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THE INTERACTION OF SPECTRIN-ACTIN AND SYNTHETIC PHOSPHOLIPIDS

II. THE INTERACTION WITH PHOSPHATIDYLSERINE

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Summary

Sonicated vesicles of phosphatidylserine and phosphatidylserine/phosphatidylcholine mixtures were recombined with spectrin-actin from human erythrocyte ghosts. Morphological properties and physicochemical characteristics of the recombinates were studied with freeze etch electron microscopy, ^{31}P NMR and differential scanning calorimetry.

Sonicated dimyristoyl phosphatidylserine vesicles show a decrease in enthalpy change of the lipid phase transition upon addition of spectrin-actin. These vesicles collapse and fuse, into multilamellar structures in the presence of spectrin-actin, as demonstrated by freeze fracturing and NMR.

Spectrin-actin cannot prevent the salt formation between phosphatidylserine and Ca^{2+}; all phosphatidylserine is withdrawn from the lipid phase transition. In contrast a protection against the action of Mg^{2+} could be observed.

Mixed bilayers of dimyristoyl phosphatidylserine/dimyristoyl phosphatidylcholine show phase separations at molar ratios above 1/1 (van Dijck, P.W.M., de Kruijff, B., Verkleij, A.J., van Deenen, L.L.M. and de Gier, J. (1978) Biochim. Biophys. Acta 512, 84—96). These phase separations can be prevented by spectrin-actin.

Ca^{2+}-induced lateral phase separations in cocrystallizing phosphatidylserine/phosphatidylcholine mixtures, can be reduced by spectrin-actin. Formation of the Ca^{2+}-phosphatidylserine salt, occurring in addition to lateral phase separation when mixtures contain more than 30 mol % phosphatidylserine, cannot be prevented by spectrin-actin.

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid.

Introduction

Several lines of evidence indicate that there are distinct interactions between spectrin-actin and natural phospholipids [1—3]. Recently hydrophobic interactions were observed between synthetic phospholipids and the spectrin-actin complex [4]. These interactions had significant effects on the thermotropic and morphological properties of phospholipid bilayers.

It is known that in the erythrocyte membrane the spectrin-actin complex is located exclusively at the cytoplasmic side, adjacent to a lipid monolayer containing specific classes of phospholipids. This inner monolayer contains all the phosphatidylserine of the erythrocyte membrane and therefore it is of importance to investigate the interaction of spectrin-actin with phosphatidylserine.

In this paper we present results from recombination studies of spectrin-actin with phosphatidylserine and phosphatidylserine/phosphatidylcholine mixtures using the techniques of differential scanning calorimetry, phosphorus nuclear magnetic resonance and freeze fracture electron microscopy.

Materials and Methods

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine was synthesized following standard procedures [5]. 1,2-Dimyristoyl-*sn*-glycero-3-phosphoserine was synthesized from 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine by enzymatic breakdown with phospholipase D in the presence of L-serine and purified on CM-cellulose as described by Comfurius and Zwaal [6]. Spectrin-actin, from here referred to as 'spectrin' was extracted from erythrocyte ghosts according to Marchesi [8] and used without further purification. If however purification was required 'spectrin' was purified on Sepharose 4B in 25 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.6, according to Ungewickell and Gratzer [7]. Recombination was carried out in an incubation buffer containing 3 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4, as described before [4]. Samples were incubated at 43.5°C, this being the optimal temperature between the transition temperature of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (38°C) and the denaturation temperature of 'spectrin' (45°C). When necessary $CaCl_2$ or $MgCl_2$ was added, after 60 min incubation, as a 100 mM solution in incubation buffer to give a final divalent cation/lipid molar ratio of 2.5. For differential scanning calorimetry (Perkin-Elmer DSC-2) the recombinate was concentrated by ultracentrifugation (50 min 150 000 × *g*, 4°C). After thermal analysis samples were dissolved in 2% (w/v) sodium dodecyl sulphate, 10 mM β-mercaptoethanol and boiled for 5 min. Phosphorus was determined as described by Bartlett [9]. Protein was determined according to Lowry et al. as modified by Higuchi and Yoshida [10]. Freeze fracturing was carried out as described [11] after addition of 25% (v/v) glycerol. Spray-freezing was performed with a Balzers apparatus as described by Bachmann and Schmitt [21]. ^{31}P NMR was performed with a Bruker WH 90 spectrometer operating at 36.4 MHz as described by de Kruijff et al. [12]. Accumulated free induction decays were obtained at 30°C or 43°C, under conditions of 3 W broad band proton decoupling, from 500 transients with a 1.7 s interpulse time using 90° pulses.

