Molecular Crystals and Liquid Crystals
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gmcl16

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Version of record first published: 21 Mar 2007.

To cite this article: W. M. Wong & P. H. Geil (1976): Dynamic Mechanical Spectroscopy of Biomembrane Systems, Molecular Crystals and Liquid Crystals, 37:1, 281-301

To link to this article: http://dx.doi.org/10.1080/15421407608084363

Mol. Cryst. Liq. Cryst., 1976, Vol. 37, pp. 281–301

Printed in Holland

Dynamic Mechanical Spectroscopy of Biomembrane Systems

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(Received September 17, 1976)

Torsional briad analysis (TBA) was used to study the molecular interactions between the membrane components, e.g. protein–lipid and lipid–lipid. Several model systems as well as red cell ghosts were examined. In various phospholipids two or more relaxation peaks, depending on the system, were observed in each of three temperature intervals between 100–360°K. These peaks have been attributed to melting, rotation of whole long segments and rotation of short segments and/or end groups of the hydrocarbon chains. It was found that these peaks were affected by the fatty acid composition, i.e., chain length and degree of unsaturation, and water content.

The TBA results from liposomes and liposome–protein systems, known to be viable models for membranes, showed that the protein affects the motion of the hydrocarbon chains in negative lipoprotein systems, but not in positive lipoprotein systems; this is interpreted as indicating that hydrophobic interactions between protein and lipid take place in the former but not in the latter system. Furthermore, it is shown that lipid–lipid interactions change in the presence of cholesterol and dicetylphosphate.

Broad relaxation peaks were observed in human erythrocytes ghosts with various degrees of hydration. The broadening of the peaks is considered due to the contribution from the various protein and lipid components of the membranes.

INTRODUCTION

Recently a variety of experiments have suggested a dynamic structure for most biological membranes[1–10] with erythrocyte cells being a notable exception.[11] In the latter the protein spectrin is assumed to form a filamentous network on the cytoplasmic side of the membrane, holding the intra-membrane proteins in place. For a review, see, for instance, Ref. 12.

Accepting the concept that the membrane components, protein and lipid, are mobile, it is necessary to consider the mechanism that permits maintenance of the physical structure and physiological function of the membrane and also permits motion of the membrane components. However, in order to

consider the mechanism, one must have knowledge of the interactions of the various components of the membrane, i.e., the lipids, proteins and other macromolecules and water. A bilayer liquid crystal type structure is often suggested for the lipid component,[12] liquid crystals being known to retain their structure in the absence of external forces. However, the deformation characteristics of a membrane can not be explained in terms of either a bilipid layer–liquid crystal structure or a filamentous network as proposed for erythrocytes.[13,14] A solution to the problem of the origin of the mechanical strength of biological membranes requires knowledge of the interactions between the various components of the membrane. In this paper we describe the application of a new technique, dynamic mechanical spectroscopy, to the study of the effect of these interactions on the motions of the molecules in biomembrane model systems and their change with temperature.

Torsional braid analysis (TBA), the method used here for the dynamic mechanical spectroscopy, is an extension of torsion pendulum studies that have been used extensively for characterizing the thermomechanical behavior of materials (see, for instance, Ref. 15). TBA has the advantage over torsion pendulum studies that it permits the characterization of semi-micro amounts of material without restriction as to their physical state. However, to date, only limited applications of either technique have been made to biological samples and, to our knowledge, none to liquid crystal materials.

In TBA the specimen is simply deposited on a multifilament glass braid whereas for the torsion pendulum it must be in the form of a film or cylinder, with, in both cases one end of the sample being set in free torsional oscillation. The period of the oscillation and its rate of decay is measured as a function of temperature, yielding values of a relative shear modulus (G' is proportional to $1/P^2$ where P is the period) and the decrement θ_{i+1}/θ_i (where θ is the twist angle for a given cycle). The decrement reflects the fractional dissipation of energy per cycle, being equal to one for a perfectly elastic material. Usually it decays exponentially, with the value of the logarithmic decrement $\Delta = 1/k \ln \theta_i/\theta_{i+k}$ (for k cycles) being plotted as a function of the measurement temperature.

Of concern here is the usefulness of this technique for characterizing various specific molecular motions in a sample and their change with temperature. At sufficiently low temperature the thermal energy kT, will be small compared to the potential energy barrier height for a specific type of motion; i.e., it will be frozen with the molecule or segment thereof only vibrating about its equilibrium position. As the temperature is increased, a temperature will be reached at which the barrier can be surmounted and a larger amplitude motion begin (e.g., 120° or 360° rotation of a methyl group). The onset of each type of motion from one minimum to the next brings about a noticeable decrease or relaxation of G′ over a small temperature interval.

The rate of movement over the barrier is temperature activated and, depending on T, can be in or out of phase with the applied mechanical energy. If out of phase some of the energy is dissipated as heat. The amount of energy that is dissipated reaches a maximum at the same T and frequency where the change in G′ due to the onset of motion is the greatest. Therefore, a maximum is shown on a plot of logarithmic decrement vs. temperature, at a constant frequency (as above) or vs. frequency at a constant temperature. Below this T interval, where the motion is out of phase, the energy is stored elastically; above the interval the energy required is negligible.

Examples of the types of mechanical spectra obtained are shown in the various figures below. Each peak in the plot of logarithmic decrement vs. temperature corresponds, presumably, to the onset of a specific type of molecular motion at the frequency of the measurement, in this case at about 1 cycle per second. Assignment of the types of motion frequently requires comparison with model materials and other types of measurements. Unfortunately, for many of the materials used here, no other low temperature measurements are available and so we are restricted to comparisons with other types of materials, particularly polymers.

As in many other types of measurements, the interpretations proposed below are not unique, with considerably more validity being possible if complimentary data was available from other techniques. Of particular interest would be x-ray, differential scanning calorimetry (DSC) and nuclear magnetic resonance (NMR) measurements as a function of temperature in the range of interest, all of which, however, are also not in themselves fully adequate. In comparison with TBA, x-ray diffraction is primarily of value in characterizing phase changes in crystalline regions and is relatively unaffected by the onset of or changes in molecular motion and interactions in disordered regions and DSC has inadequate sensitivity for some of the relaxation processes. Broadline NMR can be used to characterize molecular motions in a manner similar to TBA, but at a considerably higher frequency; this results in a shift of the relaxation processes (peaks in Δ) to higher temperature and frequently to a merging of the peaks.

