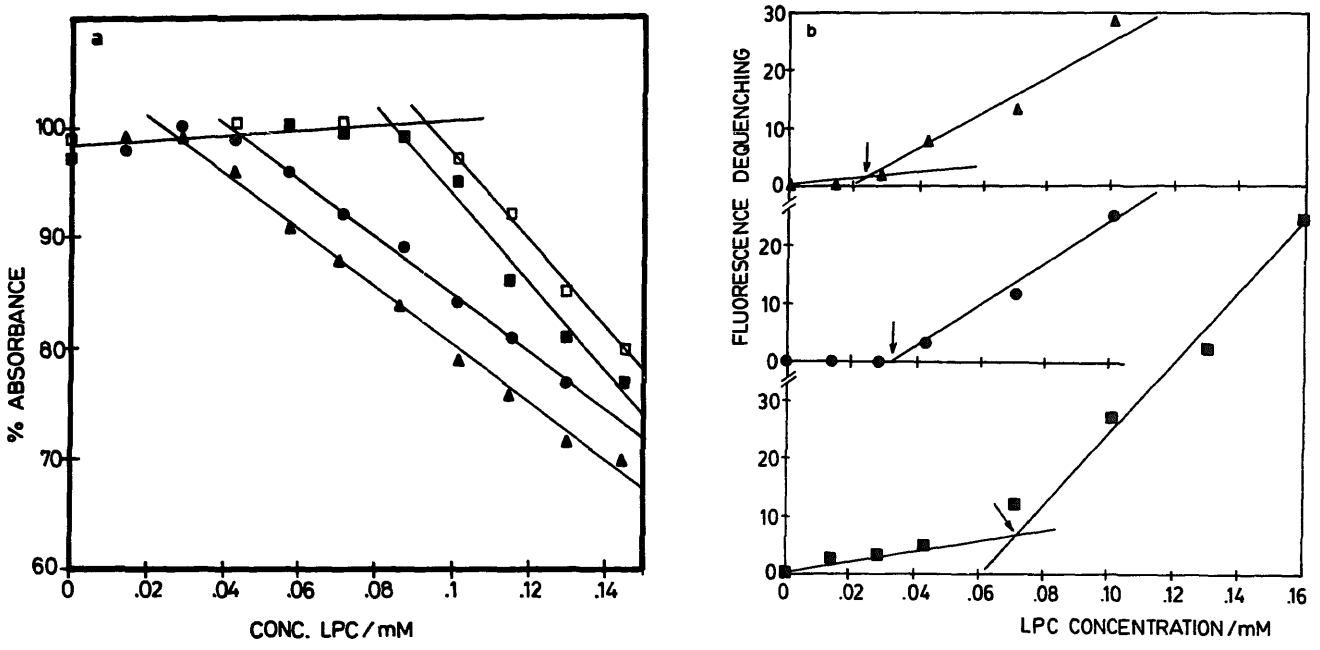

Fig. 5. Effect of lysoPC on dimyristoylphosphatidylcholine liposomes as a function of insulin concentration. (a) Changes induced by LPC on DMPC liposomes measured by absorbance changes at 450 nm in the presence of $1.21 \cdot 10^{-2}$ (□), $2.43 \cdot 10^{-2}$ (■), $3.63 \cdot 10^{-2}$ (●) and $6.05 \cdot 10^{-2}$ (▲) insulin/lipid ratios. (b) Changes induced by LPC of a dispersion containing one part of liposomes with octadecylrhodamine (8 mol%) and three parts of liposomes without the dye in the presence of $2.43 \cdot 10^{-2}$ (■), $4.78 \cdot 10^{-2}$ (●) and $7.27 \cdot 10^{-2}$ (▲) insulin/lipid ratios. Arrows indicate the critical lytic concentration.

spond to the reversible change at the phase transition. However, in the presence of LPC the hormone promotes a decrease in absorbance which is due to the disruption of the liposomes into smaller particles.

Insulin produces a decrease of enthalpy at the phase transition and induces an enhancement of the lytic action of LPC.

The enthalpic change is accompanied by a decrease in the absorbance change at the phase transition. The contribution to the absorbance of a liposome sample upon melting are the refractive index of the dispersed particles and the anisotropy of the membrane phase [17,22,26]. As the hormone has no effect on the fluorescence anisotropy the effect on the absorbance at 450 nm at the phase transition without LPC, can be due to

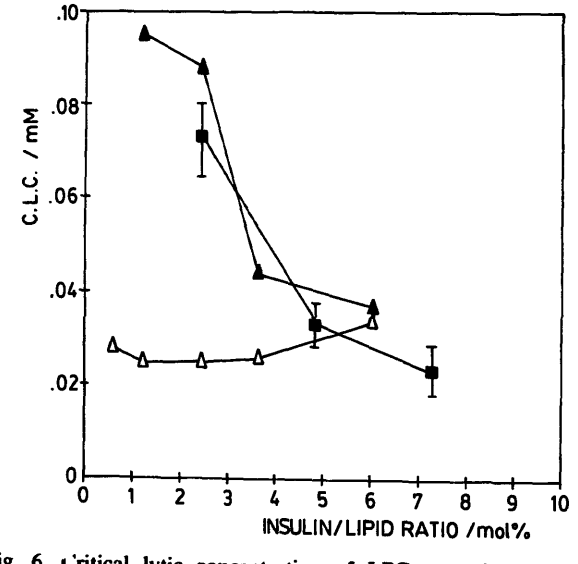

Fig. 6. Critical lytic concentration of LPC as a function of the insulin/lipid ratio on DMPC liposomes. △, CLC determined by absorbance changes as described in Fig. 5 for liposomes in the gel state (18°C). ▲, CLC determined by absorbance changes for liposomes in the fluid state. ■, CLC determined by fluorescence dequenching for liposomes in the fluid state.