Typically, sonicated lipid suspensions in 25% ^2H_2O were studied at a lipid concentration of 20 mg/ml.

Results

Phosphatidylserine

Fig. 1 shows the thermotropic behavior of dimyristoyl phosphatidylserine with 'spectrin'. 'Spectrin', when added to a suspension of sonicated lipid vesicles, induces a decrease in the lipid enthalpy change, which is proportional the the protein/lipid ratio. For instance at a protein/lipid ratio (w/w) of 1.2 the enthalpy change decreased from 8.35 kcal/mol for pure dimyristoyl phosphatidylserine to 4.8 kcal/mol for the recombinate. The transition temperature was decreased a little, depending on the protein/lipid ratio. At a ratio (w/w) of 2.5 the top of the transition peak shifted about five degrees.

In contrast to recombinates with phosphatidylcholine, which remain as vesicles, recombinates of dimyristoyl phosphatidylserine do not appear as vesicles when viewed in freeze fracture electron micrographs. The original vesicles (Fig. 2) seem to have broken up to form small flat sheets, probably coated with 'spectrin' at both sides (Fig. 3A). This is supported by ^{31}P NMR measurements which show a rapidly decreasing intensity of the narrow resonance peak following the addition of 'spectrin'. The linewidth, however, remains constant indicating that small 200—300 Å vesicles are still present [12] and that only part of the vesicles fuse to large bilayer structures. The NMR signal of these large bilayer structures, as visible by freeze fracturing, is too broad to be detected under the conditions used to record the spectrum of vesicles. The vesicle fusion after addition of 'spectrin', as detected by NMR, is much more extensive than that obtained if dimyristoyl phosphatidylcholine is used (Fig. 4). A similar result to that described above for phosphatidylserine was obtained for dimyristoyl phosphatidylglycerol vesicles (Fig. 4). Although in this case fusion of the sonicated vesicles results in large closed unilamellar

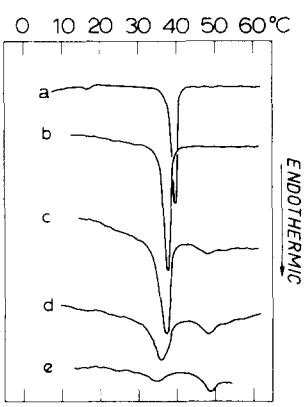

Fig. 1. Thermotropic behaviour of dimyristoyl phosphatidylserine incubated with increasing amounts of 'spectrin'. a, liposomal preparation without 'spectrin'; b—e sonicated vesicles incubated with 'spectrin'. 'Spectrin'/phospholipid ratio (w/w) of (b) 0.2, (c) 0.6, (d) 1.2 and (e) 2.5. The peak emerging at 45° C is the 'spectrin' denaturation peak.