Prior work on changes in molecular motion in biomembrane systems with temperature has primarily been at temperatures above room temperature. For instance Steim *et al.*[5–7] used differential scanning calorimetry (DSC) to study E. coli and M. laidlawii membranes; they found that broad transition peaks, due to melting of the fatty acid chains, were observed at identical temperatures slightly higher than the growth temperature, 37°C, for both the membranes and extracted membrane lipids. These results, he states,[6] are considered a verification of the Danielli–Davson model with the bilayers being rather extensive and the interaction between proteins and lipids being electrostatic. Although similar experiments on erythrocyte

ghosts have shown no transitions with conventional DSC equipment, using a highly sensitive differential heat capacity calorimeter, Jackson, *et al.*,[16] have observed several peaks at 50°C and above; they attribute these peaks to denaturation of membrane bound proteins.

Gulik-Kryzwicki *et al.*[17,18] performed a series of experiments on interaction of protein–lipid–water systems using the techniques of circular dichroism, x-ray diffraction and fluorescence. The lipids, acid phospholipid cardiolipin and phosphatidylinositol and the basic proteins, lysozyme and cytochrome C were used. The results indicated that electrostatic and/or hydrophobic interactions are present in basic protein–acid lipid systems depending on the nature and composition of the components.

Many other model systems have been used to study protein–lipid interactions in addition to synthetic lipids and purified, single component proteins. Of particular concern to us are previous investigations from this university of liposomes, spherical visicles bounded by one or more liquid crystal lipid bilayers.

Sweet and Zull used negatively charged liposomes (egg lecithin-cholesterol-dicetylphosphate) and positively charged liposomes (egg lecithin-cholesterol-stearylamine) and bovine serum albumin (BSA)[19,20] to study glucose diffusion and interactions of proteins and lipids. They suggested that the initial binding of positive BSA to negative liposome is due to electrostatic interactions with the polar portions of the lipids and that the BSA then penetrates into the apolar interior of the lipid bilayers leading to hydrophobic interactions and creating an area through which polar molecules can move easily and consequently increase the diffusion rate. Later Sogor and Zull[21–23] further studied the permeability and structure of the BSA-liposome systems. Their small angle x-ray diffraction and electron microscopy results suggested that both the negative and positive charged liposomes, with and without the protein BSA, showed the characteristic lamellar structures observed in biological membranes. The solute permeability exhibited by these lipoprotein systems followed the sequence glucose < arabinose < malonamide < glycerol, which is the same as for the native membranes. Therefore, they suggested that these lipoprotein systems are viable models for membranes. It is this system that we have used as a complex model approaching the structure of native membranes.

The biological applications to date of dynamic mechanical spectroscopy have been concerned primarily with molecular motions and interactions with water in collagen,[24] the biologically related polypeptides[25] and nylon 6.[26] For instance, the primary or α relaxation of collagen was found to shift due to the effect of aging and water. Water was also found to contribute to three additional relaxation peaks: T_{H_2O} (270°K), β_1 (180–200°K) and β_2 (150°K),[27] due to the free water, sorbed water and bound water respectively.

In this paper we describe the initial results of the application of TBA to an investigation of macromolecular motions, interactions of protein–lipid and lipid–lipid, and the effect of water on a number of individual membrane components, model systems and intact membranes. As such the results complement our examination of the protein interactions in intact membranes using the ultrastructural deformation technique.[13,14] The following materials have been used: (1) Phospholipids-sphinogomyelin (SPH), phosphatidyl serine (PS), phosphatidyl ethanolamine (PE), phosphatidyl choline (PC or lecithin) with various hydrocarbon chains including egg yolk lecithin (EYL), reduced egg lecithin (REL), dipalmitoyl lecithin (DPL) and dioleoyl lecithin (DOL); (2) Cholesterol; (3) Bovine serum albumin (BSA); (4) Liposomes—negative and positive; (5) Lipo-protein—BSA with negative and positive liposomes; and (6) Human erythrocyte ghosts (HEG).

EXPERIMENTAL

All chemicals were purchased from Supelco, Schulman and Nutritional Biochemical Companies. They were of analytical grade and used without further purification. While impurities are known to effect the relaxation behavior of synthetic polymers, e.g., the addition of low molecular weight plasticizers lowers the glass transition temperature, effects of the size noted here for different types of lipids are not expected for reasonable quantities of impurities.

The liposomes and lipoprotein systems were furnished by Drs. Sogor and Zull of the Biology Department, Case Western Reserve University. These model systems were prepared by methods similar to that originally used by Bangham.[28] A detailed description of the preparation is given in the Ph.D. thesis of Dr. B. Sogor.[21]

Four types of liposome and lipoprotein systems were examined: (1) Negatively charged liposomes of EYL-cholesterol-dicetyl phosphate having a molar ratio of 70 : 10 : 20 at pH = 3.4; (2) positively charged liposomes of EYL-cholesterol-stearyl amine having a molar ratio of 60 : 10 : 30 at pH = 8.0; (3) negatively charged liposomes plus BSA at pH = 3.4 and (4) positively charged liposomes plus BSA at pH = 8.0. The amount of protein used is to saturate the liposome.

Human erythrocyte ghosts and lipid extracted ghosts were prepared by the method of Dodge[29] and Rose and Oklander.[30]

The cryogenic torsional pendulum was built by Armeniades[31] in the Macromolecular Science Department, Case Western Reserve University. A detailed description of this equipment is given in his thesis (see also Ref. 32).

Phospholipids (except SPH), and cholesterol were deposited on the braid from chloroform solutions. SPH was deposited from methanol. For examination of the effect of water, EYL was deposited from distilled water as well as chloroform. BSA, liposomes, lipoproteins and membranes were also deposited from water or buffer. For several samples the braid was then put in a flask having the desired humidity and allowed to reach equilibrium before mounting in the specimen chamber. For most of the specimens, the braid was mounted in the specimen chamber shortly after the specimen was deposited and then vacuum dried at room temperature for one day. The chamber was then filled with helium at atmospheric pressure and the sample cooled to liquid N_2 temperatures before the measurements were begun. A few of the specimens were dried for longer times. All the specimens discussed were "one day dry" unless otherwise specified.

