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EFFECTS OF GLUCOSAMINE ON MODEL MEMBRANES

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ABSTRACT Glucosamine is one monomer of chitin and chitosan polymers, which have found applications in several fields of medicine and technology.
This paper reports a study on the modifications induced by glucosamine molecules in the thermodynamical and structural properties of model membranes made of DPPC liposomes. Such effects were detected for molar ratios of monomer to lipid greater than 1:10, by using x-ray diffraction and calorimetric techniques.
Suggestions are made on location of the molecules and on phase separation inside the bilayer, in analogy with the behaviour induced by small lipophilic molecules and cholesterol.

1. INTRODUCTION

In recent years chitin and chitosan (fig. 1) have found many applications not only in the industrial field as they are floculant for organic materials and chelating polymers for metals[1], but also in medicine and particularly in dentistry. In fact these two materials have been used as biodegradable supports for the delayed release of drugs (for instance as a subcutaneous implanted

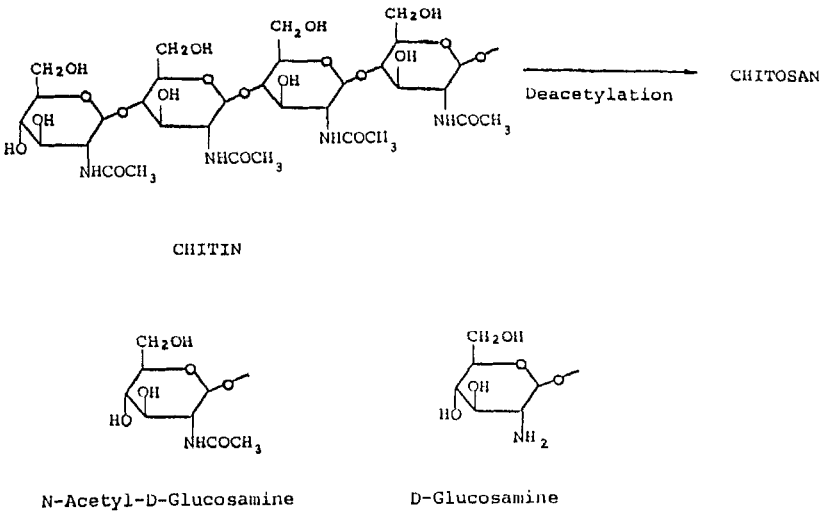

FIGURE 1 Chitin and chitosan polymers and their monomers glucosamine and acetylglucosamine.

membrane carrying pilocarpine[2]), as a surgical suture[3], as a wound healing accelerator and in the treatment of burns[4]. Moreover they were found to have an important role in immunological processes for their immunopotentianting effect[5], for their ability to inhibit the growth of tumors[6] and to activate peritoneal macrophages[7].

They have a bacteriostatic action on organisms responsible of some cutaneous pathologies[8] and exert hemostatic activity. Due to this latter property they were used in vascular grafts[9]. In dentistry we found that they accelerate healing and completely prevent fibroplasia, thus giving evidence of an important biological action[10]. In all cases, these two polymers are progressively degraded by lysozyme and absorbed.

In spite of such interesting applications in different fields of medical sciences the interaction of these polymers or their monomers

glucosamine and N-acetylglucosamine (fig.1) with membranes has been scarcely studied to date. This paper presents an investigation of the interaction of glucosamine with model membranes constituted by liposomes of dipalmitoylphosphatidylcholine (DPPC) in water. This study was undertaken as a first step to obtain information on the fate of the degradation products in vivo and on the mechanism of the interesting biological action of chitin and chitosan.

2. MATERIALS AND METHODS

2.1 Sample preparation

Synthetic DPPC (1,2(dipalmitoyl)3-sn--phosphatidylcholine) was obtained from Calbiochem (San Diego, CA, U.S.A.). Glucosamine (D(+)glucosamine hydrochloride) was obtained from Sigma (St.Louis, MO, U.S.A). Appropriate amount of glucosamine was added to the lipid in a molecular ratio R ranging from .001 to 10. For small quantities a water solution was used. The specimens were then high vacuum dried and afterwards resuspended in distilled water in a in weight ratio of lecithin to water τ= 1/7. Multilamellar liposomes were obtained by incubation of these mixtures for some hours at about 55°C and by vortexing several times.