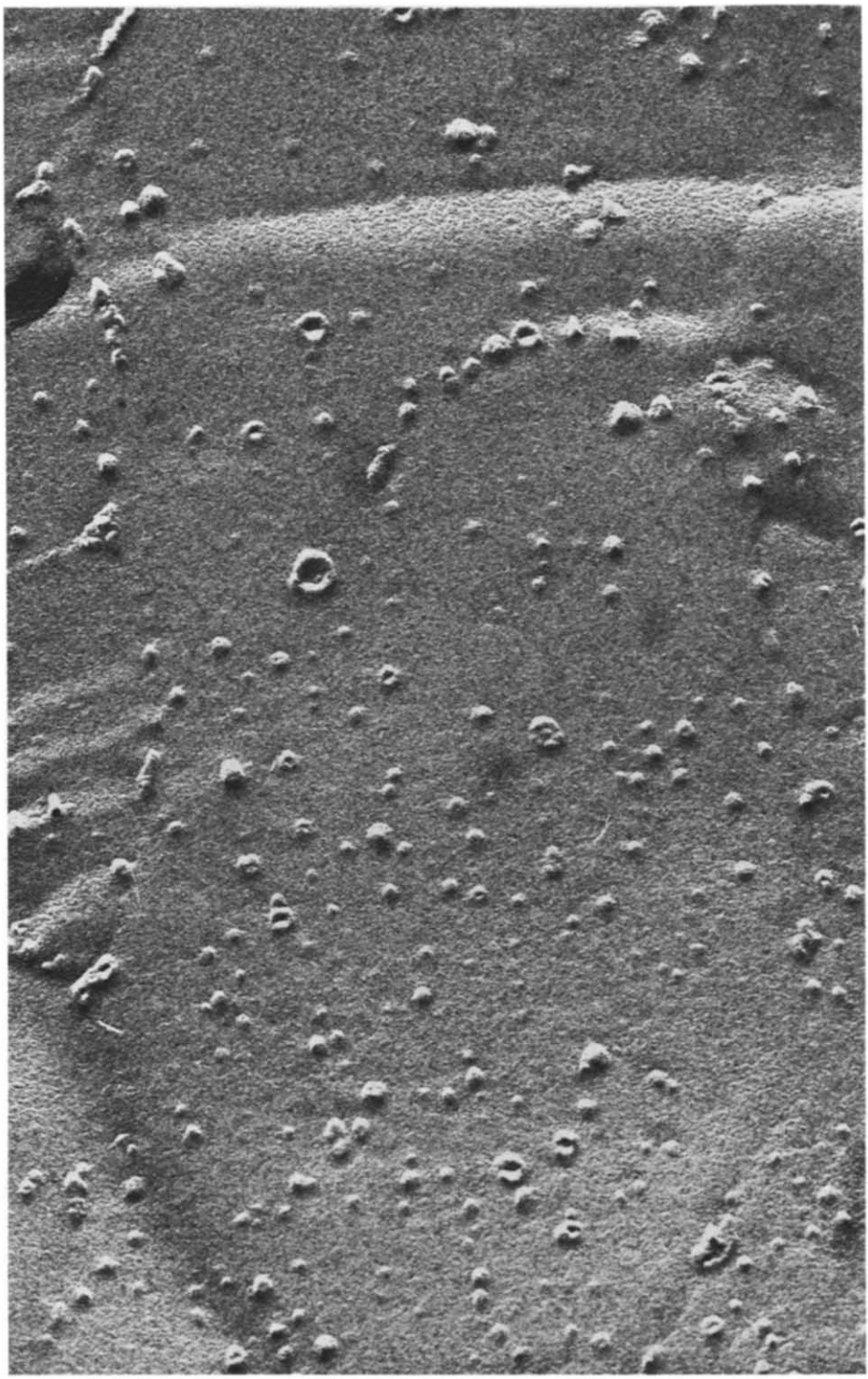

Fig. 2. Freeze fracture morphology of sonicated dimyristoyl phosphatidylserine vesicles quenched with the method of spray-freezing. Magnification about 100 000X.

