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LASER-RAMAN SPECTROSCOPIC STUDY OF EGG LECITHIN AND EGG LECITHIN–CHOLESTEROL MIXTURES

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SUMMARY

Raman spectra are presented for egg lecithin and egg lecithin–cholesterol mixtures (1:1 molar ratio). From the positions and intensities of the Raman lines near 1100 cm^{-1}, the hydrocarbon chains for egg lecithin are found to be in a liquid conformation. The addition of cholesterol appears to inhibit the formation of certain *gauche* isomers and hence causes a marked increase in chain rigidity. The results support the theory recently proposed by Rothman and Engelman (Rothman, J. E. and Engelman, D. M. (1972) *Nature New Biol.* 237, 42) for the mechanism of cholesterol–phospholipid interaction.

INTRODUCTION

The structure of biological membranes and the role of cholesterol in determining that structure are the objects of much current biophysical research. Raman spectroscopy offers several advantages for such studies. Unlike electron spin resonance spectroscopy, the Raman effect does not require the insertion of free-radical probes, which may greatly perturb the membrane lipid bilayer. In addition, spectra may be obtained from polycrystalline materials, surface films, or aqueous solutions. Comparison as to the molecular conformation in various states is therefore possible. Finally, the technique is non-destructive to samples and data may be obtained on small amounts of material.

Prior to understanding the Raman spectra of naturally occurring membranes, it is necessary to examine model membrane systems. In the only previously published Raman spectroscopic study of a membrane model system, Lippert and Peticolas[1] investigated the effect of cholesterol on the conformation of dipalmitoyllecithin multilayers. They showed that the spectral region 1000–1200 cm^{-1} is very sensitive to the conformation of the hydrocarbon chains, and were able to monitor the gel–liquid crystal transition near 40 °C under various conditions.

The next level of approach to biological complexity is to examine natural lecithins which, unlike dipalmitoyllecithin, have a mixture of fatty acids substituted in the α and β positions of the glycerol backbone. Egg lecithin has been studied by a wide variety of methods, including X-ray diffraction[2], electron spin resonance[3]

and nuclear magnetic resonance[4], and is therefore a suitable choice for a Raman spectroscopic investigation.

Reported in this paper are Raman spectra of egg lecithin and of egg lecithin–cholesterol mixtures in a 1:1 molar ratio. Samples were examined both as vesicles dispersed in aqueous media and as films cast on an aluminum surface. Information is obtained about the conformation of the hydrocarbon chains, and the results are compared with those available by other techniques. The role of cholesterol in modifying chain conformation is discussed.

MATERIALS AND METHODS

Chromatographically pure egg lecithin was obtained in chloroform solution from Lipid Products, South Nutfield, Surrey, and Σ-grade cholesterol was obtained from Sigma Chemical Co. Both were found to be free of fluorescent impurities and were used without further purification.

Egg lecithin and egg lecithin–cholesterol (1:1 molar ratio) vesicles were prepared by sonicating[5] water suspensions of weighed samples under nitrogen at 45 °C for 40 min. (In order to facilitate dispersion formation, 4% (by weight) of phosphatidylserine was added to the egg lecithin prior to suspension[5].) The resultant somewhat turbid preparations were centrifuged at 50000 × g for 10 min to remove the largest particles. Electron micrographs (× 80000) confirmed the presence of spherical vesicles of diameter 200–1500 Å. Final lipid concentrations were about 8% by weight.

Some of the egg lecithin samples were prepared by a method recently developed in this laboratory[6], which does not involve sonication Spectra in these cases were identical to those obtained when sonication was used

Films of lipid and lipid mixtures were cast from chloroform solution onto a clean aluminum surface. The solvent was evaporated under a gentle stream of nitrogen and the last traces were removed in an evacuated dessicator.

Raman spectra of vesicles were obtained on a Spex 1401 Raman spectrophotometer equipped with a Coherent Radiation model 52A argon ion laser and a photon-counting detection system. The laser was typically operated to give 200 mW of 5145 Å or 4880 Å radiation incident on the sample. The samples were contained in a 1 mm (internal diameter) capillary and the scattered light was collected at 90° to the incoming beam in the standard manner for this instrument Measurements at elevated temperatures were carried out in a cell similar to that described by Thomas and Barylski[7].

The lipid film samples were examined using a modified[8] Cary 81 Raman spectrophotometer equipped with a Spectra Physics model 256 argon ion laser. About 100 mW of 4880 Å light irradiated the sample which was tilted at 150° to the incoming beam. Scattered light was collected at 90°.