2.2 X-ray diffraction.

X-ray diffraction patterns were obtained by using a conventional powder diffractometer. Ni-filtered Cu-Kα radiation (λ=1,54Å) was used. The divergence of the primary beam impinging on the sample was about 8'.The generator - sample distance was ≈18 cm and the sample - counter distance was ≈20 cm. The sample had thickness of ≈1.5 mm and was sandwiched between two very thin aluminium sheetsfixed to a circular hole in an aluminium matrix with a diameter of ≈1 cm. The heating was obtained by a hot stage containing electrical resistors and temperature was controlled to ±.1°C by a BT 300/301 control system supplied by SMC (Grenoble, France). Data were obtained at different temperatures in the range

from room temperature to 60°C.

2.3 Differential scanning calorimetry (DSC)

Calorimetry curves were obtained by using a Perkin Elmer Calorimeter, model DSC-2C with related data processor. The scan rate both in heating and in cooling was 2.5°C· min^{-1}. Aluminium containers of 20 µl capacity for volatile samples were used.

Every sample underwent at least two complete cycles, each one consisting of a heating and a cooling scan.

3. RESULTS

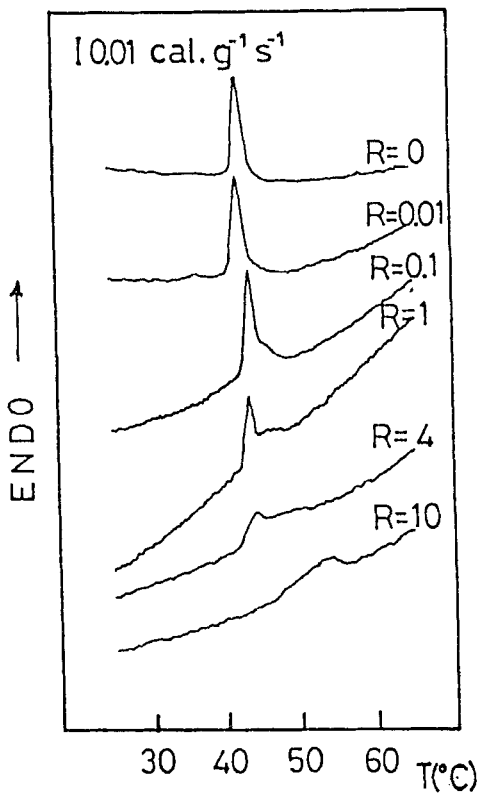

FIGURE 2 Calorimetric scans obtained heating DPPC liposomes with different glucosamine content. The ratios R (=moles of glucosamine/moles of DPPC) are reported in the corresponding patterns.

3.1 Calorimetry

Figure 2 shows the calorimetric curves obtained for liposomes by increasing the temperature from 25°C to 65°C. Every pattern is labelled with the corresponding ratio R between glucosamine and DPPC moles.

Fig.2(a) reports the curve obtained by heating the control sample, without glucosamine (R=0). A peak,

corresponding to the so-called pre-transition ($L_{\beta'}$ ->$P_{\beta'}$ transition), peculiar to synthetic lecithins with only one type of chain, is visible at ~ 36°C. A sharp transition peak corresponding to the aliphatic chain melting ($P_{\beta'}$ ->L_{α} transition), the so called main transition, is evident at higher temperature (~42°C). The remaining part of figure 2 shows the calorimetric curves obtained for increasing concentrations of glucosamine. The pre-transition peak is no more detectable for molar ratios R greater then .1. The main transition peak broadens and flattens for increasing concentration of the monomer and a shoulder appears on the higher temperature side; this shoulder progressively transforms into a broad and separate peak, moving toward higher temperature for increasing R values. At the higher molar ratio investigated, only this broad peak remains which is largerly broadened and

