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THE CONDENSING EFFECT OF GLUCAGON ON PHOSPHOLIPID BILAYERS

R.M. EPAND [a], R.F. EPAND [a], T.P. STEWART [b] and S.W. HUI [b]

[a] *Department of Biochemistry, McMaster University, Hamilton, Ontario L8N 3Z5 (Canada) and* [b] *Department of Biophysics, Roswell Park Memorial Institute, Buffalo, NY (U.S.A.)*

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Glucagon forms discoidal particles with dimyristoylphosphatidylcholine at temperatures below the phase transition. Under these conditions and at a lipid to protein molar ratio of 20 : 1, glucagon is observed to induce a closer packing of the phospholipid bilayer. Similar effects are observed upon the interaction of glucagon with dilauroylphosphatidylcholine. In the region of the phase transition the discoidal particles are observed by freeze-fracture electron microscopy to undergo end-to-end association leading to the formation of multilamellar structures containing only a few layers and having a large internal volume. Above the phase transition temperature the properties of the lipid appear to be unperturbed by glucagon according to either freeze-fracture or densitometer studies. These results further support the importance of phospholipid phase transitions in peptide-lipid interactions.

Introduction

There has been considerable interest in recent years in studies of protein-phospholipid interactions as models for the structure of biological membranes and serum lipoproteins. Measurements of changes in volume which occur upon the reaction of proteins with phospholipids yields a specific, fundamental physical parameter with which to characterize these systems. The densitometric measurements, together with morphological studies by freeze-fracture electron microscopy and ^{31}P-NMR studies provide necessary information to understand the molecular organization of the interacting proteins and phospholipids. We have applied these methods to a study of the interaction of glucagon with phosphatidylcholine. This interaction leads to the formation of a lipoprotein complex [1] which is unstable at temperatures above the phase transition temperature (T_c) of the phospholipid component [2]. In this work we have studied further the molecular organization of the glucagon-phosphatidylcholine system at temperature through the T_c of the phospholipid. The rapid freeze-quenching technique enabled us to observe the changing morphology of the complex at T_c without the possible introduction of artifacts through the use of a cryo-protectant. The striking changes in morphology and density as the phospholipid undergoes a phase transition supports our previous findings that the glucagon-phospholipid interaction is drastically affected by the structural phase of the phospholipid [2,3].

Materials and Methods

Materials

Crystalline bovine-porcine glucagon was purchased from the Elanco Corp. and used without further purification. Dilauroyl- and dimyristoylphosphatidylcholine (DLPC and DMPC) were purchased from Calbiochem. Corp. and shown to be pure by thin-layer chro-

Abbreviations: DPLC. dilauroylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; Pipes, piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid).

matography. (Silica gel H using chloroform/methanol/water (65 : 24 : 4, v/v) as solvent).

Methods

Buffer. All experiments were performed in an aqueous solution of a buffer containing 20 mM Pipes, 1 mM EDTA, 150 mM NaCl and 0.02 mg/ml NaN_3, pH 7.40.

Preparation of DLPC-glucagon complex. DLPC was suspended in buffer (15 mg/ml) by vortexing at room temperature. This suspension was cooled in an ice bath and added to glucagon crystals to give a final molar ratio of lipid to glucagon of 15 : 1. The suspension was warmed and recooled to aid the dissolution.

Preparation of DMPC-glucagon complex. The DMPC-glucagon complex was prepared in a number of ways in order to obtain different ratios of lipid to peptide and to evaluate the effect of the method of preparation on the final product.

A DMPC-glucagon complex containing approximately 45 lipid molecules per glucagon molecule was formed by dissolving a lyophilized powder of the complex which was prepared from an ammonium acetate solution as previously described [3], into the Pipes buffer used in this work. The resulting solution was centrifuged for 10 min at 5°C at 12 000 × *g* and the supernate dialyzed at 4°C against an approx. 100-fold excess of buffer. It was also found possible to prepare this complex more directly by adding a suspension of DMPC which had been vortexed above the phase transition temperature to a suspension of glucagon crystals in Pipes buffer, at 25°C. A solution resulted from warming and cooling the mixture between approx. 45 and 10°C. This solution was then centrifuged for 10 min at 12 000 × *g* at 20°C and used directly for densitometry or was dialyzed against excess Pipes buffer at 4°C as described above.