RESULTS

Several spectroscopic studies of long chain hydrocarbons[9–11] have shown that the region 1000–1200 cm^{-1} is particularly sensitive to chain conformation. This is illustrated in Fig. 1a–1d where Raman spectra of n-hexadecane and oleic acid

in the solid and liquid states are shown. Peaks in this area have been assigned[10,12] to skeletal vibrations in which alternate carbon atoms move in opposite directions along the chain. For solid hexadecane, (Fig. 1b), the band near 1081 cm^{-1} is extremely weak compared to that at 1125 cm^{-1}. This is characteristic of the all-*trans* (crystal-like) configuration of the chains. Upon the melting of hexadecane (Fig. 1a), many

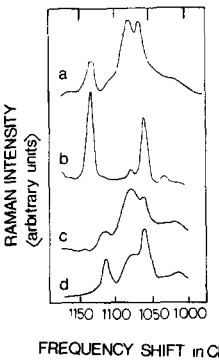


Fig 1 Raman spectra of: a, liquid hexadecane, b, solid hexadecane, c, liquid oleic acid, and d, solid oleic acid (77 °K), in the region 1000–1200 cm^{-1} Spectral slit width, about 3 cm^{-1} Excitation source, 250 mW of 5145 Å radiation

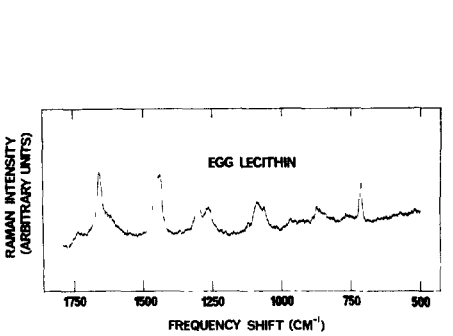
gauche isomers contribute to the intensity of these Raman lines[1,10]. This condition is reflected in the spectra by an intensity decrease of the 1125 cm^{-1} vibration and a frequency shift to 1085 cm^{-1} and intensity increase of the other mode. Similar effects occur for oleic acid. Since about 70% of the hydrocarbon chains in egg lecithin are those which occur in hexadecane or oleic acid[13], the Raman spectral properties of egg lecithin in the region 1000–1200 cm^{-1} should reflect the conformation of its hydrocarbon chains in a way similar to that in Fig. 1. The intensity ratio of $I(1125)/I(1085)$ will be used as a probe for investigating chain conformation*. This procedure has been previously used for dipalmitoyllecithin by Lippert and Peticolas[1].

The Raman spectrum of egg lecithin vesicles is shown in Fig. 2 and spectra for solid dipalmitoylethanolamine and egg lecithin films are presented in Fig. 3A and 3B. Most of the intense bands can be assigned to specific vibrations of the hydrocarbon chains (Table I). The only vibration which originates in the polar head groups is that near 720 cm^{-1} in egg lecithin and 760 cm^{-1} in dipalmitoylethanolamine. The band is assigned to a deformation vibration of the choline or ethanolamine groups respectively.

By analogy with the 1000–1200 cm^{-1} region of hexadecane and oleic acid, it appears that the hydrocarbon chains in dipalmitoylethanolamine are in a

* The indicated ratio $I(1125)/I$ (1085) is used as a measure of chain fluidity in all the systems studied It is, however, recognized that these frequencies can deviate slightly from molecule to molecule For example, in oleic acid (Fig. 1c and d), the higher frequency vibration occurs at 1115 cm^{-1} Although the molecular motion assigned to each of these vibrations is presumably the same in all systems studied, the origin of the slight differences in frequency (1–10 cm^{-1}) is by no means clear

The intensity $I(1125)$ in the current context refers to the area under the higher frequency vibration, regardless of where its peak is, while the intensity $I(1085)$ refers to the area under the lower frequency vibration



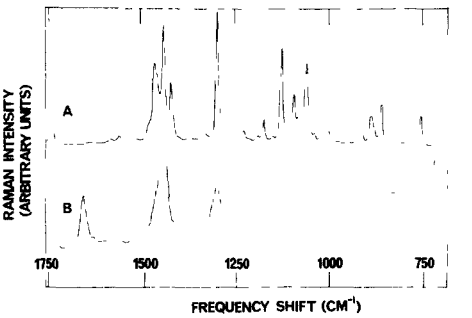


Fig 2. Raman spectrum of a sonicated H_2O suspension of egg lecithin vesicles in the region 500–1750 cm^{-1}. Spectral resolution, about 3 cm^{-1} Excitation source, 200 mW of 4880 Å radiation. Vesicle concentration, 8% by weight