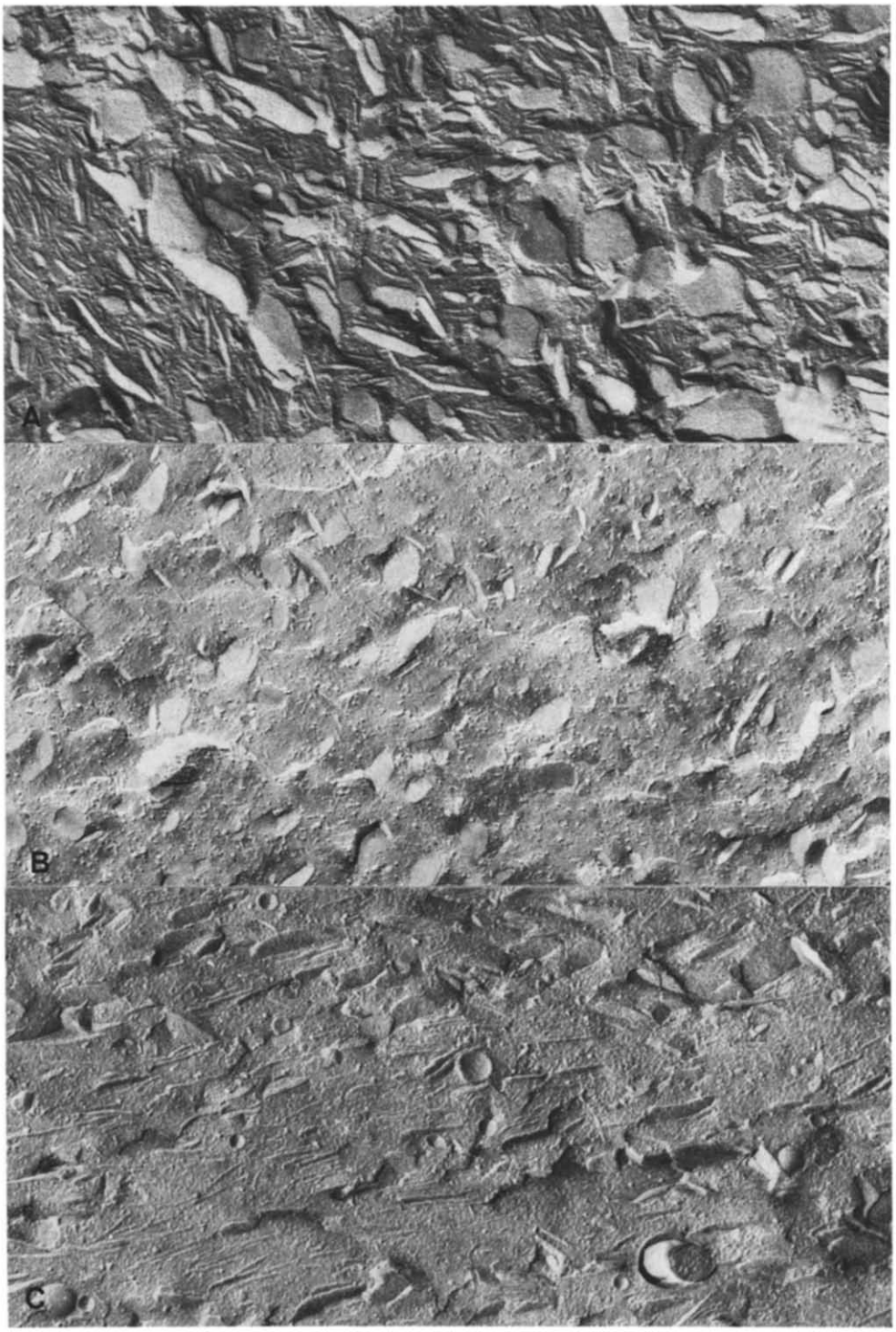

Fig. 3. Freeze fracture morphology of sonicated dimyristoyl phosphatidylserine vesicles incubated with 'spectrin' (A); incubated with 'spectrin' and subsequently with Mg^{2+} (B); incubated with 'spectrin' and subsequently with Ca^{2+} (C). Protein/lipid ratio (w/w): (A) 1.0, (B) 1.3, (C) 2.0. Magnificiation about 80 000X.

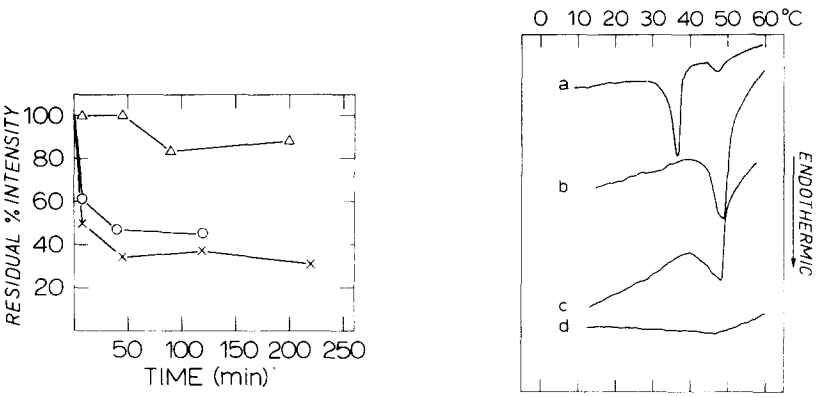

Fig. 4. Residual intensity (peak area) of the ^{31}P NMR signal of sonicated vesicles after different times of incubation with 'spectrin' at 30° C (phosphatidylcholine and phosphatidylglycerol) or 43° C (phosphatidylserine). △———△, dimyristoyl phosphatidylcholine; ○———○, dimyristoyl phosphatidylglycerol; X———X, dimyristoyl phosphatidylserine. 'Spectrin'/lipid ratio (w/w) 0.3 for phosphatidylcholine and phosphatidylserine; 0.5 for phosphatidylglycerol.