A DMPC-glucagon complex containing approximately 20 lipid molecules per glucagon molecule was formed by mixing DMPC and glucagon suspensions at 25°C followed by solubilization as described above. In the present case the maximal amount of glucagon was used which would lead to a soluble complex. Dialysis of the resulting solution at 4, 20 or 25°C led to the formation of a precipitate which could not be redissolved, therefore all of the results reported at this low lipid to protein molar ratio are for solutions which have not been dialyzed.

Glucagon concentration. The concentration of glucagon in the lipoprotein complex was determined from its absorbance at 278 nm, suitably corrected for light scattering, using a molar extinction coefficient of 7388 $cm^{-1} \cdot M^{-1}$ [3].

Lipid concentration. Phospholipid concentration was determined by total phosphorus analysis after perchloric acid ashing of the samples [4].

Densitometry measurements. All solutions used for densitometry measurements were centrifuged for at least 10 min at 10 000 rev./min to remove any particulate matter. The solutions were then degassed under reduced pressure. Density measurements were made with the use of two DMA 602 external cells using a DMA 60 measure unit in the phase lock loop mode (A. Paar K.G., Graz, Austria). The lipoprotein solution was placed in one of the cells and buffer or dialyzate placed in the other. In the use of dialyzed samples, the dialyzate contained only traces of peptide or phosphate. The details of the measuring system and the methods used to calculate apparent partial specific volumes are described elsewhere [5,6].

Freeze-fracture electron microscopy. 0.1 μl aliquots of the solution containing the DMPC-glucagon complex were sandwiched between 75 μm thick copper foils and incubated for 3 min at selected temperatures prior to being plunged into liquid propane. The samples were accelerated into liquid propane by a guillotine device similar to that of Costello and Corless [7]. Our device differs from that of Costello's in that it employs an environmental chamber where the sample can be incubated at 100% humidity and a wide range of temperatures prior to freezing. After freezing the samples were freeze-fractured in a Polaron E-7500 Freeze-fracture module at −120°C under a vacuum of $5 \cdot 10^{-7}$ torr or better. Replicas were cast by resistance evaporation, floated off in distilled H_2O, transferred to 100% Clorox, washed three times in distilled H_2O and mounted on bare 400 mesh Hex grids (Polaron Instruments, Inc. Doylestown, PA). Representative micrographs were taken with a Siemens 101 electron microscope.

Phosphorous NMR. ^{31}P-NMR spectra were obtained on a Bruker WH-90 Fourier Transform spectrometer operating at 36.4 MHz. Spectra were usually obtained in the presence of broad band proton decoupling (8 W). To measure the magnitude of the observed signal, however, proton decoupling was elimi-

nated to avoid Overhauser effects and an internal standard of sodium pyrophosphate was included. The concentrations of pyrophosphate and lipid were determined by phosphate analysis and the areas of their resonance lines were measured by cutting out the peaks from duplicate copies of the spectra and weighing them. Generall 5000 pulses of 90° were acquired and transforms involving 8 K data points were employed. For the measurement of signal areas a delay time of 5 s, which is more than twice the T_1 values we have measured for the signals in our system, was included so that the measured peak area would be relatively independent of the relaxation rate. A Bruker B-ST 100/700 temperature controller was used to maintain sample temperature with ±1°C.