Fig 3 A, Raman spectrum of solid (polycrystalline) dipalmitoylethanolamine in the region 700–1750 cm^{-1}. Spectral slit width, 4 cm^{-1} Excitation source, 50 mW of 4880 Å radiation B, Raman spectrum of egg lecithin film in the region 700–1750 cm^{-1}. Spectral slit width, 7 cm^{-1} Excitation source, 75 mW of 4880 Å radiation

TABLE I

RAMAN SPECTRUM OF EGG LECITHIN VESICLES

Relative intensities in parentheses with strongest line fixed at (10). ν (X–Y) = X–Y bond stretching vibration; def = bond angle deformation vibration; sh = shoulder

Frequencies (cm^{-1})	*Tentative assignment*
720 (7)	Head group def
770 (1)	
845 (sh)	
872 (3)	CH_2 def
890 (1)	
954 (0)	
967 (1)	= C–H def (out of plane)
1062 (4)	
1085 (6)	ν (C–C)
1123 (1)	
1261 (4)	= C–H def (in plane)
1298 (6)	CH_2 twist
1370 (1)	CH_2 wag
1445 (10)	CH_2 def.
1658 (10)	ν (C–C) + H_2O
1731 (2)	ν (C–O) ester

predominantly *trans* conformation (crystalline), while those in egg lecithin are in a liquid state. These conclusions are consistent with those previously obtained from X-ray diffraction studies (ref. 2; and Torbet, J. and Wilkins, M. H. F., unpublished). It is further of interest to note that from the similarity of Figs 2 and 3B, the conformation of the chains in the film of egg lecithin is similar to that in the vesicles.

The effect of cholesterol on the conformation of the hydrocarbon chains of egg lecithin is illustrated in Fig. 4a–c, where the 1000–1200 cm^{-1} regions are shown for egg lecithin and egg lecithin–cholesterol (1:1 molar ratio) at 25 and 70 °C, respectively. The increased intensity near 1125 cm^{-1} upon cholesterol addition,

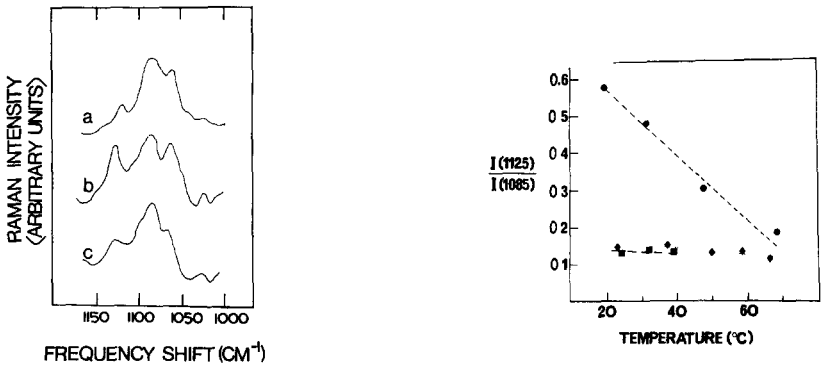


Fig 4 Raman spectra in the region 1000–1200 cm^{-1} for: a, egg lecithin vesicles at 25 °C, b, egg lecithin–cholesterol vesicles (1:1 molar ratio at 25 °C) Weak background due to cholesterol vibrations near 1128 and 1090 cm^{-1} has been subtracted out in b and c. c, Egg lecithin–cholesterol vesicles (1:1 molar ratio at 70 °C.)