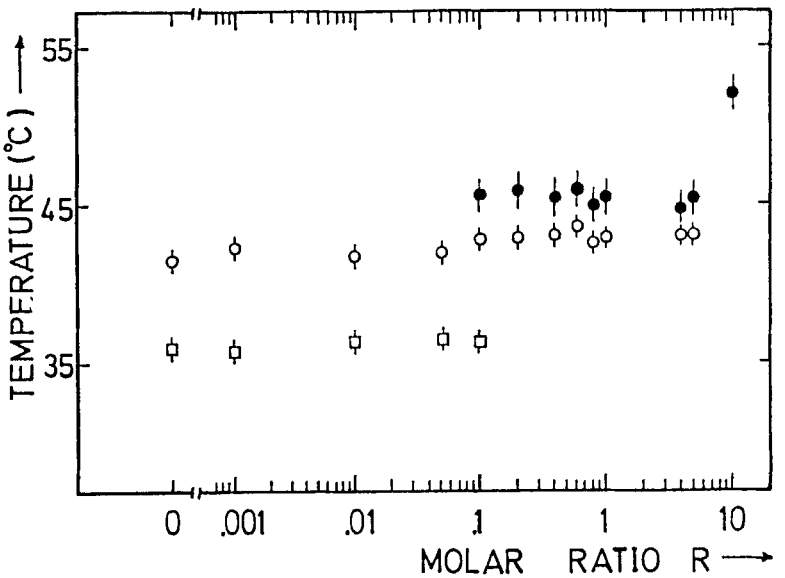

FIGURE 3 Peak temperatures vs. molar ratio R:□ maximum of the pre-transition peak; o maximum of the main transition; • center of the broad peak or shoulder.

shifted in temperature.

Fig.3 reports the temperatures corresponding to the maximum of the pre-transition (□) and main transition (o) peaks as a function of the molar ratio R. Pre-transition is not detectable for R>.1. The temperature of the narrow main transition peak does not vary greatly in the range $0 \leq R \leq 10$, at the most increasing for higher glucosamine content. For molar ratios $R \geq 0.1$ the estimated position of the center of the shoulder transforming into a large peak is also reported in the figure. A shift greater then 7 °C is observed at the highest R value.

Full width at half maximum (FWHM) of the narrow main transition calorimetric peak is reported in fig.4: the average value of the

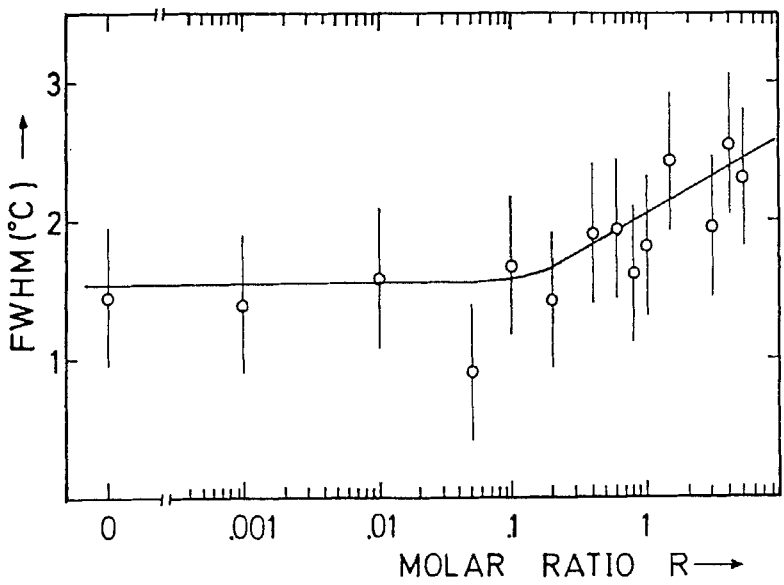

FIGURE 4 Full width at half maximum (FWHM) of the narrow calorimetric peak at the main transition as a function of the molar ratio R. The line is a guide for the eye.

heating and cooling scans of the first two complete cycles is reported. A broadening of the peak appears with increasing glucosamine concentration, in particular for molar ratios $R > 10^{-1}$

Entalpy variations in the temperature region around the main transition were also considered. The total transition entalpy ΔH_{TOT} is defined as the sum of the entalpy ΔH_N of the narrow peak and the entalpy ΔH_L of the larger peak. Their values were evaluated after striping peaks by hands. Fig. 5a show the ratio $\Delta H_L / \Delta H_{TOT}$ as a function of R. The ratio is different from zero for $R \geq 0.1$ and increases with increasing R values.