Results

Densitometry

If glucagon and lipid were to combine without perturbing the volume of either component then the apparent partial-specific volume of the lipoprotein complex formed, ϕ_c, is given by:

$$\phi_c = \chi_1 \phi_1 + \chi_2 \phi_2 \quad (1)$$

where the ϕ_n are the apparent partial specific volumes and the χ_n are the weight fractions, the subscripts 1 and 2 referring to lipid and glucagon, respectively. The partial specific volume of the complex formed in this manner may be calculated from our measurements of the temperature dependence of the density of DLPC and DMPC and from the partial specific volume of glucagon calculated by the method of Zamyatnin [8] which agreed well with values calculated by the method of Cohn and Edsall [9]. We were not able to directly measure the partial specific volume of glucagon because of the limited solubility of the peptide in the absence of phospholipid. We have also assumed that the temperature dependence of the partial specific volume of glucagon is similar to that of other proteins, varying by about $6 \cdot 10^{-4}$ ml/g per °C [10,11,12]. From these data we were thus able to calculate the hypothetical temperature dependence of the apparent partial specific volume, assuming no volume change on mixing of the components. These appear as solid lines in Figs. 1–3. In the case of the 50 : 1 DMPC-glucagon complex, the experimental

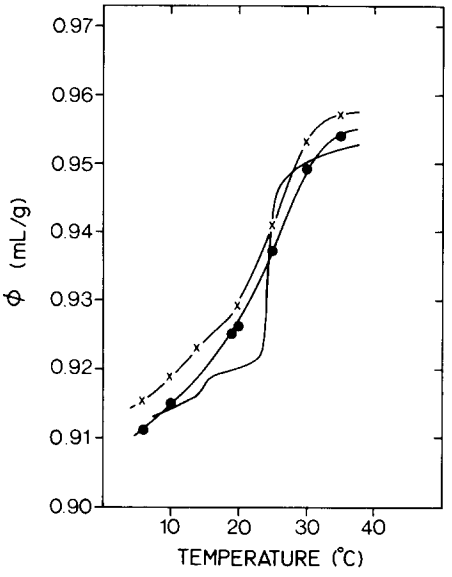


Fig. 1. Temperature dependence of the apparent partial specific volume, ϕ, of dimyristoylphosphatidylcholine-glucagon at a molar ratio of 52 : 1. Solute concentration 1.3% in 20 mM Pipes, 1 mM EDTA, 150 mM NaCl and 0.02 mg/ml NaN_3, pH 7.40. Solid curve, calculated (see Results); X——X, heating scan; •——•, cooling scan.

curves are in reasonable agreement with the calculated curve (Fig. 1) although glucagon does abolish or broaden the premelt transition of 16°C and it significantly broadens the main transition at around 24°C. The heating and cooling curves are equal within experimental error as is the second heating curve. However, repeated heating and cooling or storage at 4°C for a day leads to some sharpening of this transition. There was little effect of the method of preparation of this complex on the final result.

The densitometry results are quite different when the ratio of DMPC to glucagon is 20 to 1 (Fig. 2). There appears to be a small increase in volume as a result of the initial interaction of peptide and lipid. Subsequent slow cooling to low temperatures however, results in a marked decrease in volume which is largely reversible on reheating and recooling. In particular, the partial specific volume of the complex below 20°C shows a 2 to 3% decrease in volume compared to the sum of its constituents. This is similar to the volume changes which occur at the main phase transition for pure phospholipids (Ref. 6 and references therein). DLPC also shows this condensation

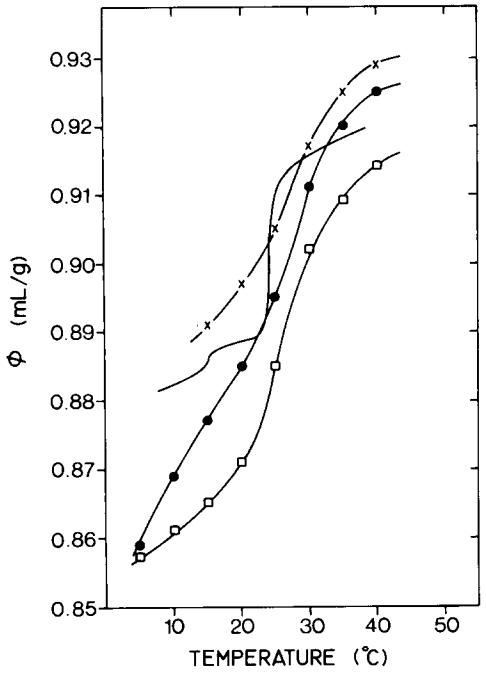


Fig. 2. Temperature dependence of the apparent partial specific volume, ϕ, of dimyristoylphosphatidylcholine-glucagon at a molar ratio of 19 : 1. Solute concentration 1.3% in 20 mM Pipes, 1 mM EDTA, 150 mM NaCl and 0.02 mg/ml NaN_3, pH 7.40. Solid curve, calculated (see Results); X——X, first heating scan; •——•, cooling scan; □——□, second heating scan.