Since the specimens were deposited on the braid in air at room temperature the possibility of oxidation was of concern. The time required to deposit the specimen depends on the solvent used, extending from 30 minutes for most of the organic solvents to 2–3 hours at the most. Egg yolk lecithin specimens air-dried for 2 hours, air-dried for 24 hours, and dried under nitrogen were chosen to test for oxidation. Gas Chromatography (using a Packard 417) results showed no significant (the percent of unsaturated hydrocarbon chains decreased from 50% to 49%) amount of oxidation for EYL air-dried for 24 hours as compared to the nitrogen dried sample.

RESULTS AND DISCUSSION

Lecithin (PC) is one of the major phospholipids in biological membranes. It makes up about 25% of the total phospholipids in human erythrocytes. In order to see the effect of different types of hydrocarbon chains on the molecular motions and/or phase transitions, several different lecithins, EYL, RFL, DPL, and DOL cast from chloroform, were investigated. Their relaxation spectra are shown in Figure 1. Relaxation peaks were observed at ca. 324°, 285°, 200°, 155–160 and 120°K. For the purpose of convenience, these relaxation peaks are separated into three regions, labeled α, β, γ. It is noted that several of the samples have peaks at even higher temperatures; if measurements were carried to sufficient temperature additional peaks, including melting of the lipid, should be observed. The α peak represents the high temperature melting of the hydrocarbon chains occurring in the 260° to 300°K temperature range. The γ peaks (often two peaks) correspond to the 120° to 160°K range with the β peak or peaks being in the medium temperature 160° to 260°K region. Some overlap in the temperature ranges is observed depending on the chemical structure of the sample. For most of

our samples, as discussed below, it was possible to assign one of the relaxation peaks to T_m (i.e., α) on the basis of prior work in the literature using DSC or x-ray diffraction measurements. The mechanism of the β and γ peaks, first reported here, are discussed further below. We first consider the results themselves in terms of changes in peak position with changes in chemical structure, water content, interaction, etc.

As shown in Figure 1, both DPL and REL show approximately the same thermal behavior with α peaks at 324°K (DSC measurements showed DPL melts at ca. 329°K[33,34]), β at 230°K, γ_1 at 155–160°K and γ_2 at 120°K.

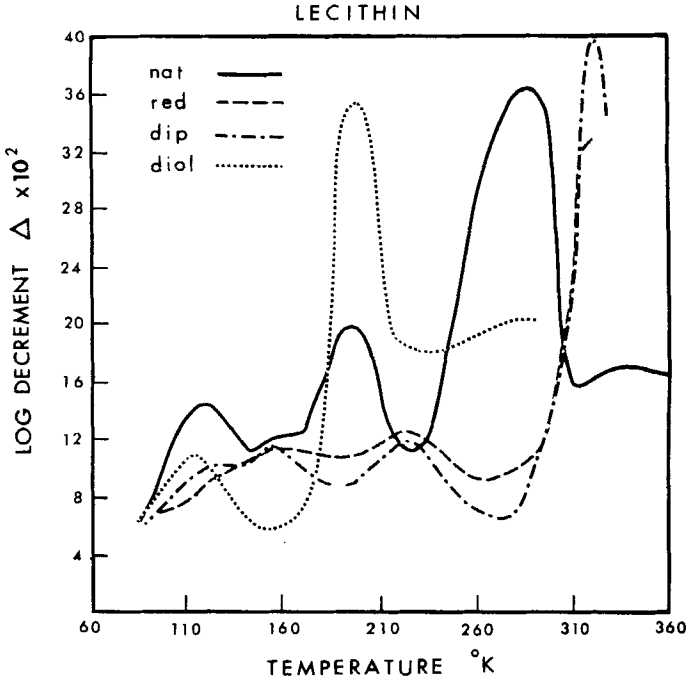

FIGURE 1 TBA spectrum of four lecithins cast with chloroform; natural egg yolk lecithin, reduced egg lecithin, dipalmitoyl lecithin and dioleoyl lecithin.

Dry DPL, in which both hydrocarbon chains are the same length, 16 carbons, and fully saturated (16 : 0) was shown by Chapman[33,34] to be lamellar in structure with hexagonal packing of the fatty acid chains at room temperature. REL, which has two different fatty acid chains (16 : 0) and (18 : 0), may have the same crystalline structure as DPL. EYL, although a mixture, has primarily one saturated chain (16 : 0) and one unsaturated chain (18 : 1), was shown by Tardier and Luzzati[35] to have different phases at room temperature depending upon the water content, e.g., a three dimensional cubic

phase, or a one dimensional lamellar phase with the chain either in liquid-like conformation or packed in hexagonal and square lattices. The latter was observed for anhydrous conditions with 6% acetyltrimethyl-ammonium bromide added; increasing water lead first to the hexagonal form and then the L_β and L_α form. The α and β dynamic mechanical peaks for this sample were shifted to lower temperatures, 285°K and 200°K. The γ_1 and γ_2 remained in the same temperature regions as those of DPL and REL, i.e., at 155–160°K and 120°K. The hydrocarbon chains of DOL are both unsaturated (18 : 1). The α peak, we suggest below, shifted to a much lower temperature, 200°K. Neither the β nor γ_1 peaks were observed. The γ_2 was found at 120°K, the same temperature as that observed for the other lecithins. No information was available concerning the structure or conformation of this lecithin.

As pointed out above, the α relaxation peak is considered to represent the melting of the hydrocarbon chains of the phospholipids. At this temperature, the phospholipids transform from the crystal to a liquid crystal phase; long range order of the polar heads is retained but the packing of the hydrocarbon chains is disrupted. This transition is characterized in x-ray diffraction by a change from, e.g., the 4.15 A peak characteristic of hexagonal packing, to a 4.6 A diffuse peak identical to the observed in liquid paraffins. Our TBA data showed that the α relaxation of the DPL (REL), EYL and DOL followed the trend that the more unsaturation the lower the melting temperature of the hydrocarbon chains. It is for this reason, and the height of the peak, that the 200°K peak of DOL rather than the peak at ca. 285°K is assigned as α. The melting temperatures of DPL and EYL are compatible with published DSC results.[33,34]

The relaxation peaks found at 340°K for EYL and 285°K for DOL, which occur at temperatures higher than the side chain melting temperatures (as shown by DSC for EYL), are believed to relate to motion resulting in changes in the ionic network of the lipids.