Fig. 5. a, thermotropic behaviour of dimyristoyl phosphatidylserine vesicles incubated with 'spectrin'. b and c, as a with subsequent addition of Mg^{2+}; b, first scan; c, second and following scans; d, as a with subsequent addition of Ca^{2+}. Protein/lipid ratio as in Fig. 3.

vesicles [4] instead of flat sheets, the changes in the NMR spectrum due to this fusion are the same as for phosphatidylserine. So, the effect of 'spectrin' on negatively charged phospholipids is distinct from the effect on a neutral phospholipid such as phosphatidylcholine.

Heat denaturation of 'spectrin' for 10 min at 60° C has the following effects: The amount of phospholipid that precipitates with the 'spectrin' during ultracentrifugation of the recombinate is considerably smaller than with non-denatured 'spectrin'. The enthalpy change of the phase transition of the lipid in the recombinate is decreased in similar way as with non-denaturated 'spectrin'. If denaturation is carried out after recombination of 'spectrin' with phosphatidylserine the enthalpy change of the lipid phase transition of the recombinate, like found for phosphatidylglycerol recombinates [4], has increased about 0.5 kcal/mol with respect to the non-denaturated recombinate.

Purification of 'spectrin' according to Ungewickell and Gratzer [7] does not change the enthalpy change of the phase transition of the phosphatidylserine recombinate significantly with respect to a recombinate containing the crude, low ionic strength extract.

Phosphatidylserine/phosphatidylcholine mixtures

Mixtures of dimyristoyl phosphatidylserine and dimyristoyl phosphatidylcholine do not show cocrystallization at all mixing ratios. Above a 1 : 1 molar ratio of phosphatidylserine/phosphatidylcholine, phase separation occurs [13]. Upon addition of 'spectrin' to lipid mixtures, above lipid molar ratios of 1 : 1, the biphasic behaviour is absent and only one single thermotropic peak is seen with an intermediate transition temperature.

Divalent cations

Negatively charged phospholipids, such as phosphatidylserine and phos-

phatidylglycerol, form insoluble salts with Ca^{2+} and Mg^{2+}. When these divalent ions are added, in a sufficient concentration, to liposomes of phosphatidylserine, the liposomes collapse and so called cochleated or cylindrical structures are formed [13,14,19]. These structures have a thermotropic phase transition at much higher temperatures and with a larger ΔH than divalent ion-free lipid. Sonicated vesicles of phosphatidylserine yield similar results upon addition of Ca^{2+} or Mg^{2+}, although the conversion to the Mg^{2+} salt is completed only after repetitive scanning through the phase transition temperature.

If Ca^{2+} is added to dimyristoyl phosphatidylserine after recombination with 'spectrin', the phase transition at 38°C disappears, as in the absence of 'spectrin' (Figs. 5a and d). Apparently, 'spectrin' cannot prevent the formation of the Ca^{2+}-phospholipid salt complex as it can with phosphatidylglycerol [4]. In contrast, if Mg^{2+} is added to the recombinate, the phase transition at about 45°C does not disappear, even after repetitive scanning. After one scan through the phase transition, the shape and area of the thermotropic peak remain constant (Figs. 5b and 5c). This means that because of the presence of 'spectrin' the major part of the lipid cannot be converted to the Mg^{2+}-lipid salt. The presence of Mg^{2+} increases the transition temperature of the recombinate by about ten degrees which was found also for the 'spectrin'-Mg^{2+}-phosphatidylglycerol complex [4]. After heating above 45°C the cooperativity of the thermotropic transition decreases, but the enthalpy change remains the same (Fig. 5c). If some Mg^{2+}-lipid salt is formed in the calorimetric sample, as indicated by the decrease in ΔH, this does not take part in the phase transition at about 45°C. As a consequence of this the calculated enthalpy change of the calorimetric sample, on the basis of its total phosphorus content, may be smaller than the actual ΔH of the 'spectrin'-lipid complex (Fig. 6).

