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THE INTERACTION OF SPECTRIN · ACTIN AND SYNTHETIC PHOSPHOLIPIDS

C. MOMBERS, P.W.M. VAN DIJCK, L.L.M. VAN DEENEN, J. DE GIER and A.J. VERKLEIJ [a]

Laboratory of Biochemistry and [a] *Institute of Molecular Biology, State University of Utrecht, Padualaan 8, Utrecht (The Netherlands)*

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Summary

Using differential scanning calorimetry and freeze fracture electron microscopy interactions were studied betwen lipids and a spectrin · actin complex isolated from human erythrocyte membranes. With dispersions of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol and mixtures of these two compounds, which for experimental reasons were chosen as the lipid counterpart, such an interaction could clearly be deduced from changes in the temperature and the enthalpy of the phase transition. Furthermore it was demonstrated that the interaction with this membrane protein protects the bilayer against the action of Ca^{2+} and Mg^{2+} and prevents fusion of lipid vesicles which easily occurs in some of the systems when divalent ions were added to the pure lipid vesicles.

Introduction

In recent years the role of the spectrin · actin complex in the erythrocyte membrane has become an intriguing problem (see reviews refs. 1—5). This complex accounts for about 30% of the total erythrocyte membrane protein. It is located exclusively at the cytoplasmic side of the erythrocyte membrane [6] and can easily be released in dissociation form free of lipid from the erythrocyte ghost [7]. Upon sodium dodecyl sulphate polyacrylamide gel electrophoresis spectrin is visible as bands I and II with molecular weights of 250 000 and 230 000 daltons respectively and actin as band V with a molecular weight of 43 000 daltons [3].

It has been suggested that the spectrin · actin complex is comparable with the actomyosin of muscle tissues and forms a contractile apparatus under the membrane which governs in an undefined way the shape of the erythrocyte. Further, it was proposed that spectrin polymerizes into a filamentous network

under the membrane, restricting the lateral diffusion of the integral membrane proteins [8—10]. This idea was criticized by Tilney and Detmers [11] in that in their opinion filaments are not present in situ. They proposed that spectrin and actin exist as an anastomozing network, i.e. a "mesh of unpolymerized material on the cytoplasmic surface of the erythrocyte membrane" restricting the lateral diffusion of intrinsic proteins.

The network models only consider protein-protein interactions between the spectrin · actin complex and intrinsic membrane proteins and do not consider possible spectrin · actin-lipid interactions, as found in recombination studies by Schubert [12] and several other authors [13,14]. Schubert [12] showed that spectrin · actin recombined with sonicated lipid from human erythrocytes had similar binding properties to spectrin · actin with respect to the native erythrocyte membrane. Sweet et al. [13] and Juliano et al. [14] have given evidence for a hydrophobic binding of spectrin to phospholipid vesicles, especially those containing negatively charged phospholipids, resulting in an increase in permeability of those vesicles for monovalent ions or glucose, although the optimal interaction conditions seem to be at variance with those of Schubert [12].

In order to characterize these protein-lipid interactions we studied the recombination of spectrin · actin with sonicated vesicles of well-defined synthetic phospholipids. The influence of spectrin · actin on the thermotropic properties of the phospholipids was studied with differential scanning calorimetry. Morphological changes were followed with freeze-fracture electron microscopy.

Materials and Methods

Spectrin · actin isolation. Human erythrocyte ghosts were prepared according to Dodge et al. [15]. The ghosts were extracted for 15 min at 37°C with 10 volumes 0.1 mM disodium EDTA in twice distilled water (pH 8.5 with NaOH) according to Marchesi's method [16]. The extract was immediately centrifuged at 100 000 × *g* for 1 h (4°C). The 100 000 × *g* supernatant was concentrated by ultrafiltration to a final protein concentration of approximately 1 mg/ml and dialyzed against 10 mM Tris (pH 8.5 with HAc), 0.1 mM disodium EDTA, at 4°C.

This supernatant was stored at 4°C and used without any further purification. On sodium dodecyl sulphate polyacrylamide gel electropherograms the protein preparation was shown to contain spectrin (bands 1, 2, 2.1 etc.), actin (band 5) and some band 4.1 or 4.2 protein [3]. Multimers of spectrin proved to be absent. The protein solution did not contain any phosphate.

