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LYSOLECITHIN–CASEIN INTERACTIONS

I. NUCLEAR MAGNETIC RESONANCE AND SPIN LABEL STUDIES

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SUMMARY

The interactions between lysolecithin and acid-, α_{s1}-, β-, and κ-caseins have been studied by high resolution NMR spectroscopy and by the ESR spin-labelling technique. The NMR measurements (at 60 and 220 MHz) indicate that the alkyl chains of the lysolecithin interact with the protein whilst the motion of the choline groups remains unaffected.

When increasing amounts of lysolecithin are added to caseins, the aromatic amino acid resonances (220 MHz spectrum) become sharper, suggesting that the protein structure either deaggregates or becomes looser.

Addition of caseins to lysolecithin micelles containing spin probes in their alkyl chain region results in a broadening of the ESR spectra of the spin probes and an increase in their correlation time. This suggests a reduction in motion in the chain region of the lysolecithin molecules and supports the NMR data. The ESR data also indicate the stoichiometry of the interaction between lysolecithin and caseins.

INTRODUCTION

With the exception of the serum lipoproteins, very few investigations have been undertaken on water-soluble lipoproteins. Because of the difficulties inherent in the study of complex natural lipoprotein systems, a series of studies on model lipoprotein complexes has been undertaken in this and other laboratories[1–4].

The interaction between phosphatidylserine and bovine serum albumin has been studied turbidimetrically[5] and the binding of fatty acid salts to the same protein has been studied by electrophoresis[6]. More recently the binding of lysolecithin to serum albumin has been followed by high resolution NMR spectroscopy[7]. The interactions of lysolecithin with human red cell apoprotein, serum albumin and acid casein have been compared using a combination of NMR and spin labelling[8]. NMR has also been used to study the aggregation properties of and the molecular motions present in the three main fractions of acid casein[9].

In this paper we describe the results of an investigation into the interactions between lysolecithin and the caseins using high resolution NMR and the ESR spin-labelling technique described by STONE *et al.*[10].

MATERIALS AND METHODS

Crystalline lysolecithin (*ex* egg) was purchased from Messrs. Koch-Light Ltd. Sodium dodecyl sulphate was purchased from BDH Ltd. The acid casein and α_{s1}-casein were prepared (from Ayrshire skimmilk) by previously reported methods[11]. The β- and κ-caseins (Ayrshire) were gifts from Dr. L. Irons (of this laboratory). [$^2H_4$]urea was obtained from Beta Scientific, Ltd.

NMR spectroscopy

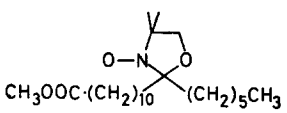
High resolution NMR spectra were recorded on a Perkin–Elmer R12 60-MHz spectrometer (at 34°) and on a Varian HR-220, 220-MHz spectrometer (room temperature). All the spectra were single-scan recordings. Chemical shifts are expressed in ppm relative to the sodium salt of 3-(trimethylsilyl)-1-propane sulphonic acid (TSS) at 10 ppm (τ scale).

All samples were run either in ^2H_2O or buffered ^2H_2O. For the 220-MHz spectrometer the samples were first lyophilised from ^2H_2O to remove as much residual H_2O as possible and then made up in ^2H_2O phosphate buffer (0.08 M NaCl, 5.65 mM Na_2HPO_4, 3.05 mM NaH_2PO_4, indicating p^{2}H 6.7). Spectral assignments are based on previous studies[9,12].

ESR spectroscopy

ESR spectra were obtained at 9.5 GHz on a Varian V-4502 X-band spectrometer.

The spin probe, the *N*-oxyl-4',4'-dimethyloxazolidine of methyl 12-ketostearate (I) was prepared by published methods [13,14].



(I)

Spin probe samples were prepared by dissolving the probe in 1 % lysolecithin solution and adding the appropriate quantity of protein. The final concentration of the nitroxide probe in all ESR experiments was 10^{-4} M.

The reorientational correlation times (τ_c) of the nitroxide probes were calculated by the method of KIVELSON[15]. Isotropic motion of the probe and Lorentzian lineshape are assumed for these calculations.