Clear identification of molecular mechanisms associated with the relaxation peaks of the phospholipids at temperatures lower than the melting temperature of the hydrocarbon chains suffer from lack of knowledge concerning the conformation and any significant experimental data for comparison. At the present time it is only possible to compare the results with those obtained for synthetic polymers and a few biological macromolecules.

Many polymers, such as polyethylene, polyamides, polyesters and long side chain polyolefins show a γ relaxation peak in the 100–160°K range[36] at ca. 1 hertz. At least one γ relaxation peak is always observed for polymers containing linear $(CH_2)_n$ sequences, where $n > 3$ or 4. Schatzki[37] showed that olefins with long unbranched side chains exhibited a γ relaxation at 103°K (200 hertz). He proposed that this relaxation is due to rotation of the terminal

ethyl group in amorphous regions. Olf and Peterlin[38] suggested the presumably similar motion in polyethylene probably involves full rotation of $(CH_2)_n$ segments in the amorphous regions. Illers[39] first showed that this relaxation has two components, the higher temperature one being labeled γ_1 and the one at lower temperature γ_2. γ_2 is suggested to occur in relatively short sequences, with $n = 3$ or 4, while for γ_1 longer sequences are needed. The activation energy of the γ_1 and γ_2 relaxations are 15 and 7 Kcal/mole. More recently, Boyd and Breitling[40] proposed a crankshaft type motion, different from the crankshaft motion originally described by Schatzki[37] and Boyer[44], for the γ process. Their conformational analysis of a single isolated segment revealed that the minimum energy path for this motion has two potential barriers of 11 and 7 Kcal/mole leading to two relaxation times (or temperatures). The crankshaft motion, presumed to occur in amorphous or crystal defect regions, involves two rotating bonds which are coplanar or nearly colinear; bonds 1–5 change from a TTGTT sequence to GTG'TG, where T is trans 0°, G is gauche $+120°$ and G' is gauche $-120°$.

The γ_2 and γ_1 peaks have now been resolved for several polymers. Typical temperatures reported are as follows: 108°K and 152°K for linear polyethylene,[42] 114°K and 142°K[42] and 135°K and 155°K for branched polyethylenes, 115°K and 157°K for nylon 66,[42] and 118°K and 140°K for nylon 11.[42] The intensity ratio of the γ_1 and γ_2 peaks has been found to vary from 0.5 to 4.0 for different polymers. Illers pointed out that the relative intensity depended on the morphology of the polymers; for example, the γ_1 was found missing for PE single crystal and stress oriented samples.[39,43,44] He suggested that structural restraints prevented the motion involved in γ_1. From this he concluded that the γ_2 peak, usually located at about 110–120°K, resulted from the end group rotation of short segments (N = 3, 4) and/or simple crankshaft motions while the higher temperature, larger activation energy γ_1 peak is due to longer segments. Thus the two γ peaks are considered by Illers to result from different types of motion whereas Boyd and Breitling's model involves only a single type of motion.

It is noted that all lecithins examined showed relaxation peaks at 120°K and 155–160°K except DOL which showed one peak at 120°K only. These peaks are suggested to result from similar mechanisms to those of γ relaxations in synthetic polymers. Variations in relative height of the peaks may also be due to differences in morphology, about which nothing is known at present. The explanation for the absence of γ_1 in DOL may be related to the fact that its hydrocarbon chains are both unsaturated: rotation of the long segments may be restricted whereas short segment motion, γ_2, is permitted.

In addition to the γ process, some amorphous or partially crystalline polymers exhibit β relaxations at higher temperatures than the γ relaxation; these involve the rotational motion of side groups, e.g., —COOR and

—OCOR of polymethylacrylate the polyvinylesters. Crystalline polymers, at temperature about 50° below the melting temperature, exhibit an α relaxation associated with the crystalline regions. Among others, Takayanagi[45] has proposed that this relaxation involves translation and torsional oscillation motions of segments along and about the chain axis while Tuijnman[46] suggested that it is the result of rotational motion of complete planar zig-zag segments about the chain axis.

The 230°K peak, located between the α and γ_1 peaks of DPL and REL, labeled β, we suggest is associated with rotational motion of the entire saturated hydrocarbon chains in the crystalline regions, i.e., it is similar in mechanism to the α process of crystalline polymers. This would correspond to a transition from, for instance, an orthorhombic lattice to hexagonal packing. The same mechanism we suggest, explains the 200°K peak of EYL; the lower temperature of this peak compared to DPL is suggested to be due to the rotational motions of the unsaturated hydrocarbon chains occurring at lower temperature than the saturated chains. In DOL, in which both hydrocarbon chains are unsaturated, both the β and γ_1 relaxations are missing; we suggest this is due to actual melting occurring when the chains start to move.

Thus, in summary, we suggest the following relaxation assignments: α-melting of hydrocarbon chains; β-rotation of entire hydrocarbon chains, resulting, for instance, in hexagonal packing; and γ-local motion of short segments and ends of the hydrocarbon chains.

Lecithins, as well as other phospholipids, can undergo conformational changes in the presence of water. Tardier and Luzzati,[35] showed that egg yolk lecithin, at water contents from zero to 10% and temperatures between −30°C and 150°C, exhibits many crystalline phases, L_α, L_β, H'_β, P_δ and C, etc., depending on the water content and temperature of the lecithin.

Small[47] examined egg yolk lecithin at various water contents and in the temperature region from 0° to 250°C. He found that lecithin shows both liquid and liquid–crystal phases when the water content is greater than 45%; at lower water content, crystal and liquid–crystal phases were present. However, detailed descriptions of the phases were not given.

Water would be expected to significantly affect the mechanical properties of lecithins. DSC studies,[48] for instance, suggested that water lowers the phase transition temperature of distearoyllecithin from ca. 355°K to 335°K for water contents of 0 to 40%. A water peak was also found at 0°C for water contents above 25%.