Before incubation of this spectrin · actin preparation, further referred to as "spectrin", with phospholipid vesicles, the protein was dialysed overnight at 4°C or 3 h at 24°C against "incubation" buffer (10 mM Tris, pH 7.4 with HAc, 0.1 mM disodium EDTA, 150 mM NaCl).

Phospholipids. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine and 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol were synthesized as described before [17, 18].

Recombination. Lipid vesicles were prepared by suspending 3 to 4 μmol of phospholipid in 2.0 ml "incubation" buffer at 45°C followed by sonication

under nitrogen at 0°C for 1—4 min with a Branson sonicator (tip-probe, power-setting 4) and centrifugation (30 min, 25 000 × *g*) to remove titanium and residual liposomes. The vesicles were incubated with 1.5 mg "spectrin" for 60 min at 37°C, followed by a second incubation period of 30 min in the presence or absence of Ca^{2+} or Mg^{2+}.

Differential scanning calorimetry. For thermal analysis the recombinate was collected by ultracentrifugation (60 min, 150 000 × *g*, 4°C). The differential scanning calorimetric analyses were performed as described before [18]. Deviations from the normal procedure will be outlined in the legends of the figures. After thermal analyses samples were dissolved overnight in 3 ml 50 mM Tris/acetate, 70 mM sodium dodecyl sulphate pH 8.5 and if necessary boiled for 5 min or sonicated in a bath type sonicator. Phospholipid contents were determined as described by Bartlett [19]; protein was determined by the method of Lowry with bovine serum albumin in 70 mM sodium dodecyl sulphate as a reference [20].

Freeze-fracture electronmicroscopy. The morphology of the samples was studied by freeze-fracturing electron microscopy on samples concentrated by ultracentrifugation (60 min, 150 000 × *g*, 4°C).

Before quenching, glycerol was added up to 30% (v/v) to prevent freeze damage. The samples were quenched in a mixture of solid and liquid nitrogen and fractured as described before [21]. Electron micrographs were made with a Siemens Elmiskop 1^A.

Results and Discussion

In differential scanning calorimetry studies on "spectrin"-lipid interactions the choice of the lipid part is limited by the following requirements: i) the phospholipids have to be homogeneous in species to obtain well-defined thermotropic peaks; ii) the lipid phase transitions of the phospholipids have to be lower than 40°C in order to be able to incubate "spectrin" with phospholipids above their transition temperatures without having "spectrin" denaturation. In the present study we have chosen 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol as a neutral and a negatively charged phospholipid which meet these requirements.

To enable maximal interaction, the lipid dispersions were sonicated prior to the incubation with "spectrin". After the incubation with "spectrin" freeze fracture electron microscopy pictures (Fig. 1A) showed vesicles with diameters ranging from 200—1000 Å. These vesicles, relative large in comparison with protein-free sonicated vesicles (van Dijck, P.W.M. et al., in preparation) could be concentrated by ultracentrifugation, whereas the vesicles incubated without "spectrin" did not form a pellet under the conditions which were used.

Fig. 2 shows the calorimetric scan of "spectrin" incubated samples of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine in comparison with the scan of the pure lipid bilayer as determined on a sample of unsonicated liposomes. The transition peak is broadened and in the presence of excess of protein, reached at a protein/lipid ratio of 0.29 (w/w), a decrease in the enthalpy of transition (ΔH) from 6.8 to 5.0 kcal/mol could be observed. Ca^{2+} and Mg^{2+} concentrations, tested up to a molar ratio of Mg^{2+}/lipid = 2, appeared to have no effect on the