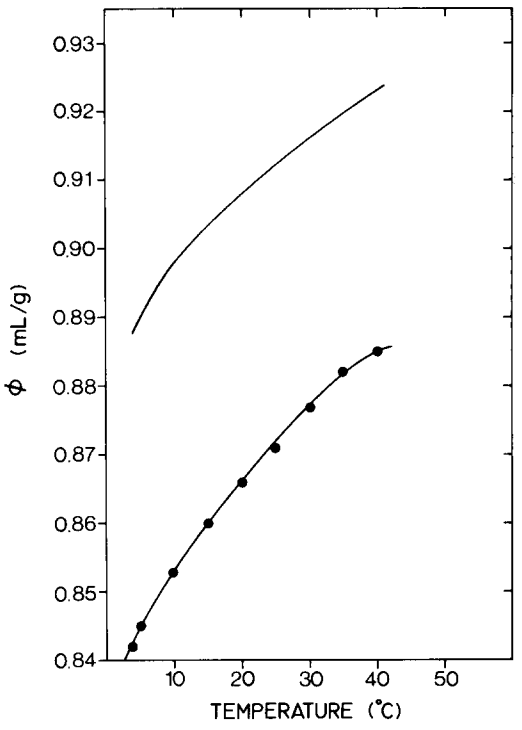
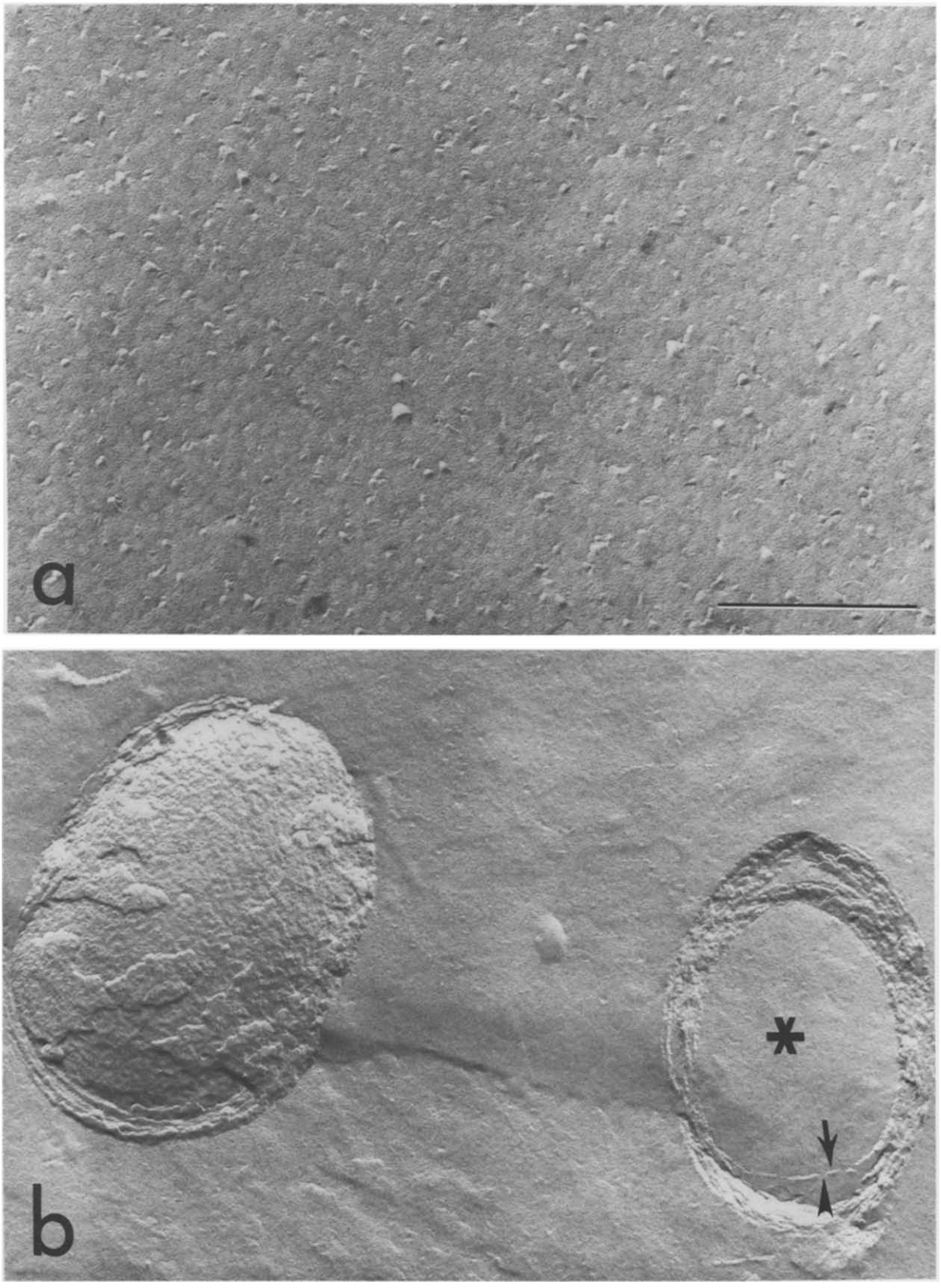