Freeze fracturing of 'spectrin'-Mg^{2+}-phosphatidylserine recombinates shows

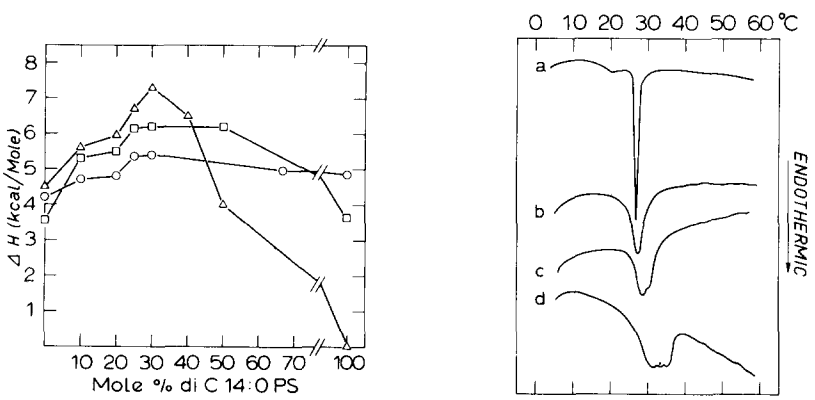

Fig. 6. Enthalpy changes of sonicated mixtures of dimyristoyl phosphatidylcholine (di C14:0 PC) and dimyristoyl phosphatidylserine (di C14:0 PS) incubated with 'spectrin' (○———○), dimyristoyl phosphatidylcholine/dimyristoyl phosphatidylserine with 'spectrin' and Mg^{2+} (□———□), dimyristoyl phosphatidylcholine/dimyristoyl phosphatidylserine with 'spectrin' and Ca^{2+} (△———△). At all incubations the protein/lipid (w/w) ratio was about 1.3.

Fig. 7. Thermograms of a dimyristoyl phosphatidylcholine/dimyristoyl phosphatidylserine mixture (molar ratio, 4 : 1). a, liposomal preparation; b, sonicated vesicles preparation incubated with 'spectrin'; c, sonicated vesicles preparation incubated with 'spectrin' and Mg^{2+} or d, incubated with 'spectrin' and Ca^{2+}. 'Spectrin'/lipid (w/w) ratio, 1.1.

flat sheets, comparable with those of the 'spectrin'-phosphatidylserine recombinate (Fig. 3B). 'Spectrin'-Ca^{2+}-phosphatidylserine recombinates, though having no phase transition, show similar structures (Fig. 3C), which are clearly different from the cochleated structures described for pure Ca^{2+}-phosphatidylserine complexes [14].

Dimyristoyl phosphatidylserine and dimyristoyl phosphatidylcholine are not mixable at all molar ratios [15]. Mixed bilayers containing more than 50 mol % phosphatidylserine show lateral phase separation, i.e. both lipids segregate in the plane of the bilayer. This phase separation is indicated by a double thermotropic peak in calorimetric scans [15]. Mixtures containing less than 50 mol % phosphatidylserine show only one thermotropic peak. However, addition of Ca^{2+} to these latter one-phasic systems induces phase separation [15]. This Ca^{2+}-induced phase separation consists of two processes. First a gradual lateral phase separation occurs resulting in a doubling of the thermotropic peak, and, when the phosphatidylserine content is high enough (more than about 30 mol %) structural phase separation also occurs, i.e. bilayers break up to form the phosphatidylserine-Ca^{2+} salt [15].

The formation of the Ca^{2+} salt and the structural phase separation which follows, result in a decrease of the enthalpy change of the measured phase transition. This is because the method used to determine lipid-phosphorus in the calorimetric sample cannot discriminate between the Ca^{2+} salt and the uncomplexed lipid [15].

As mentioned above, addition of 'spectrin' to dimyristoyl phosphatidylserine/dimyristoyl phosphatidylcholine (1 : 4) mixtures yields a one phasic system. Subsequent addition of Ca^{2+} results in a decrease of the cooperativity of the phase transition (Fig. 7), but this decrease is less than in the absence of 'spectrin' [15]. The recombinate system appears to remain almost one-phasic in the presence of Ca^{2+} as the thermotropic phase transition peaks are less well separated and phase separation cannot be shown by freeze fracture techniques, i.e. the band pattern next to smooth fracture faces, typical for segregated phosphatidylcholine, is not visible in the presence of 'spectrin' (Fig. 8).

Fig. 6 shows that for recombinates with increasing phosphatidylserine concentrations in the presence of Ca^{2+} the enthalpy change of the measured phase transition rapidly decreases above a concentration of 30 mol %. This can be explained by the formation of a Ca^{2+}-lipid salt with a very high transition temperature. However, like in Fig. 8C, the freeze fracture morphology of this salt is different from the typical cochleated structures that would be formed in the absence of 'spectrin'. Probably there is also an interaction of 'spectrin' with phosphatidylserine if the lipid is in the Ca^{2+} salt form. Also with Mg^{2+} phase separations are induced in phosphatidylserine/phosphatidylcholine mixtures, but they are less extensive than with Ca^{2+} (Figs. 7 and 8). Mixtures containing less than 50 mol % phosphatidylserine, show a single phase transition when Mg^{2+} is added in the presence of 'spectrin'. So, at similar concentrations the effect of Mg^{2+} on phosphatidylserine is quite distinct from the effect of Ca^{2+}. Here also the ΔH of the measured transition increases, at first, in comparison with the reference lipid, but at higher phosphatidylserine/phosphatidylcholine ratios it decreases again maybe as a result of the formation of a phosphatidylserine-Mg^{2+} salt by part of the lipid (Fig. 6).