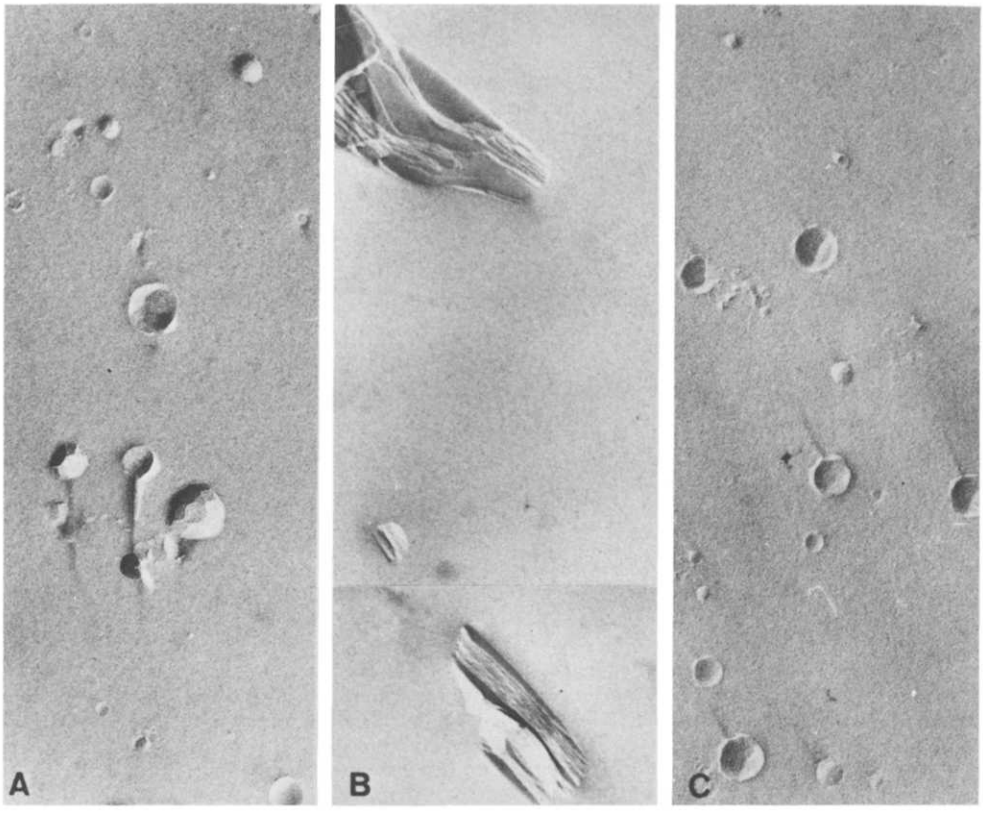

Fig. 1. Freeze fracturing morphology of sonicated 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol incubated with "spectrin" (A); incubated with Ca^{2+} (B); incubated with "spectrin" and subsequently with Ca^{2+} (C). ×75 000. (Protein/lipid = 0.27 (w/w), Ca^{2+}/lipid = 2.0).

transition temperature and the ΔH values both of the pure 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and of the protein-lipid recombinate.

Of considerably more interest in this respect are the observations made on the 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol. In earlier studies [18] we showed that Ca^{2+} and Mg^{2+} affect the liposomal structure in dispersions of this negative lipid. A molecular reorientation was found to lead to the formation of stacked lamellae (Figs. 1B and C). Therefore it is striking that we found that the "spectrin" incubated vesicles were insensitive to Ca^{2+} and Mg^{2+} concentrations and that no stacked lamellae could be formed. Apparently the protein-lipid interaction has an effective protective action on the vesicles structure of 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol. In control experiments we incubated the vesicles with human serum albumin and subsequently with Ca^{2+} and found that this protein is incapable of fullfilling the protective action. Stacked lamellae were formed as in case of protein-free vesicles.

Fig. 3 shows the calorimetric behaviour of 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol with increasing amounts of "spectrin". The following observations could be made: i) "spectrin" induces a significant broadening of the transition;