The effect of water on the TBA spectrum of EYL cast from water is shown in Figure 2; the spectrum for the chloroform cast sample from Figure 1 is also shown for comparison purposes. They are seen to differ considerably in both the α and β relaxation regions with all of the relaxation peaks shifted considerably with water content. For the water cast samples, the γ_2 peaks

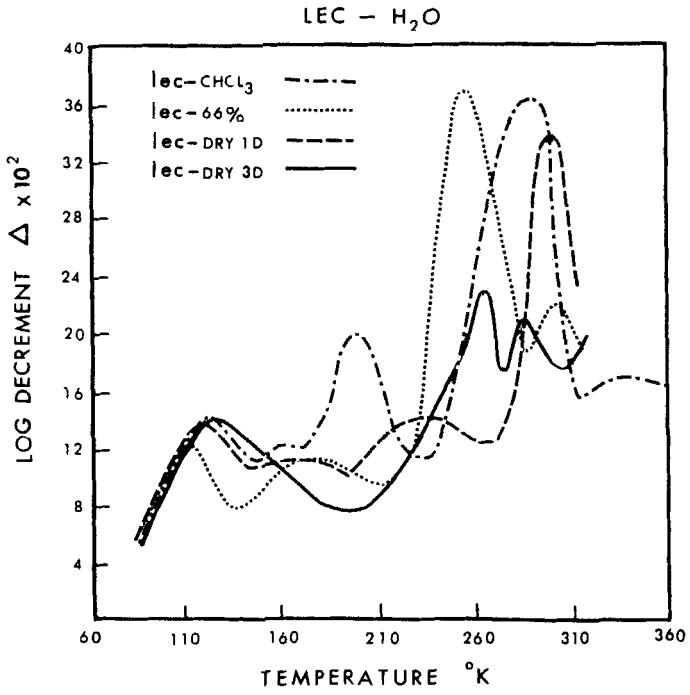

FIGURE 2 TBA spectrum of egg lecithin with different degrees of hydration (66% wet, one-day-dry and three-day-dry) plus the sample cast with chloroform.

shifted from 110° to 130°K as the samples became drier. The γ_1 peak for the chloroform cast sample corresponds in temperature to the one-day-dry sample; it is possible that the chloroform cast sample may have picked up some moisture before being placed in the pendulum chamber, or residual chloroform may be present giving rise to similar effects. The γ_1 peaks are located at ca. 175°K for the sample equilibrated at 66% relative humidity (66% wet) and the one-day-dry samples; both peaks are quite broad, for the 66% wet sample the γ_1 peaks extends from 140° to 210°K. The broadening of these peaks is suggested to result from overlapping of the inherent γ_1 peak (ca. 150°K in chloroform cast samples) and a peak due to bound water. The γ_1 peak of the three-day-dry sample may have merged with the γ_2 peak, forming the broad peak at 130°K.

The β relaxation peak in the one-day-dry sample is suggested to correspond to the peak at 235°K; in the 66% wet sample, this peak may be the shoulder on the strong free water peak at 260°K. In the three-day dry sample, the β peak may also be a shoulder at about 240°K. One notes that, in comparison with the chloroform cast sample, the β peaks in all of the water cast samples

are at a higher temperature (ca. 235° vs 200°K) and of lower intensity. This may be due to a different packing of the hydrocarbon chains in the chloroform cast sample or, possibly, a plasticization effect by residual chloroform.

The α relaxation peaks of EYL show drastic changes with changes in water content. We suggest that this can be attributed to the phase changes that Tardier and Luzzati[35] and Small[47] have shown. The α peak of the one-day-dry sample is located at 305°K. Two peaks with similar intensity, at 265°K and 285°K, are seen for the three-day-dry sample (as the sample gets drier). Luzzati showed that for less than $\frac{1}{2}\%$ water, the L_δ phase is present in the vicinity of 300°K with the P_δ phase being seen above ca. 325°K; at intermediate and lower temperatures a mixture of phases is seen. For a water content of 12% the C phase is seen from 250°K up to ca. 330°K; slight changes in water content (higher or lower) significantly lower the temperature at which this is the only phase seen. Although the temperatures of these phase changes observed by Tardier and Luzzati are about 30° higher than those at which we observe peaks, it is possible that they are due to similar phenomena. Needed are both x-ray and mechanical measurements on identical samples.

The 66% wet sample showed an α peak at 310°K, but the intensity is much lower; (the 260°K peak is attributed to free water). We interpret this as indicating a much lower crystalline content in this sample. This result is in accord with Small's data, i.e., lecithin with water contents higher than 45% showed a mixture of liquid and liquid crystal phases.[47] The lower degree of crystallinity may also explain the low temperature of the γ_2 peak.

In addition to PC, the phospholipids PE, PS and SPH are found to a significant extent in membranes. These four lipids make up more than 90% of the total phospholipids in erythrocytes. The TBA spectrum of chloroform cast PE, PS, and methanol cast SPH plus that of PC, are shown in Figure 3. All four of the samples have γ_2 peaks in the temperature region of 110° to 120°K; the intensity of this peak is higher in PC than the others, suggesting a large number of mobile units per unit weight in this material than in the others. The γ_1 peak is found only for PC. It may, however, be present as shoulders on the β peaks of PSH and PE. Single β peaks are found at 200°K for PC, 220°K for PE and 235°K for PS, two β peaks are observed for SPH, at 200°K and 250°K. The α peaks are observed at 290°K of PC, 310° for PE, 330°K for PS and above 360°K for SPH. One can see that the α and β relaxations temperatures of these phospholipids are correlated, i.e., both the α and β peaks move to higher temperature in the order of PC, PE, PS and SPH.