Fig 5 Temperature variation of the I (1125)/I (1085) intensity ratio for egg lecithin–cholesterol (1:1 molar ratio). - - - - -, Vesicles suspended in H_2O; . . . , egg lecithin vesicles in H_2O; and - - - egg lecithin–cholesterol (1:1 molar ratio) mixtures dissolved in chloroform

TABLE II

I(1125)/I (1085) RATIO FOR VARIOUS SUBSTANCES

Substance	*Temperature* (°C)	$I(1125)/I(1085)$
Liquid hexadecane	25	0 30
Solid hexadecane *	5	20
Liquid oleic acid	25	0 15
Solid oleic acid *	−195	1 0
Egg lecithin vesicles	25	0 16
Egg lecithin–cholesterol	25	0 58
Vesicles	70	0 20
Dipalmitoylethanolamine	25	2.62

* The high ratio for solid hexadecane reflects the rigidity of the packing in the crystal. The lower ratio for solid oleic acid illustrates the effect upon the packing of a bend already present in the chain

accompanied by an intensity drop and sharpening of the 1085-cm^{-1} band, are indicative of a rigidification of the hydrocarbon chains, as previously discussed. However, the $I(1125)/I(1085)$ intensity ratio does not approach that in dipalmitoylethanolamine, (Table II), which indicates that the chains are not completely in the *trans* form. Chapman and Penkett[4] have arrived at somewhat similar conclusions from NMR spectral data, but the Raman technique directly illustrates the contribution of *trans* and *gauche* isomers to the chain conformation.

Upon heating egg lecithin–cholesterol to 70 °C, the intensity ratio drops (Fig. 4c) and the spectrum resembles that of egg lecithin. The chains have therefore apparently partially regained their liquid conformation. The thermal behaviour of the Raman lines is illustrated in Fig. 5, where the $I(1125)/I(1085)$ ratio is plotted as a function of temperature. The indicated ratios are corrected for the presence of weak cholesterol bands near 1128 and 1090 cm^{-1}, respectively. This is accomplished by using cholesterol bands near 600 cm^{-1} as a measure of its intensity. Control experiments consisted of egg lecithin–cholesterol mixtures dissolved in chloroform. Vesicles do not form and the hydrocarbon chains are seen from Fig. 5 to be in a liquid conformation.

DISCUSSION AND CONCLUSIONS

The state of phospholipid hydrocarbon chains upon cholesterol addition has been described by Chapman[14] as the "intermediate fluid condition". Rothman and Engelman[15], in their recently proposed model of phospholipid–cholesterol interaction, have attempted to define this condition in molecular terms. They state that "the intermediate fluid condition of the hydrocarbon chains probably corresponds to an ordered upper half" (*i.e.* the half nearest the headgroups) and "a more disordered lower half". The cholesterol is proposed to insert itself nearly. parallel to the chains so that its ring region is tightly packed with the upper half of the chain. The tail region of cholesterol interacts with the lower region of the hydrocarbon which can then assume *gauche* conformations, as it is not tightly packed.

The Raman spectroscopic evidence as outlined previously supports the above mechanism The bands due to the hydrocarbon chains show that the contribution of gauche isomers to the spectra is significantly reduced upon cholesterol addition and that the relative contribution of *trans* isomers is increased. The chains are not, however, in crystalline form as occurs in dipalmitoylethanolamine (Fig. 3A) at room temperature The effect of cholesterol addition, as predicted by Rothman and Engelman[15], is to prohibit the formation of some, but not all, *gauche* isomers and hence cause a decrease in chain fluidity. Presumably, those *gauche* isomers which are sterically forbidden are those which involve bends in the tightly packed upper half of the chain At 70 °C, the chains gain more rotational mobility and few *gauche* isomers are restricted

Lippert and Peticolas[1] observed effects similar to those just described for dipalmitoyllecithin above its transition point of 40 °C. Below this temperature, however, cholesterol causes a marked increase in chain fluidity. These observations are once again in complete accord with the Rothman–Engelman description[15]. The insertion of cholesterol into a "crystalline" phospholipid results in a loosening up of the close-packed structure. The formation of *gauche* isomers in the lower half of the hydrocarbon chains becomes a sterically permitted process. In the current study of egg lecithin, all spectra were obtained above the transition point and no situation occurred in which cholesterol increased chain fluidity

The utility of the Raman effect for direct measurement of lipid vibrational motions in aqueous media has been illustrated in the current work. It is possible to obtain Raman spectra of aqueous solutions only because interference from the

Raman-active vibrations of water is slight. This situation is in complete contrast to infrared spectroscopy, as water is highly opaque throughout most of the region of interest. Only limited regions of the vibrational spectrum are accessible *via* infrared absorption studies, which then must be carried out in ^2H_2O solution.

Although the suitability of biological materials for Raman spectroscopic studies decreases as sample complexity increases, several further studies appear feasible. These include the role of protein in shaping membrane structure and the effect of small molecules (*e.g.* anaesthetics) on model membrane systems.

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