The anisotropic g-tensors and hyperfine couplings of the nitroxide probe were measured in glycerol at −120°. The Hamiltonian parameters calculated from these quantities were $b = 3.3 \cdot 10^8$ sec^{-1} and $\Delta\gamma = 4.6 \cdot 10^4$ sec^{-1}·Gauss^{-1} (see EDELSTEIN *et al.*[16]).

Hyperfine couplings were measured between the low-field and the centre lines of the spectra to within ± 0.05 Gauss, against standards of the 2,4-dinitrophenylhydrazone of 2, 2, 6, 6,-tetramethyl-4-piperidone nitroxide in water and dodecane (16.16 Gauss and 14.30 Gauss, respectively)[17].

RESULTS AND DISCUSSION

Nuclear magnetic resonance spectroscopy

The 60-MHz NMR spectra of lysolecithin (1 % in ^2H_2O) with increasing concentrations of κ-casein are shown in Fig. 1. Similar series of spectra showing the same general features were obtained with whole acid casein, α_{s1}-casein and β-casein. It can be seen from Fig. 1, that when the protein is added, the alkyl-chain signal (8.7 τ) of the lysolecithin broadens whilst the choline signal (6.7 τ) remains unaffected. Fig 2 shows a plot of the line-width at half-height ($\Delta\gamma$ ½) of the alkyl chain signal of lysolecithin with the different casein fractions. The linewidth of the choline signal remains unaffected.

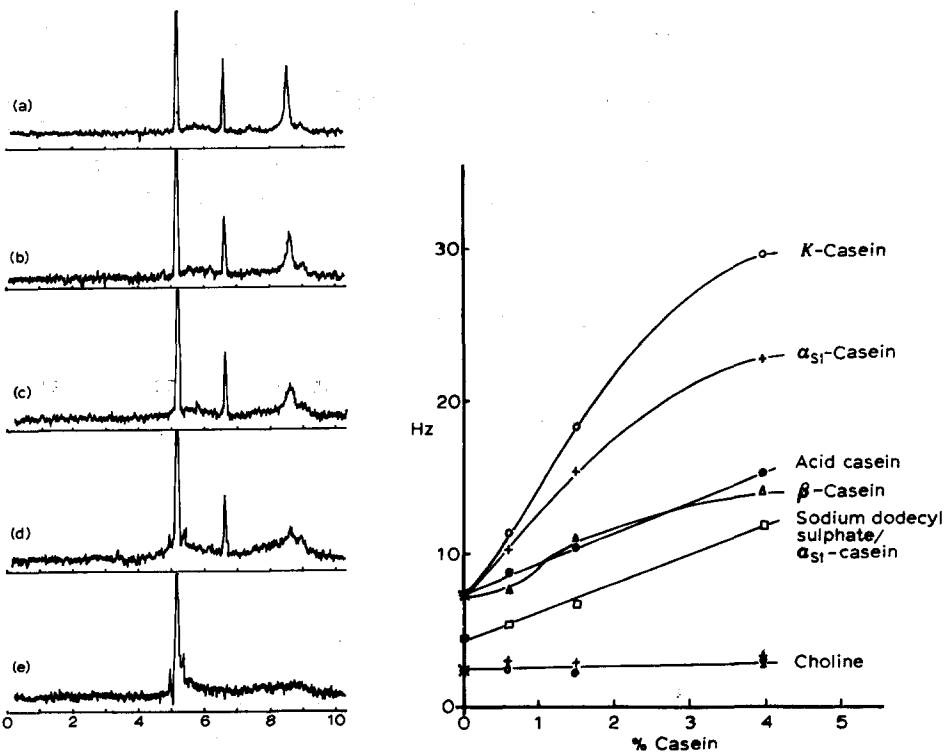


Fig. 1. 60 MHz NMR spectrum of 1 % lysolecithin in ^2H_2O with (a) 0 %, (b) 0.6 %, (c) 1.5 %, (d) 4.0 % κ-casein; (e) 4.0 % κ-casein alone.

Fig. 2. Graph of linewidths ($\Delta\ \gamma\ ½$) of alkyl chain and choline resonances of lysolecithin (and sodium dodecyl sulphate) *vs.* % casein concentration.