Data show that total entalpy of unit mass of sample decreases for increasing glucosamine concentration. As, however, the transition could be attribuited only to the lipid part, total entalpy for unit mass of DPPC is reported in fig.5b for different molar ratios. Reported values are the average of heating and cooling processes. A decreasing of transition entalpy for higher concentration is evident.

In particular molar ratios $R \approx 0.1$ can be regarded as critical also for what concerns this entalpy variations, as it was the case for $\Delta H_L / \Delta H_{TOT}$ (fig. 5a), for the narrow peak width (fig. 4) and for the entalpy variation ΔH_N for unit mass of DPPC, reported in fig. 5c.

3.2 X-ray diffraction

X-Ray diffraction patterns give information about the layer thickness in the low angle region, and about the interchain distances in the high angle region.

Layer thickness D were obtained by using the Bragg law: $n \cdot \lambda = 2 \cdot D \cdot \sin(\alpha/2)$. Where α is the angular positions of the low angle peaks, λ is x-ray wavelength and n is the order of reflection.

Liposomes of pure lecithin in excess water are known to exhibit an increase in thickness D from about 65 Å to about 70 Å in correspondence of the pre-transition[11].

A similar increase was also observed in our case for all those samples showing a pre-

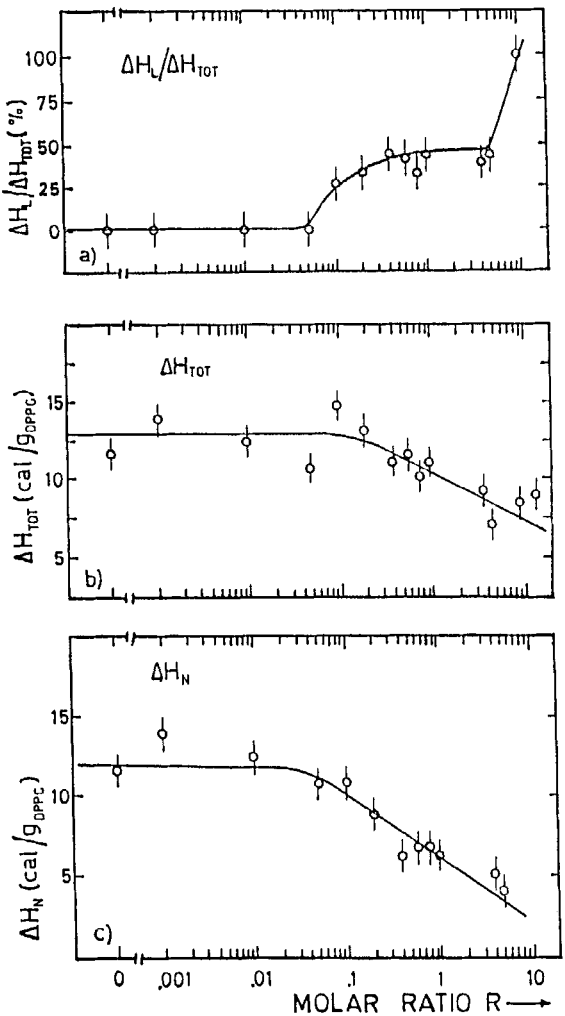

FIGURE 5 Transition entalpy for the large peak ΔH_L for the narrow peak (ΔH_N) and their sum ΔH_{TOT} at the main transition:

a- $\Delta H_L/\Delta H_{TOT}$ ratio

b- ΔH_{TOT} per unit mass of DPPC

c- ΔH_N per unit mass of DPPC

The lines are guides for the eye.

transition calorimetric peak, i.e. those characterized by R≤0.1. The behaviour of D for samples which did not show any calorimetric pre-transition peak was also investigated. An increase in layer thickness at temperature around those characteristic of the pre transition was observed also in these cases, as shown in fig.6 for a sample with R=0.8.