All of these phospholipids are from natural sources. PC was obtained from egg yolk, while PS, PE and SPH were extracted from bovine brains. Heterogeneity of chain length and especially the degree of unsaturation of the chains are expected, particularly for the latter three samples. These

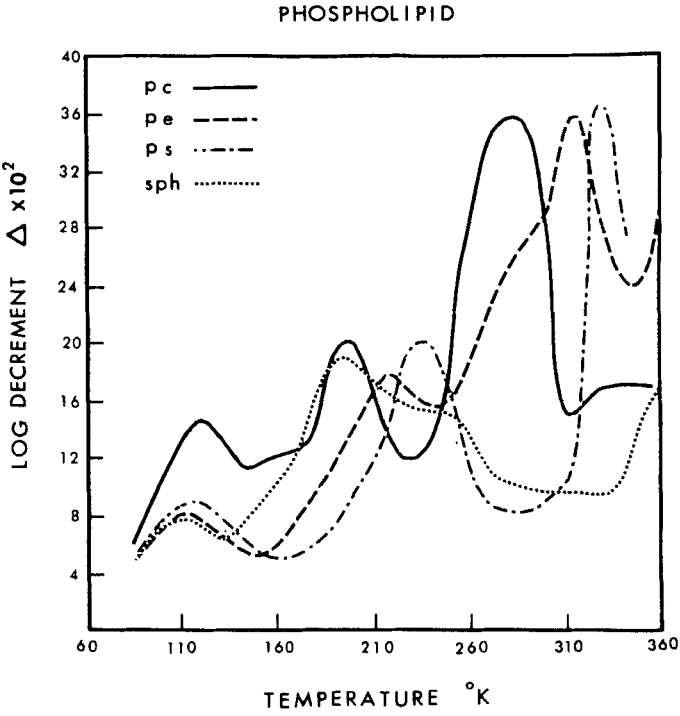

FIGURE 3 TBA spectrum of four different phospholipids: Phosphatidyl choline is from egg yolk; phosphatidyl ethanolamine, phosphatidyl serine and sphingomyelin are from bovine brain. All lipids are cast from chloroform except shingomyelin which was cast from methanol.

phospholipids are known to contain hydrocarbon chains of various degrees of unsaturation, i.e., 18 : 1, 20 : 4 and 22 : 6, etc., whereas the egg yolk PC contains primarily 16 : 0 and 18 : 1 side chains. This can explain why the intensity of the γ_2 peaks are lower than that of PC; the density of mobile chain ends will be lower due to both the longer chain length and large number of double bonds. Furthermore, the high unsaturation of the chains of these lipids would reduce or eliminate the γ_1 peak because the number of $(CH_2)_n$ sequences with $n \geq 3$ or 4 is less in these lipids than PC.

As in the case of the previous materials we interpret the β peaks as being due to the onset of hydrocarbon chain rotation. The two β relaxation peaks for SPH may be due to two distinct side chain rotation temperatures. Why this should occur in SPH and not the other materials is not known.

It is noted that very little x-ray work has been carried out on dry phospholipids obtained from natural sources. Baer, Palmer and Schmidt[49] have studied the lipids from nerve tissues. They concluded that the molecules are packed in a lamellar structure; wide angle spacings of 4.2 Å corresponding

to the spacing in the hexagonal phase were found at room temperature for all the lipids examined, e.g., lecithin (PC), cephaline (PC, PS) and SPH. PC, represented by EYL here, may have the L_δ conformation as suggested by Tardier and Luzzati.[35]

In our samples it is noted that PE, PS and SPH all have α peaks above room temperature with PC having its α peak close to room temperature, in agreement with the x-ray observation suggesting a hexagonal or cubic lattice at room temperature. Presumably this lattice is due to rotation of the chains, with their lateral packing being determined by the lattice spacings of the polar heads (closer packing would result in a hexagonal lattice). The low temperature shoulder in the PE α peak may be due to a crystal–crystal transition, or the melting of a low temperature phase. A small peak is also observed in the vicinity of 300°K in the SPH, it may also represent either melting of a low temperature, separate phase, or a "crystal–crystal" transition before hydrocarbon chain melting occurs above 360°K. It is obvious from the spectra that considerable, distinct differences can be observed for these phospholipids, despite their impurity; as pointed out above clear identification of the molecular mechanism for the various peaks will require the application of x-ray diffraction and other techniques.

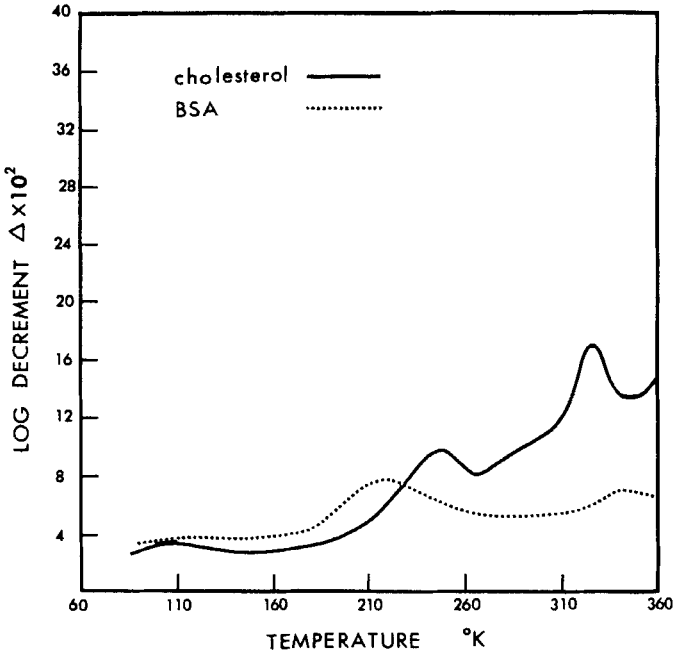

FIGURE 4 TBA spectrum of cholesterol cast from chloroform and bovine serum albumin cast from distilled water.

Cholesterol is the primary non-phospholipid lipid in membranes, especially in plasma membranes, myelin and erythrocytes. Cholesterol, which makes up 25% of the total lipid of erythrocytes, has been shown not only to affect the molecular motions of phospholipids, as indicated above, but also to affect the permeability of membranes.[50–53] Many experimental results, i.e., electron spin labeling[54] and x-ray diffraction[55] have shown that the effect of cholesterol depends upon its concentration. Its relaxation spectrum is shown in Figure 4, along with that of BSA, for comparison with the spectra from liposome and lipoprotein systems shown in Figure 5.