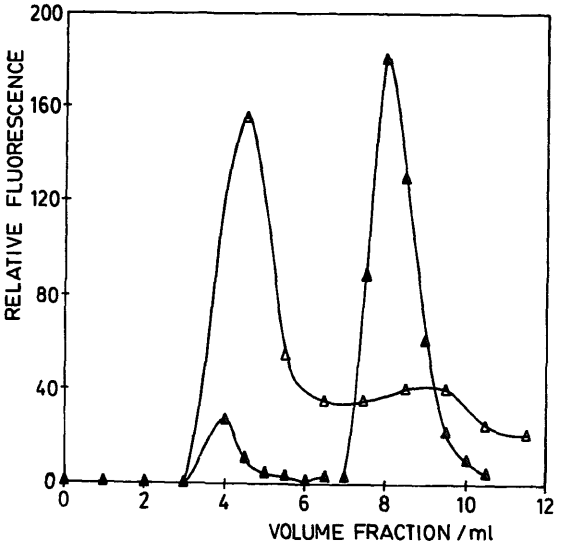

Fig. 7. Effect of lysophosphatidylcholine on the size of liposomes treated with insulin. △, liposomes incubated with 4 mol% insulin and eluted previously to the lysoderivate treatment ▲, liposomes eluted after the treatment with 0.1 M lysoPC at 30°C in the presence of 4 mol% insulin.

changes in the refractive index of the particles [17,21]. Thus the enthalpic decrease of Fig. 4 is related to the perturbations in the membrane interface with no effect on the hydrocarbon core. The hormone does not displace the transition temperature denoting that there is no effect on the packing of the cooperative units in the range 1–2.5 mol% for insulin/lipid ratio as confirmed by the unchanged values of anisotropy

This insulin/lipid ratio corresponds to a total phenylalanine concentration of 0.08 mM. Peptides of phenylalanine and glycine have been shown to promote a very slight decrease in the transition temperature of DMPC (less than 0.1°C) only in a range of 12 mM concentration [24] for which a 8.19 cal/mol enthalpic change has been found.

However, insulin promotes a decrease in area, proportional to the enthalpy at the main transition, with a negligible change in the peak width. As the hydrocarbon core is not altered, it can not be ruled out that the cooperative units contributing to the transition would belong to those regions of the bilayer near the headgroups [28].

Previous calorimetric work has demostrated that phenylalanine-containing peptides induce a phase separation in the plane of the bilayer at peptide/DMPC liposome mixtures with 15 mol% peptide containing phenylalanine. Thus, the amino acids of the protein would act as impurities in the membrane surface inducing phase boundaries with a negligible effect on the hydrocarbon core as suggested by the anisotropy assays but enhancing the LPC action.

In this regard it is worth discussing the results of Figs. 2 and 3. When liposomes are displaced from the gel to liquid crystalline phase across the transition temperature in the presence of the lysocompound and then they are returned to the original gel state, the results are quantitatively different if insulin is present in the medium. In the absence of insulin, when the temperature is decreased the absorbance value returns to one which is lower than that obtained at the same temperature before the warming up of the preparation but higher than that found at high temperatures. This indicates that not all the liposomes are lysed during the gel to liquid crystalline cycle at this LPC concentration. Experiments performed at the same lysoPC concentrations in the presence of insulin showed that absorbance decreases much more, attaining values lower than those corresponding to the liquid crystalline state. This suggests that lysocompounds in the presence of insulin can act at the phase boundary appearing when membrane goes from the liquid crystalline to the gel state.

The lytic effect of LPC is more effective when gel and liquid crystalline states coexist in the membrane [7]. This coexistence has been induced by inserting into the membrane, proteins that span the membrane bilayer such as glycophorin and band 3 protein. In these cases proteins would induce a gel-like structure around them promoting a phase boundary where lysocompound can intercalate [7,8].

The present results show that insulin promotes the action of lysoPC. Since insulin seems to interact mainly with the membrane interface without affecting the apolar region [19], the LPC action would be due to changes in the headgroup region of the lipid bilayer. It must be recalled that the lytic effect is also enhanced by the osmotic shrinkage of liposomes in the gel state. In this case, the appearance of hydrophobic sites on the membrane surface were observed without alterations in the anisotropy of the hydrocarbon core [9].

In conclusion, an aqueous soluble protein such as insulin induces an enhancement of the lytic action when it interacts with membranes in the liquid crystalline state.

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