Fig. 3. Temperature dependence of the apparent partial specific volume, ϕ, of dilauroylphosphatidylcholine-glucagon at a molar ratio of 13 : 1. Solute concentration 1.4% in 20 mM Pipes, 1 mM EDTA, 150 mM NaCl and 0.02 mg/ml NaN_3, pH 7.40. Solid curve, calculated (see Results); •——•, heating or cooling scan.

effect upon interaction with glucagon (Fig. 3) although in this case the volume of the lipid-peptide complex never returns to the calculated value for the sum of the components, even at higher temperatures. This may result, in part from a very marked broadening of the phase transition of DLPC in the presence of glucagon as has been previously suggested [13].

Freeze-fracture micrographs of glucagon-DMPC complex quenched from below the phase transition of the complex show ellipsoidal structures (Fig. 4a) in agreement with previous results [1]. When these same solutions were warmed and quenched from a temperature within the transition range they reveal multilamellar structures (Figs. 4b and 4c). It is noteworthy that these multilamellar structures contain but a few layers surrounding a relatively large aqueous compartment (* in Fig. 4b). Cross fractures of some of the lamellae suggest that the lamellae consist of smaller subunits that are disk-like in shape (arrow in Figs. 4b and 4c). Some of the disk-like subunits appear to be separated by aqueous space (arrowhead in Fig. 4b) or continuous with a structure out of the fracture plane or below the resolution of the replica. Samples quenched from above the phase transition appear as multilamellar vesicles and have a surface texture that is characteristic of some phospholipids in the L_α phase (Fig. 4d). This texture may be the result of a slow freezing artifact. Controls consisting of DMPC vesicles were quenched from the same temperatures as the glucagon-DMPC complex (see legend for Fig. 4). The controls appeared as multilamellar vesicles with surface textures consistent with those reported for DMPC [14] and similar in appearance to Fig. 4d.