High angle diffraction from pure lecitin liposomes in the $P_{\beta'}$ phase is characterized by a narrow peak at $\alpha \approx 21°$, which corresponds to a parameter of 4.8 Å in the hexagonal arrangement of the hydrocarbon chains. Chain melting at the $P_{\beta'} \rightarrow L_{\alpha}$ transition corresponds to the disappearance of that peak and a large diffusive peak, centered at $\alpha \approx 19.5°$, remains at higher temperatures. Low concentrations of glucosamine do not alter sensibly this behaviour, while higher concentrations induce a permanence of the narrow peak in a wider range of temperatures.

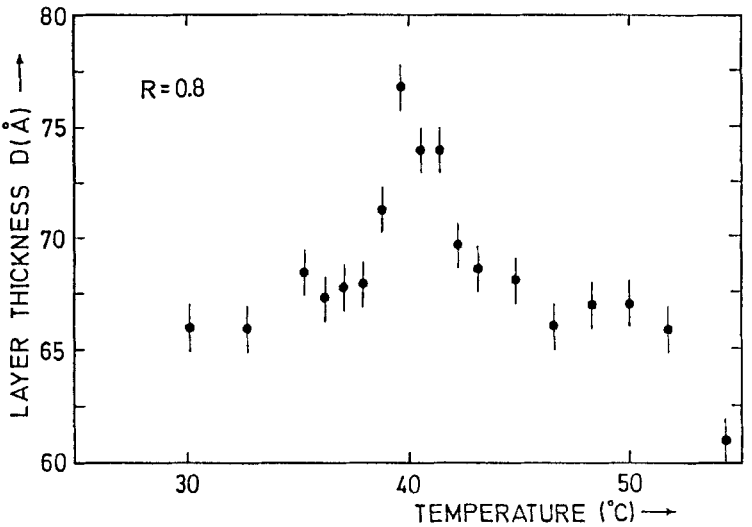

FIGURE 6 Layer thickness D as a function of temperature for liposomes containing glucosamine in the molar ratio R=0.8.

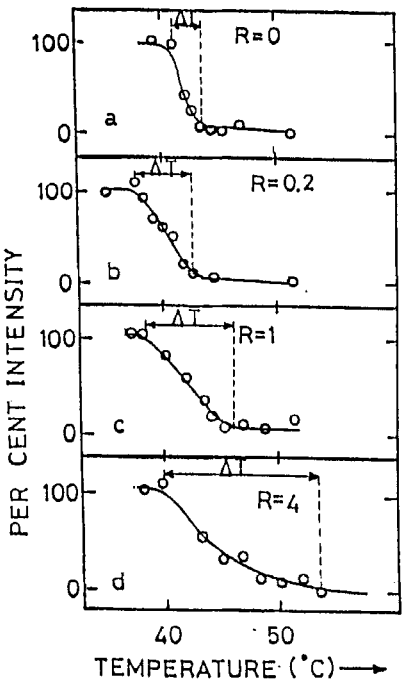

FIGURE 7 Relative intensity at $\alpha \approx 21°$: 100%= highest value of intensity; 0%= intensity in the L_α phase. Control sample (a) and DPPC liposomes containing glucosamine in a molar ratio R=.2 (b), R=1 (c) and R=4 (d). The lines are guides for the eye

In a more quantitative way, fig.7 shows the maximum intensity of the peaks at $\alpha \approx 21°$ as a function of temperature, for different values of molar ratio R. The temperature range Δt in which this intesity varies from the highest value to zero increases for increasing glucosamine concentration, as reported in fig.8.

4. DISCUSSION

Rising of a shoulder close to the main transition peak was already observed in liposomes in which external molecules were added. Jain and Wu[12] suggested that the kind of modification induced in the thermodynamical behaviour is related to the location of the additive along the bilayer.