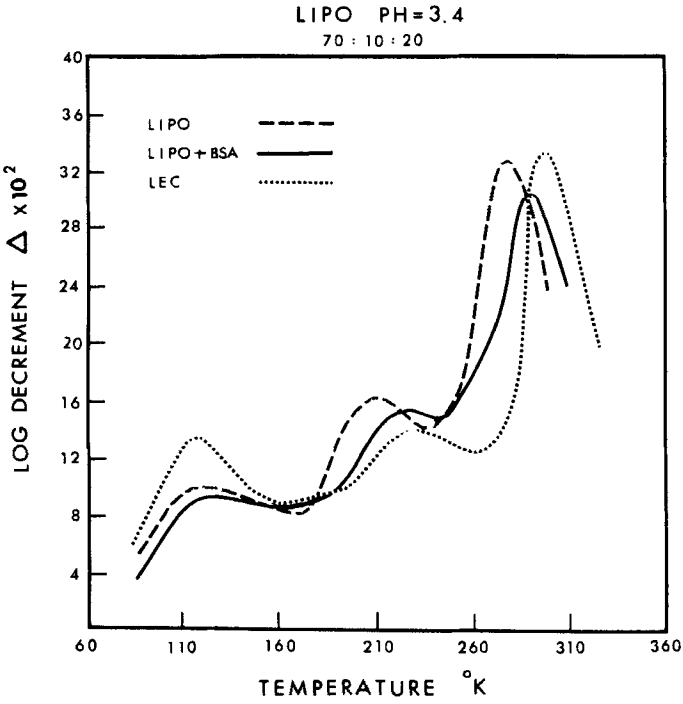

FIGURE 5 TBA spectrum of dry-negatively charged liposomes and lipoproteins; for comparison purposes, egg yolk lecithin cast from distilled water is also included.

Three relaxation peaks are observed for cholesterol, at ca. 110°, 240° and 330°K. Cholesterol, having a different crystalline structure from phospholipids, exhibits a melting temperature at ca. 420°K; therefore, the relaxation peaks observed may be considered to result from motions of the hydrocarbon portions, or crystal–crystal phase transitions.

BSA shows relatively small relaxation peaks at 220°K and 340°K, with there possibly also being a small peak in the γ_2 temperature range (i.e.,

110°K). The origin of the 210°K peak may be the same as that in synthetic polymers, the onset of rotation of some of the side chains, while the peak at 340°K may correspond to denaturation. Although further TBA study of various proteins would be of interest (as shown already for collagen) here we are primarily concerned with the effect of BSA on the lecithin molecular motions in liposomes. It is seen that the intensity of the BSA (and to some extent the cholesterol) relaxations are relatively low with respect to those of the various phospholipids. Therefore, changes in the liposome spectra with the addition of BSA can be attributed to molecular interactions rather than simple addition of the spectra of the individual components.

The relaxation spectrum of negatively charged liposomes (EYL-cholesterol-dicetylphosphate, having molar ratio 70 : 10 : 20) and lipoprotein (BSA plus negatively charged liposomes) at pH = 3.4, along with the one-day-dry EYL sample for comparison purposes (this most closely resembles the sample conditions of the liposomes) are shown in Figure 5. The negatively charged liposomes have γ_1, β and α peaks at 120°, 205° and 272°K; while the lipoproteins have similar peaks at 130°, 225° and 292°K. The one-day-dry EYL peaks are at 120°, 225° and 300°K. Comparison of the spectrum of this liposome with the EYL sample, both cast from water, shows that the α and β relaxation peaks of EYL are at higher temperatures than those of the liposome.

All of the relaxation peaks of the negatively charged liposomes were shifted to higher temperatures in the presence of BSA, the α and β peaks temperatures approaching those of the EYL alone. We thus suggest that the BSA affects the motions of the hydrocarbon chains of these negatively charged liposomes, although it is possible that it selectively binds the cholesterol, reducing its influence on the EYL. If it affects the hydrocarbon chains, the BSA would appear to be incorporated in the hydrophobic regions of the lipid bilayers. This finding gives support to the hydrophobic interactions between protein and lipid in this system suggested by Sweet and Zull[19,20] in order to explain the increase in the glucose diffusion rate of liposomes when BSA is added. Although the addition of cholesterol alone to EYL results in a closer packing of the hydrocarbon chains[50,51,54] and thus presumably to higher α and β transition temperatures, the opposite effect is seen here and in DSC measurements (α only).[55] Some of the effect observed here may also be due to the fact that our liposomes contain 20% dicetyl phosphate.

It is important to point out that additional electrostatic interactions can not be ruled out; our TBA was limited to a temperature region too low to see any significant motions of the ionic network of the phospholipids. In addition denaturation of the BSA, resulting in a change in the interactions, may occur. Sweet and Zull have shown that electrostatic interactions also take place between positive BSA (BSA is positive at pH = 3.4) and negatively

charged liposomes; the binding of BSA to these liposomes depends on the ionic strength.

As pointed out above, in positively charged liposomes (EYL-cholesterol-stearylamine, with molar ratio 60 : 10 : 30) at pH = 8.0, Sweet and Zull originally suggested that only electrostatic interactions take place; BSA had no effect on glucose diffusion. Sogar and Zull,[22] however, later suggested that the same type of interactions may occur in both negatively and positively charged lipoprotein systems but conclusive evidence was not presented. TBA was applied to both types of systems. The relaxation spectrum of the positive liposomes and lipoproteins are shown in Figure 6. Relaxation peaks were observed for both liposomes and lipoproteins, at 120°, 200°, 250° and 280°K. As can be seen, no significant difference was observed in terms of the number, intensity and location of the relaxation peaks. This indicates that the molecular motions of the hydrocarbon chains of the liposomes are not affected by the presence of the protein. Thus, based on the TBA results, we conclude that hydrophobic interactions are not involved in the binding of BSA to these positively charged liposomes.