The ^{31}P spectrum below 23°C of the glucagon-DMPC complex at a lipid to peptide ratio of 20 : 1 showed a single narrow line, typical of small phospholipid particles such as sonicated unilamellar vesicles. At 20°C, resonance signals from all of the


a
b

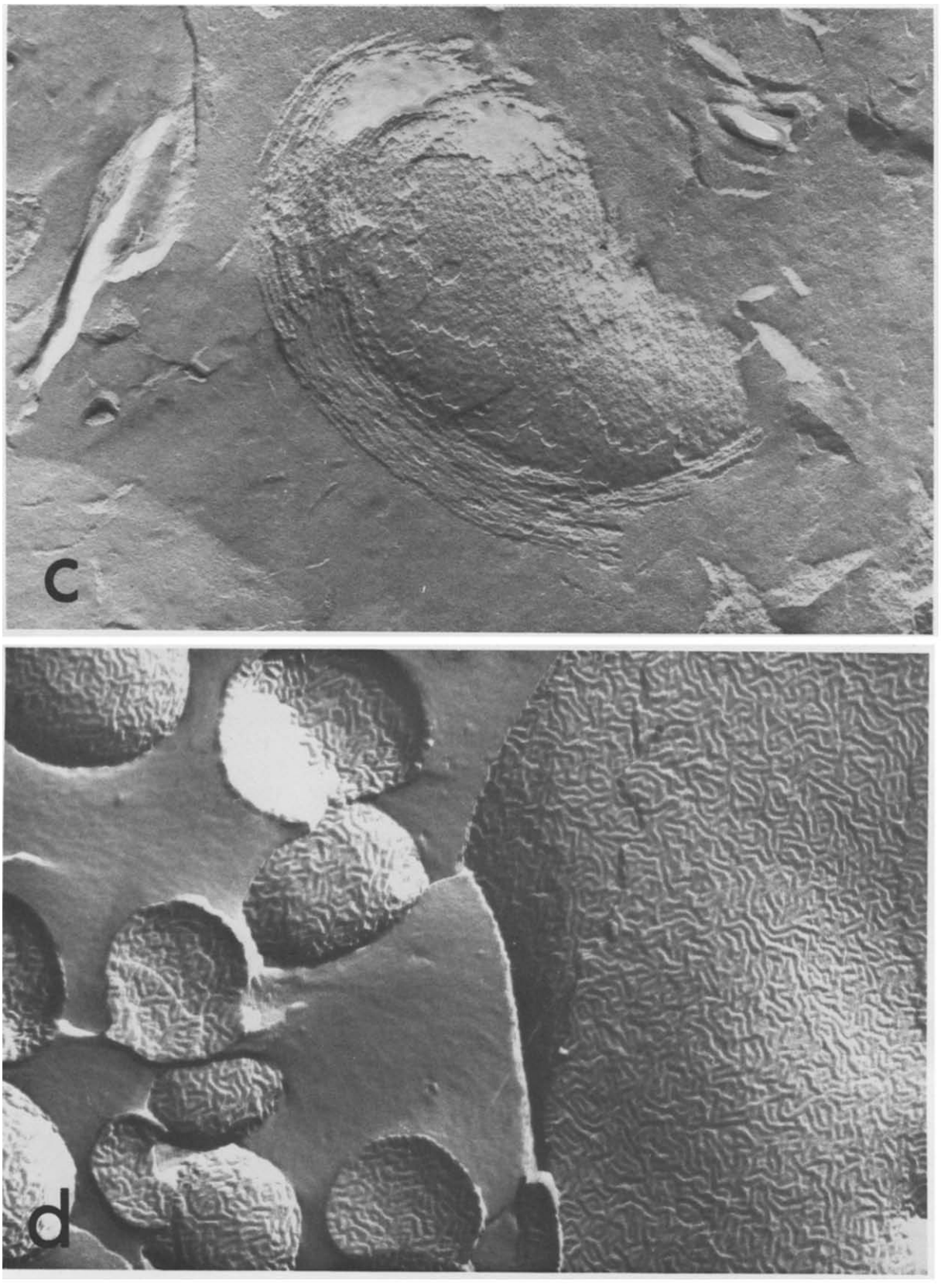


Fig. 4. Freeze-fracture micrographs of glucagon-DMPC complex quenched from: (a) 15°C; (b and c) 28°C and (d) 40°C. Bar = 0.5 μm. Similar results obtained with samples of 50 : 1 and 20 : 1 molar ratios of lipid to peptide.

phospholipid were observed. Above the transition temperature the spectra were typical of multilamellar structures with a low-field shoulder although the presence of some smaller vesicles was also observed.