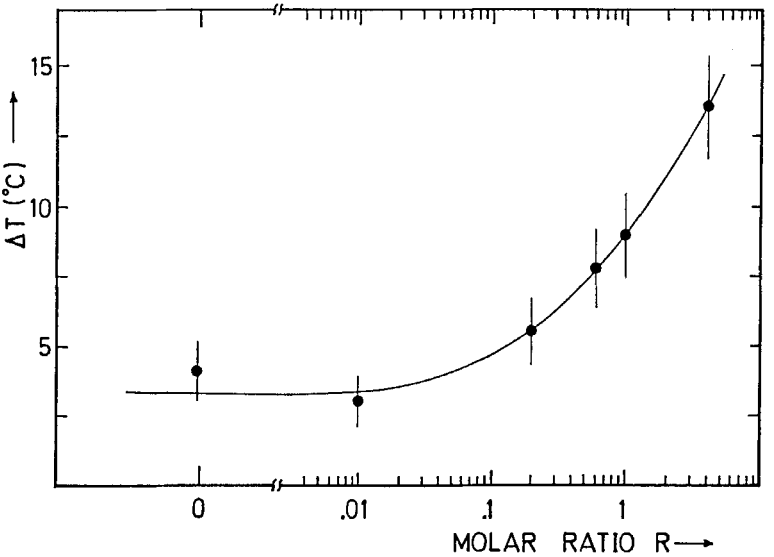

FIGURE 8 Temperature range ΔT, corresponding to the variation from the highest intensity to zero of the peak at α≈21°, as a function of the molar ratio R.

In particular lipophilic small molecules when located in the hydrophobic-hydrophilic interface produce a shoulder either on the low temperature side (type B↓) or on the high temperature side (type B↑) of the main transition peak. In both cases the area of the shoulder increases while that of the narrow peak decreases by increasing additive concentration,the sum of the two areas being costant for low concentrations. Our data on hydrophilic monomer correspond to a behaviour similar to that of B↑ type.

High sensitivity DSC studies on the effect of cholesterol in DPPC liposomes also showed the rising of a broad peak at temperatures slightly higher than that of main transition.[13] As in our case, this peak broadens and shifts to higher temperature when the entalpy of the narrow peak

reaches the zero value. The presence of two peaks was considered as due to a lateral phase separation into domains with different cholesterol content. As in our case, pre-transition calorimetric peak disappears at low concentration.

On the other hand our x-ray data present an increase in layer thickness characteristic of the pre-transition also for R $\geq$ 0.1, where no calorimetric peak of pre-transition was detected. To explain this difference one can consider that x-ray data were obtained at constant temperature and therefore in a more equilibrated state with respect to DSC data, which were obtained at constant rate of heating. Anyhow a difference between calorimetric data obtained for R $\geq$ 0.1 and those obtained for R $<$ 0.1 indicates that processes involving different rates occur in the two cases.

Fluidification of the membrane as it happens with other additives, as for instance alcools or anaesthetic[14,15], does not occur after adding glucosamine. In fact the main transition temperature as obtained from calorimetry measurements does not decrease, in case it increases for high concentrations, (fig.3) and moreover x-ray diffraction data (fig. 7) show that melting of the chains occurs in a wider temperature range for higher content of monomer and in the region of the calorimetric shoulder.

5. CONCLUSIONS

Model membranes of DPPC liposomes in water do not produce detectable variations by adding glucosamine monomers in a molar ratio R less than about 0.1. For greater R values changes are observed in the structural data and thermodynamical characteristic of the system. These modifications are interpreted as due to the location of glucosamine molecules at the hydrophobic - hydrophilic interface of the bilayer.

In analogy with the behaviour induced by the larger molecule of cholesterol, separation of phases with different content of glucosamine

cannot be excluded.

A progressive decrease of the main transition temperature for increasing additive content, indicating an induced fluidification of membranes, which could have strong effects from a physiological point of view, and which was observed in the case of other additive molecules, was not observed in the present investigation.

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