The broadening of the alkyl-chain signal of lysolecithin arising from an increase in correlation times for motion of the $(CH_2)_n$ protons and similar to that observed when lysolecithin is added to bovine serum albumin[7] or erythrocyte membrane[18], strongly suggests that interactions (presumably of a hydrophobic nature) are taking place between the alkyl chains of the lysolecithin and the apolar amino acids of the casein molecules. The fact that the choline signal remains unaffected suggests that this group can rotate freely and is not involved in interaction with the casein.

A similar broadening of the alkyl chain proton resonance is observed when α_{s1}-casein is added to sodium dodecyl sulphate (see Fig. 2). In this case however, there is no resonance from the polar region of the molecule to use as a "control".

It is clear that the alkyl chains of lysolecithin are bound either much less strongly or to a smaller extent by β- and acid casein than by α_{s1}- and κ-casein. The explanation for this may be connected with the fact that α_{s1}- and κ-casein have the ability to form a mixed α_{s1}–κ-micelle (or aggregate) stabilised by hydrophobic bonding[19]. (The hydrophobic nature of this stabilisation is confirmed by the inability of a variant (α_{s1}-casein-A), which has a sequence of seven apolar amino acids missing, to form the mixed α_{s1}–κ-micelle[20].) It is also possible for the α_{s1}- and κ-caseins to form mixed micelles or aggregates with lysolecithin micelles. As β-casein is reported to exist as a very loose association of about thirty molecules[21] with the individual amino acid residues possessing a large amount of independent motion[9], alkyl chains of lipids might not be expected to bind very strongly. Acid casein is a complex mixture of α_{s1}-, β- and κ-caseins stabilised by hydrophobic bonding[22]. The relatively weak binding of lysolecithin to acid casein could arise from the fact that the "strong" binding sites of the α_{s1}- and κ-casein in the acid casein are shielded by protein–protein interaction and are not available to the lysolecithin.

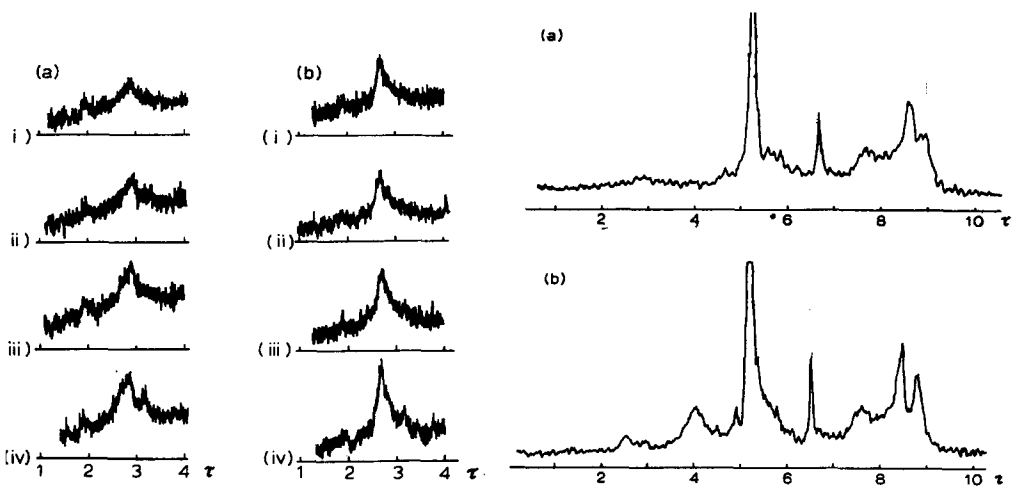


Fig. 3. Aromatic amino acid regions (220 MHz) of (a) α_{s1}- and (b) β-casein (5 %) with (i) 0, (ii) 0.5, (iii) 1.0 and (iv) 2.0 % lysolecithin in buffered ^2H_2O.

Fig. 4. (a) NMR spectrum (60 MHz) of 1 % lysolecithin and 4.0 % α_{s1}-casein in ^2H_2O and (b) in the presence of 8 M [$^2H_4$]urea.

Fig. 3 shows the aromatic regions (1–4 τ) of 220 MHz spectra of (a) 5 % α_{s1}-casein and (b) 5 % β-casein with increasing amounts of lysolecithin. The assignments of the aromatic amino acid peaks are as follows; histidine 1.9 τ; phenylalanine 2.8 τ; histidine and tyrosine *meta* to OH, 3.1 τ. The effect of adding increasing quantities of lysolecithin to α_{s1}-, β-(and also κ-)casein is to sharpen and increase in intensity the aromatic amino acid resonances from the protein. This enhancement of resolution in the aromatic regions is reminiscent of that produced by the addition of urea to the protein solutions[9]. It strongly suggests that deaggregation of the protein, or at least a

loosening of the structure of the aggregate, takes place when lysolecithin binds to the protein.