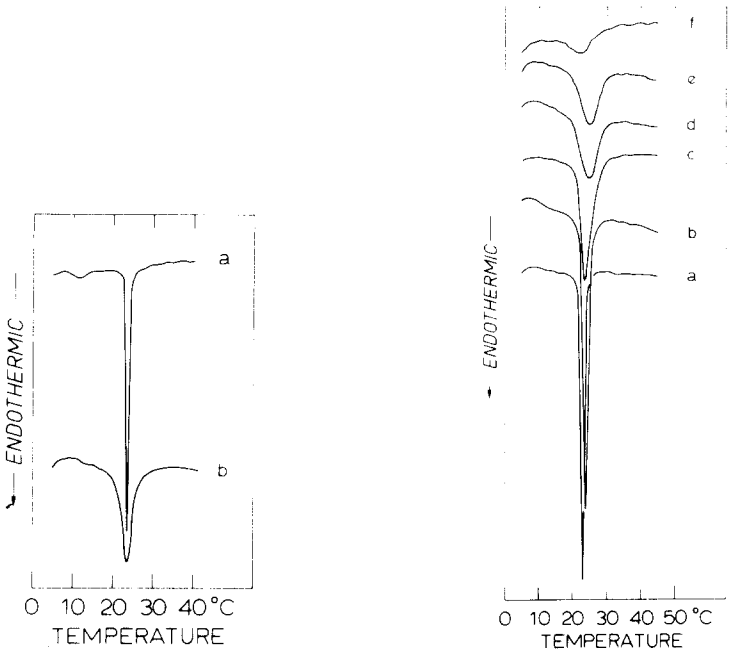

Fig. 2. Thermotropic behaviour of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine in the absence (a) and presence (b) of "spectrin". (a) Liposomal preparation; (b) vesicles incubated with "spectrin" as described in Materials and Methods (protein/lipid = 0.27 (w/w)).

Fig. 3. Influence of increasing amounts of "spectrin" on the thermotropic behaviour of 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol. (a) Liposomal preparation without "spectrin"; (b—f) vesicles incubated with "spectrin" and concentrated by ultracentrifugation; "spectrin"/phospholipid ratios (w/w) of (b) 0.01, (c) 0.05, (d) 0.11, (e) 0.16 and (f) 0.40.

ii) the ΔH decreases gradually with increasing "spectrin" concentrations to a minimum of 3.0 kcal above "spectrin"/lipid ratios of 0.4 (w/w) (compare also Fig. 4).

The physical state of the paraffin chains proved to be very important in achieving the lipid-protein interaction. Incubations at temperatures below the transition point (23°C) followed by a heating scan in the differential scanning calorimetry apparatus resulted in patterns typical for the pure lipid unaffected by the protein. So a fluid state of the paraffin core of the bilayer is required for interaction between "spectrin" and phosphatidylglycerol. From this result it can be tentatively concluded that during the combination of the "spectrin" and the vesicles in the liquid crystalline condition hydrophobic interactions are involved.

Upon scanning of "spectrin"-recombinates to above 45°C a "spectrin" denaturation peak could be seen which resulted in a sharpening of the lipid peak and a small increase in the ΔH value of about 0.5 kcal/mol in the following scan ("spectrin" itself showed a comparable peak). Also freezing of the sample resulted in a small increase of the ΔH value. An analysis of "spectrin"-phosphatidylglycerol recombinates by sodium dodecyl sulphate polyacrylamide, gel electrophoresis (after ultracentrifugation) pointed out that all components of the original protein extract were presented in the pellet.

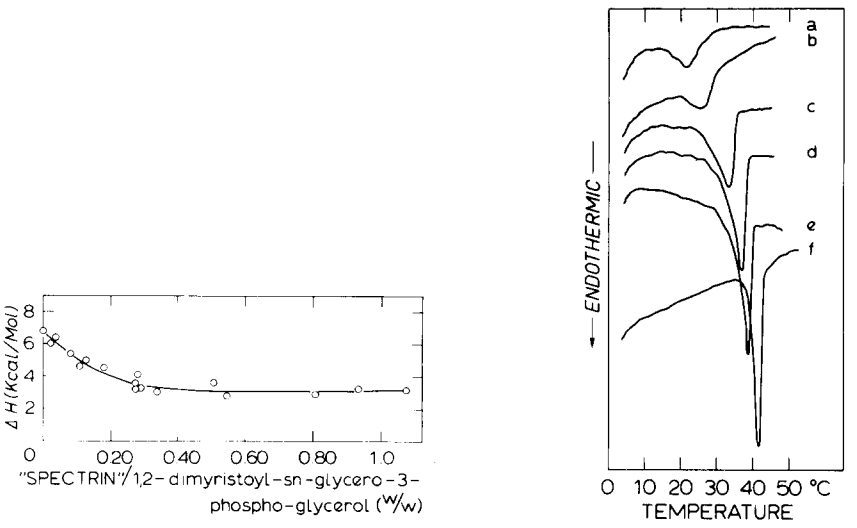

Fig. 4. Influence of increasing amounts of "spectrin" on the enthalpy of 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol.