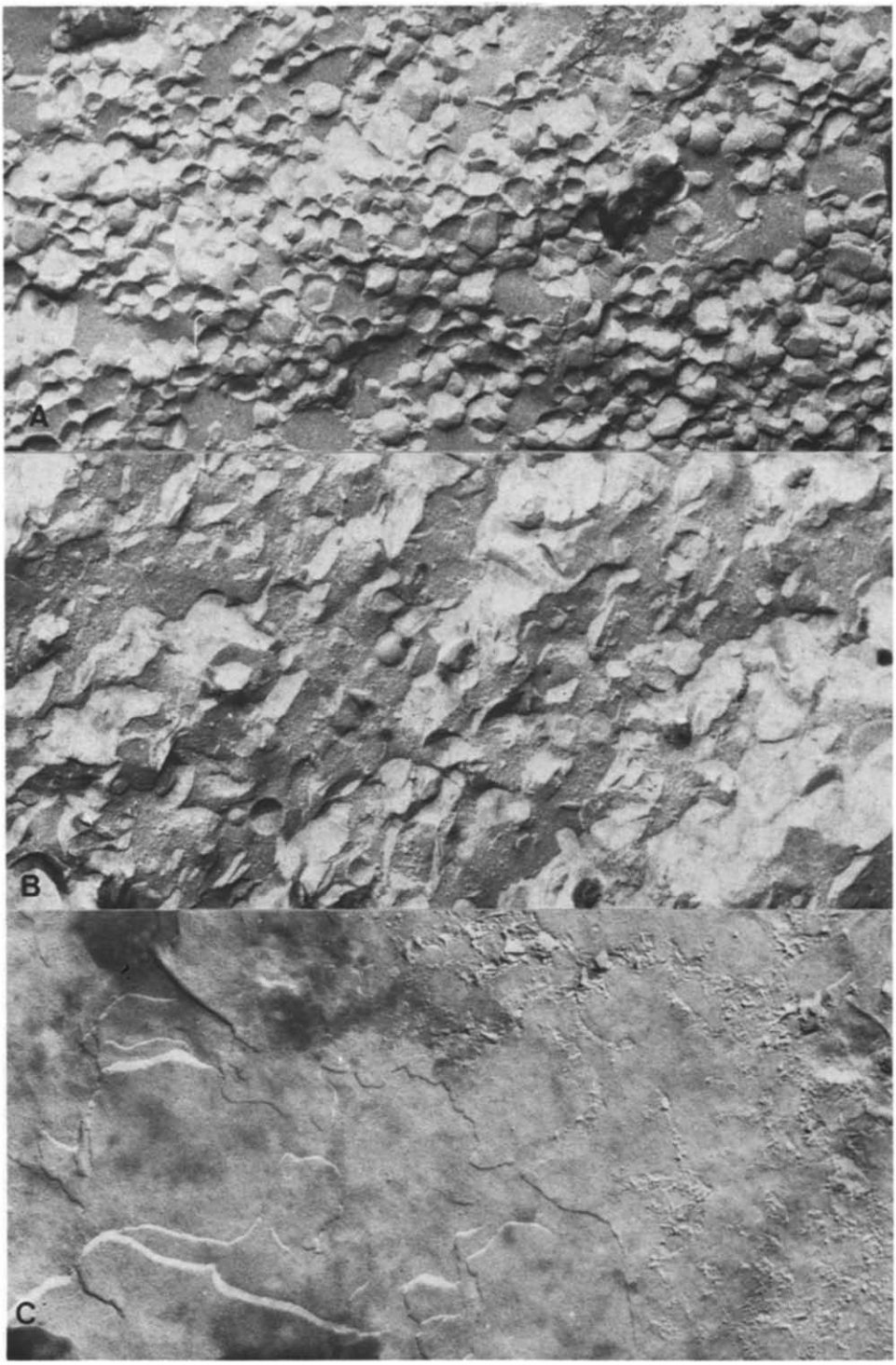

Fig. 8. Freeze fracturing of a sonicated mixture of dimyristoyl phosphatidylcholine/dimyristoyl phosphatidylserine (molar ratio, 4 : 1) incubated with 'spectrin' (A); with 'spectrin' and Mg^{2+} (B); with 'spectrin' and Ca^{2+} (C). Magnification 57 000×.