The effect of adding 8 M [$^2H_4$]urea to a mixture of α_{s1}-casein (4 %) and lysolecithin (1 %) in ^2H_2O is shown in the 60-MHz spectra in Fig. 4. The resonances of both the lysolecithin and the protein are sharpened by the addition of urea. As urea is known to disrupt water structure and hydrophobic bonds, its effect in this case is probably to break down both the protein and the protein–lysolecithin aggregates into their constituents. The sharpening of the aromatic amino acid resonances in the 60-MHz spectrum (an effect only seen at 220 MHz with lysolecithin) suggests that a greater deaggregation of the protein occurs with urea than is found with lysolecithin.

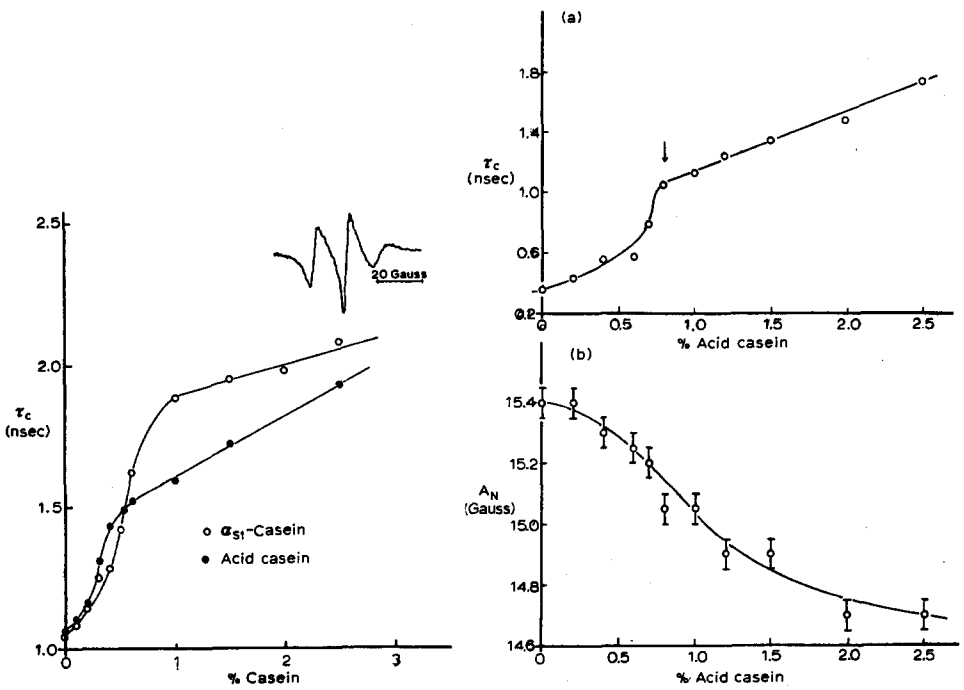


Fig. 5. ESR spectrum of spin probe (I) in 0.5 % lysolecithin; and 2.5 % acid casein in water (inset). Graph of τ_c for spin probe in 0.5 % lysolecithin *vs.* acid casein concentration. Graph of τ_c of spin probe in 0.5 % lysolecithin *vs.* α_{s1}-casein concentration.

Fig. 6. (a) Graph of τ_c of spin probe in 0.5 % sodium dodecyl sulphate *vs.* acid casein concentration. (b) Graph of hyperfine coupling of spin probe in sodium dodecyl sulphate *vs.* acid casein concentration.

Electron spin resonance spectroscopy

The effect on the reorientational correlation time (τ_c) of probe (I) in 0.5 % lysolecithin solution on the addition of acid casein is shown in Fig. 5. The hyperfine coupling of the probe in 0.5 % lysolecithin solution was 14.6 Gauss; this value was maintained on the addition of protein. The hyperfine coupling of the spin probe in lysolecithin micelles[23] as well as NMR paramagnetic broadening experiments suggest strongly that the spin probe is located in the alkyl chain region of the lysolecithin micelles. The increase in τ_c of the probe on the addition of acid or α_{s1}-casein to the lyso-

lecithin solution may then be attributed to a decrease in the motion of the probe in the alkyl chain region of the lysolecithin.