Discussion

The interaction of glucagon with phospholipids is accompanied by a negative change in volume under certain conditions. This may result either from a volume change in the peptide or in the phospholipid. We believe that it is unlikely that volume changes in glucagon, between the free and lipid bound states, contributes significantly to this effect. In general it is found that proteins undergo volume changes of less than 10^{-2} ml/g even during extensive conformation change [8] and in addition glucagon is a minor component of the lipoprotein complex. It would require a very large change, of at least 10%, in the apparent specific volume of glucagon to account for the observed decrease in volume. Alternatively it only requires a 2.5% volume change in the lipid component. Therefore, although the glucagon may contribute to the observed volume change, the largest effect is a condensation of the lipid bilayer induced by glucagon. This condensation effect is observed at a 20 : 1 lipid to glucagon molar ratio, while at 50 : 1 the density of the lipid is relatively unperturbed by glucagon. Similarly, it has been observed that the fluorescence of pyrene in DMPC is perturbed by glucagon only at low lipid-protein ratios [3]. This may result from the lipid condensation effect reducing the lateral diffusion of pyrene in the bilayer. The 20 : 1 ratio used in this work represents the minimum amount of lipid needed to solubilize glucagon while the 50 : 1 ratio represents the minimum amount of glucagon needed to solubilize the phospholipid.

The freeze-fracture results clearly demonstrate that the morphology of the glucagon-DMPC mixtures varies as a function of temperature and lipid phase. We have confirmed that the structure of the glucagon-DMPC complex below the phase transition is an oblate ellipsoid as had previously been described. The appearance of the structures at temperatures within the phase transition range suggest that as the glucagon-DMPC complex is heated through the transition the disk-like particles associate in such a way as to line up end-to-end and form multilamellar structures as the glucagon is dissociating from its strong interaction with gel phase phospholipid. That the multilamellar structures consist of only a few layers suggest that there is a curvature constraint placed on the disk-like subunits when they are assembling to form the multilamellar vesicles. This constrain suggests that the glucagon is still in some way associated with lipid as the multilamellar structures are formed. The open-ends in the intermediate multilayer structure (Fig. 4b) indicate that individual ellipsoidal complexes are still joining the shells. The vesicles quenched from temperatures above the phase transition resemble multilamellar vesicles of DMPC in the absence of glucagon. This indicates and confirms previous reports that the glucagon is no longer strongly interacting with the DMPC at these temperatures.

All our experimental evidence indicates that glucagon and DMPC dissociate at temperatures above T_c. Glucagon exists as individual molecules in solution and as self-associated units while DMPC forms multilamellar vesicles, as shown by freeze fracture and NMR results. Their total volume, therefore, equals the sum of the volumes of the individual entities. At temperatures below T_c, DMPC is no longer in an extended bilayer form. The gel state phospholipid molecules in each ellipsoid disk are surrounded by glucagon molecules and result in the closer packing among phospholipid molecules. This denser structure may result in the insensitivity of the ratio of trans to gauche bonds to changes in temperature below the phase transition temperature [15]. The increased number of gauche bonds observed may be required to accommodate glucagon to the bilayer structure. The ability of phospholipid bilayers to undergo transitions to different gel states has been shown for stearoylsphingomyelin [16], glucocerebrosides [17] and dipalmitoyl- and distearoylphosphatidylcholine [18]. In the case of sphingomyelin it was shown that the more highly ordered gel state had a higher melting temperature. A statistical mechanical description of phospholipid phase transitions has recently been proposed which indicated that more densely packed bilayers have higher melting temperatures [19]. This may explain why the major effect of glucagon on the phase transition of DMPC is to raise it to somewhat higher temperatures in addition of causing the transition to broaden (Figs. 1 and 2 and Ref. 20). Our findings further substantiate the importance of structural phases of lipids in their interactions with proteins.

Acknowledgment

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