Fig. 5. Effect of Ca^{2+} on the thermotropic behaviour of 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol incubated with "spectrin" (ratio "spectrin"/phospholipid 0.4 (w/w)). Ca^{2+}/phospholipid ratio; (a) 0.0, (b) 0.09, (c) 0.26, (d) 0.59, (e) 1.26 and (f) 33.26.

Figs. 5 and 6 show the detailed differential scanning calorimetry scans of 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol incubated with "spectrin" and subsequently with various concentrations of Ca^{2+} and Mg^{2+}. The thermotropic

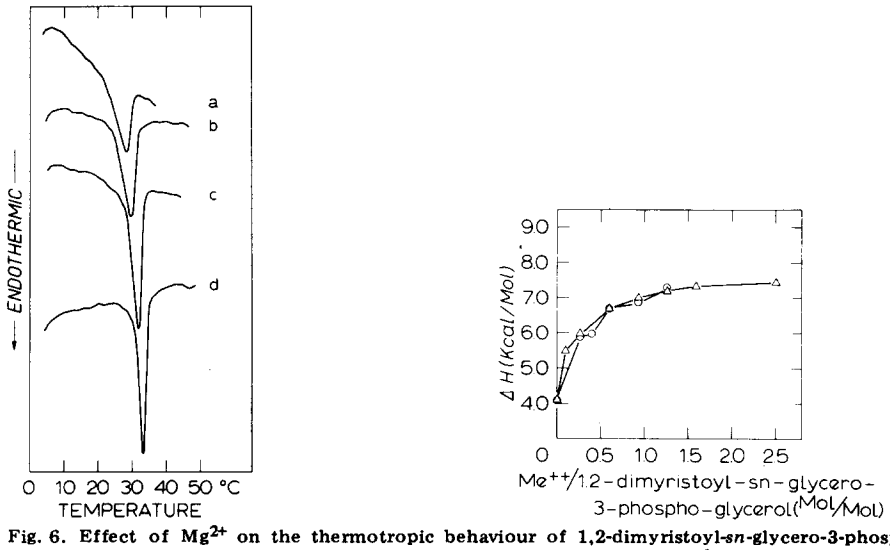

Fig. 6. Effect of Mg^{2+} on the thermotropic behaviour of 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol incubated with "spectrin" (ratio "spectrin"/phospholipid 0.4 (w/w)). Mg^{2+}/phospholipid ratio; (a) 0.26, (b) 0.59, (c) 1.26 and (d) 4.24.

Fig. 7. Influence of increasing amounts of divalent ions on the enthalpy change of 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol incubated with "spectrin". △, Data obtained with Ca^{2+}; ○, data obtained with Mg^{2+}.

characteristics can be summarized as follows: i) the transition temperature of the phospholipid · "spectrin" · Me^{2+} complex increases gradually with increasing Me^{2+} concentrations up to 39°C for Ca^{2+} and 31°C for Mg^{2+}; ii) the enthalpy change (ΔH) at the transition temperature increases to a limiting value of 7.5 kcal/mol both in the presence of Ca^{2+} and Mg^{2+} (compare Fig. 7). The shifts are strongly restricted compared with changes that are induced by the divalent ions in the pure lipid system. Without "spectrin" the limiting values for the transition temperatures are 89°C and 71°C and for the ΔH values 15.2 and 12.4 kcal/mol for addition of Ca^{2+} and Mg^{2+} respectively [18]. In control experiments in which the 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol was incubated with human serum albumin instead of "spectrin" no significant deviation from the pure system could be observed.