The lack of any strong immobilisation of the probe due to binding to the protein (as is observed when the probe is added to erythrocyte membrane apoprotein[8]) suggests that the micellar character of the lysolecithin is at least partially maintained. This view is supported by the observation that there is no change in the hyperfine coupling of the probe when casein is added to lysolecithin. This evidence is however, inconclusive as the caseins are loosely structured proteins[9] and it is also conceivable that the apolar regions of the protein might be very similar in polarity characteristics to the interior of the lysolecithin micelle.

The graph of τ_c of the spin probe in 0.5 % sodium dodecyl sulphate against acid casein concentration is shown in Fig. 6a. The motion of the spin probe in sodium dodecyl sulphate is somewhat more rapid than in lysolecithin. Addition of acid casein to sodium dodecyl sulphate reduces the motion to about the same level as in lysolecithin and casein, suggesting that immobilisation due to hydrophobic bonding occurs to similar extents in both systems.

The location of the spin probe is of some interest in the sodium dodecyl sulphate–acid casein system. The variation of the hyperfine coupling of the spin probe in sodium dodecyl sulphate with casein concentration is shown in Fig. 6b. We have already postulated[23] that in the sodium dodecyl sulphate micelles the spin probe folds about carbon -12 and lies with its nitroxide group protruding into the water, thus giving rise to the anomolously high hyperfine coupling of 15.4 Gauss. Addition of acid casein to the sodium dodecyl sulphate causes a reduction in the coupling of the probe indicating that the nitroxide group moves to a less polar environment. An expansion of the structure of the sodium dodecyl sulphate micelle would be necessary to achieve this change in polarity.

Closer examination of Fig. 6a reveals that there is a fairly sharp increase in τ_c on the initial addition of acid casein to sodium dodecyl sulphate followed by a more gradual approximately linear increase thereafter. It has been demonstrated by analytical ultracentrifugation[24] that when sodium dodecyl sulphate was added to acid casein, the sedimentation coefficient decreased with increasing protein concentration until a molar ratio of sodium dodecyl sulphate to casein of about 40 to 1 was reached. (Closer examination of this data suggests that this ratio is in fact nearer 50 to 1). At higher ratios no further decrease in sedimentation coefficient was observed. It was suggested that above this ratio any additional sodium dodecyl sulphate in the system existed as separate micelles of sodium dodecyl sulphate. The linear region of the plot in Fig. 6a, begins at the sodium dodecyl sulphate/casein ratio of 54 to 1 based on an average molecular weight of 25000 per protein unit. In the non-linear region we may assume that the observed τ_c arise from probe molecules in two separate environments, *i.e.* in sodium dodecyl sulphate micelles and in sodium dodecyl sulphate/ casein aggregates of molar ratio of approximately 54 to 1.

Comparing the plots in Fig. 5 we can derive similar information for the lysolecithin–casein systems. Using the same average molecular weight value for acid casein (25000 per unit), and a molecular weight of 500 for lysolecithin, the ratio derived from Fig. 5 for lysolecithin and acid casein is about 40 to 1. Similarly using a molecular weight value of 28000 per unit for α_{s1}-casein, the ratio derived for lysolecithin and α_{s1}-casein is about 28 to 1.

The broadening in the NMR signal of the alkyl chain protons of lysolecithin when casein is added is strongly indicative of a hydrophobic interaction between the lysolecithin alkyl chains and the apolar amino acids of the casein. This interpretation is supported by a corresponding decrease in motion of the spin probe in the chain region of lysolecithin. The relative freedom of the choline groups indicates that the polar head groups of the lysolecithin molecules are not involved in interaction with the casein.

The 220-MHz data is consistent with deaggregation or loosening of the structure of the protein when lysolecithin is added. This effect is however, small compared with that of 8 M urea.

The spin probe data predict the stoichiometry of the interactions between lysolecithin and acid and α_{s1}-casein. Further work on the stoichiometry and the sizes of the aggregates formed between lysolecithin and α_{s1}-casein is the subject of the following paper[25].

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