Discussion

Recombination of 'spectrin' with phospholipids at sufficient protein/lipid ratios can most easily be achieved by adding 'spectrin' to sonicated lipid suspensions. Handshaken systems are not suitable as only a little amount of protein can be trapped between the bilayers. An additional advantage of using sonicated vesicles in recombination studies is that fusion processes and changes in size can be readily followed using ^{31}P NMR.

Disadvantages, however, of sonicated lipid systems are encountered in comparing enthalpy changes of recombinates to the lipid reference. Enthalpy changes and heat absorption profiles are different for handshaken liposomal and sonicated lipid systems because of the strong curvature of sonicated lipid vesicles [16,17]. As described above fusion processes can occur when sonicated vesicles are incubated with 'spectrin'. This presents the problem of choosing a proper lipid reference enthalpy change. We have taken the handshaken liposomal system as a lipid reference, as sonicated vesicles of negatively charged phospholipid fuse or collapse upon addition of 'spectrin' and loose their curvature (see above and ref. 4). At low protein/lipid ratios the heat absorbance curves and enthalpy changes of the recombinates closely resemble those of handshaken liposomes in the absence of 'spectrin'.

The decrease in enthalpy change of phosphatidylserine recombinates when the protein concentration is enhanced is in the same order of magnitude as that for phosphatidylglycerol recombinates [4]. A comparable decrease of the transition temperature is also found. It is clear from the data obtained from both calorimetric and NMR techniques, which show the difference between phosphatidylserine and phosphatidylcholine, that negatively charged lipid polar headgroups are important for the hydrophobic interaction of 'spectrin' with phospholipid. On the basis of the enhanced interaction with negatively charged lipid and the observed changes in thermotropic properties of recombined lipids, it seems likely that both electrostatic as well as hydrophobic interactions are involved in binding 'spectrin' to lipid. Thus 'spectrin' can be classified as a group II protein as described by Papahadjopoulos et al. [18]. This group includes proteins such as basic myelin protein and cytochrome *c*.

With respect to the interaction of 'spectrin' and the negatively charged phospholipid phosphatidylserine it can be noted that denaturation of 'spectrin' results in a decreased binding to the lipid. Also it is demonstrated that purified 'spectrin' gives a similar result as the crude spectrin-actin, in that it reduces the enthalpy change of the phase transition of the phosphatidylserine to an equal extent.

Although 'spectrin' binds divalent ions rather avidly, Ca^{2+} and Mg^{2+} do not seem to be involved in 'spectrin'-lipid binding, as there is also an interaction in the presence of EDTA. Moreover, no effect is seen on the enthalpy change or the transition temperature of the phase transition (see Results), if divalent cations are added to 'spectrin' recombinates with neutral phospholipids. So, if there is an interaction between the lipid-bound 'spectrin' and Ca^{2+}, this does not have any thermotropic effect. This suggests that the effect of Ca^{2+} on phosphatidylserine or phosphatidylglycerol-spectrin-actin recombinates is primarily on the lipid moiety. The differences in the effects of Ca^{2+} and Mg^{2+} can also be

accounted for by the lipid, as phosphatidylserine has been shown to interact more strongly with Ca^{2+} than with Mg^{2+} [19]. Ca^{2+}-induced lateral phase separations in phosphatidylserine/phosphatidylcholine mixtures, below the molar ratio 1 : 4, are prevented by 'spectrin', and at higher ratios lateral and structural phase separations are reduced by 'spectrin'. The molar ratio of phosphatidylserine to phosphatidylcholine plus phosphatidylethanolamine in the inner monolayer of the erythrocyte membrane is approx. 1 : 4. Therefore it can be suggested that spectrin has a stabilizing effect also in the natural membrane. Oxidation of spectrin SH-groups in intact erythrocytes as described by Haest et al. [20] has been shown to be accompanied by an enhanced availability of phosphatidylserine and phosphatidylethanolamine for phospholipase A_2 degradation. This corroborates the hypothesis that spectrin stabilizes the bilayer against structural rearrangements and that spectrin may be an important factor in maintaining lipid asymmetry in the erythrocyte membrane.

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