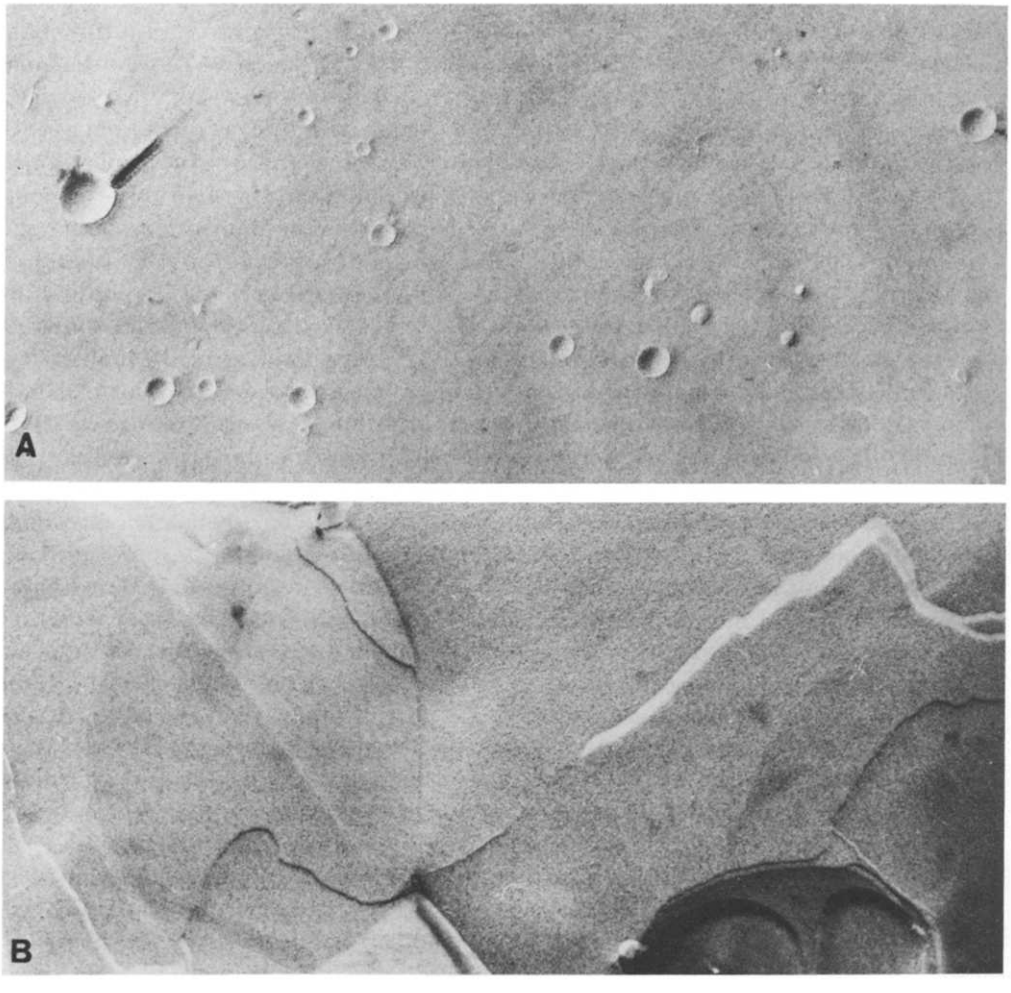

Fig. 8. Freeze fracture morphology of vesicles of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine/1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (equimolar) (A) after incubation with "spectrin" and subsequent addition of Ca^{2+}; (B) after incubation without "spectrin" and subsequent addition of Ca^{2+}. ×75 000. (Protein/lipid = 0.27 (w/w), Ca^{2+}/lipid = 2.0)

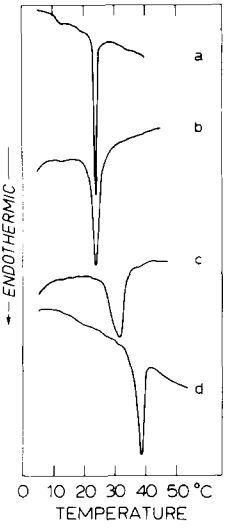

Fig. 9. Thermotropic influences of "spectrin" and/or Ca^{2+} on an equimolar mixture 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine/1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol. (a) Lipid mixture alone (liposomal); (b) vesicles incubated with "spectrin"; (c) as (b) and subsequently treated with Ca^{2+}; (d) pure lipid vesicles incubated with Ca^{2+} (protein/lipid = 0.27 (w/w), Ca^{2+}/lipid = 2.0).