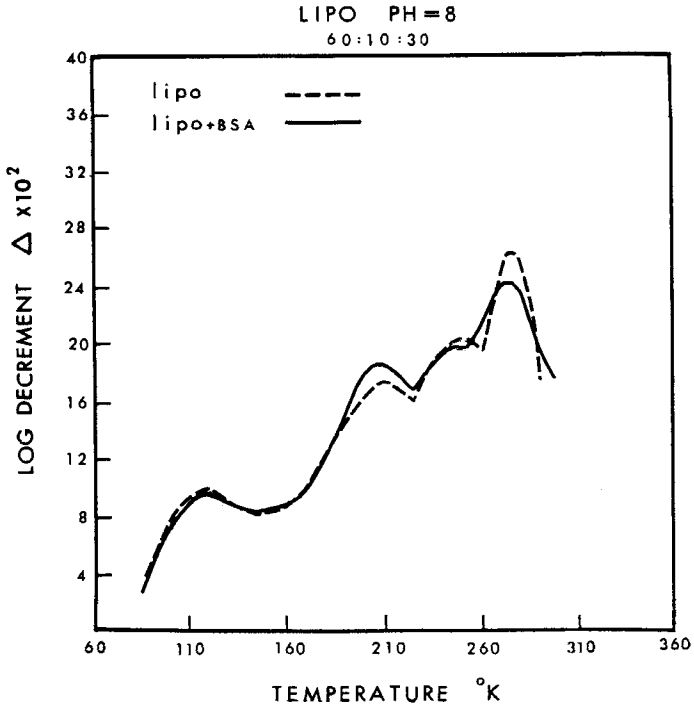

FIGURE 6 TBA spectrum of dry, positively charged liposomes and lipoproteins.

The fact that the positively and negatively charged liposomes have similar spectra in the α and β temperature regions, with a change in the β region from that of EYL alone, suggests that the hydrocarbon chain motions involved in these peaks are affected in a similar manner by the charge containing lipids and cholesterol. A change in the α temperature region is seen, a high temperature peak (260°K) being present in the positive systems which is not present in the negative systems. This, we suggest, can be attributed to details of the phase changes occurring during the hydrocarbon chain melting.

The TBA spectra of intact human erythrocyte ghosts with different degrees of hydration and the ghosts with the lipid extracted are shown in Figure 7. One can see that the relaxation peaks of the ghosts are affected by the dehydration as the sample progresses from 100% wet, 66% wet, one-day-dry to three-day-dry. The γ_2 relaxation was suppressed in the hydrated, 100% and 66% wet samples. The γ_1 relaxation, on the other hand, was found only in the 100% and 66% wet samples; this is similar to the situation for 100% wet negatively charged liposomes (not shown). Note that the height of the peaks are independent of the total amount of sample on the braid.

The intense water peak of the 100% wet sample obscures the β relaxation of

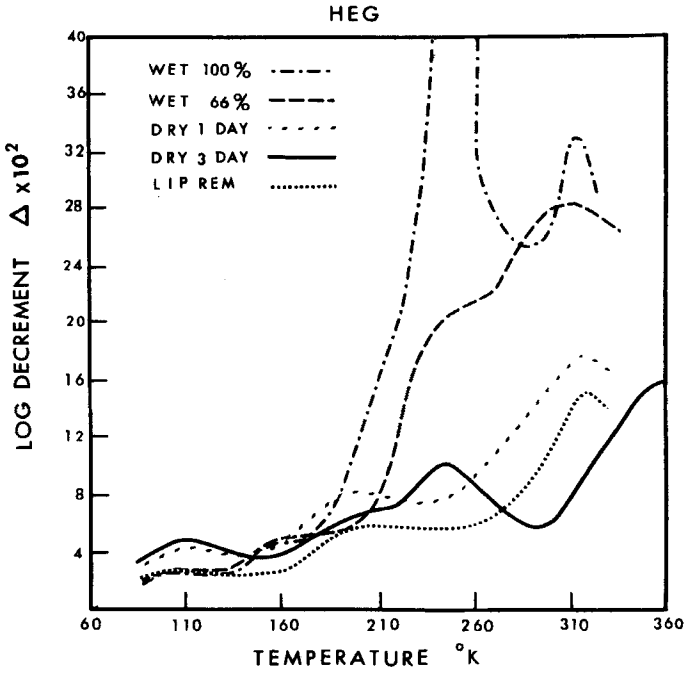

FIGURE 7 TBA spectrum of human erythrocyte ghosts of different degrees of hydration: 100% wet, 66% wet, one-day-dry, and three-day-dry, and the ghosts with lipids extracted.

the ghosts; as the sample becomes drier the intensity of this free water peak decreases, and the β peak can be seen at 200°K for the one-day-dry sample (ca. the same as in the one-day-dry negatively charged liposome, although lower in intensity), and at 240°K for the three-day-dry sample. The α peak found at ca. 310°K in all except the three-day-dry sample (where it is at ca. 350°K) is believed to result from melting of the hydrocarbon chains of the lipids and/or denaturation of the membrane proteins. All of the relaxation peaks in the samples of various degrees of dryness are very broad; this broadening we attribute to the presence of many different proteins and lipids in the native membranes.

The intensity of the α, β and γ peaks of the lipid extracted ghosts is relatively low, the peaks being similar to those in BSA; there is little or no contribution from the lipids that give rise to the major portion of the molecular motions observed in the other samples. Thus, although the peaks from the membranes are both relatively small and broad, one observed peaks in the same temperature ranges as in model systems, with similar effects due to hydration.

In conclusion, the TBA studies on membrane components, model systems and native membranes at temperatures between 100–360°K showed α, β and γ peaks. The α relaxations, which correspond to the melting of the hydrocarbon chains, are in general agreement with the DSC measurements. The β relaxations are considered to be due to the onset of rotation of the whole hydrocarbon chain while the γ relaxations are suggested to be due to rotation of short segments and/or end groups. All these relaxations were found to depend not only on the fatty acid composition, i.e., degree of unsaturation, but on the amount of water present and on interaction with other components. For example, cholesterol and dicetyl phosphate shifted the peaks of EYL to lower temperature while the protein, BSA, shifted them to higher temperature. Our TBA results strongly support the suggestion of both hydrophobic and electrostatic interactions of the proteins and lipids in biological membranes. Based on the influence of water on the α relaxation, the results suggest that water is present in and affects the organization of the interior of the hydrocarbon core of the lipid regions, as well as affecting the organization and interaction of the more polar regions. In addition, the results suggest that TBA, being a sensitive tool for examining molecular motions and the effect on them of interactions with other macromolecules, should be useful to study the effect of hormones and drugs on molecular mobility in membranes.

Acknowledgement

We would like to thank Dr. T. Yau, Case Western Reserve University for permission to use and assistance with the gas chromatography equipment. This work was supported in part by the National Institute of Health (grant GM-16131).

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