The protective action of "spectrin" was also apparent in experiments with mixtures of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol. Figs. 8 and 9 concern an experiment with these phospholipids in a molar ratio of 1.5. If Ca^{2+} were added to a sonicated vesicles dispersion of this lipid mixture the dispersion became milky. The freeze fracture pictures (Fig. 8) showed large multilayered liposomes, which must be the result of extensive vesicle fusion. If, however, the vesicles were preincubated with "spectrin" this Ca^{2+}-induced fusion was prevented completely. Fig. 9 shows the calorimetric scans of these samples and it can be noticed that also in the mixture "spectrin" restricted the shift in transition temperature that could be brought about by the divalent ion.

Summarizing, it can be concluded that "spectrin" spontaneously interacts with the synthetic bilayers which are considered in this study and that this protein-lipid interaction stabilizes the planar bilayer orientation of the lipid molecules. From the present study and the observations of Schubert [12] it can be concluded that both hydrophobic and polar interactions are involved in the lipid-protein interaction underlying this stabilization. Comparable types of lipid-protein interaction have been described for cytochrome *C* and A1 protein [22]. It is tempting to speculate that in the erythrocyte membrane besides "spectrin"-protein interactions "spectin"-lipid interactions also play a functional role. In view of the location of "spectrin" and the known asymmetric distribution of the phospholipids over the inside and outside of the membrane [23,24] it will be of importance to explore in future studies the possible interaction between "spectrin" and phosphatidylserine/phosphatidylethanolamine mixtures.

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References

1 Guidotti, G. (1972) Annu. Rev. Biochem. 41, 731—752
2 Juliano, R.L. (1973) Biochim. Biophys. Acta 300, 341—378
3 Steck, T.L. (1974) J. Cell Biol. 62, 1—19
4 Singer, S.J. (1974) Annu. Rev. Biochem. 43, 805—833
5 Marchesi, V.T., Furchtmayr, H. and Tomita, M. (1976) Annu. Rev. Biochem. 45, 667—698
6 Nicolson, G.L., Marchesi, V.T. and Singer, S.J. (1971) J. Cell Biol. 51, 265—271
7 Marchesi, V.T. and Steers, E. (1968) Science 159, 203—204
8 Elgsaeter, A. and Branton, D. (1974) J. Cell Biol. 63, 1018—1030
9 Elgsaeter, A., Shotton, D. and Branton, D. (1976) Biochim. Biophys. Acta 426, 101—122
10 Yu, J. and Branton, D. (1976) Proc. Natl. Acad. Sci. U.S. 73, 3891—3895
11 Tilney, L.G. and Detmers, P. (1975) J. Cell Biol. 66, 508—520
12 Schubert, D. (1973) Hoppe-Seyler's Z. Physiol. Chem. 354, 781—790
13 Sweet, C. and Zull, J.E. (1970) Biochem. Biophys. Res. Commun. 41, 135—141
14 Juliano, R.L., Kimelberg, H.K. and Papahadjopoulos, D. (1971) Biochim. Biophys. Acta 241, 894—905
15 Dodge, J.T., Mitchell, E. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119—125
16 Marchesi, V.T. (1974) Methods Enzymol. XXXXII, 275—277
17 Van Deenen, L.L.M. and de Haas, G.H. (1964) Adv. Lipid Res. 2, 168—229
18 Van Dijck, P.W.M., Ververgaert, P.H.J.Th., Verkleij, A.J., van Deenen, L.L.M. and de Gier, J. (1975) Biochim. Biophys. Acta 406, 465—478
19 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466—468
20 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
21 Ververgaert, P.H.J.Th., Elbers, P.F., Luitingh, A.J. and van de Berg, H.J. (1972) Cytobiology 6, 86—96
22 Papahadjopoulos, D., Moscarello, M., Eylar, E.H. and Isac, T. (1975) Biochim. Biophys. Acta 401, 317—335
23 Bretscher, M.S. (1972) J. Mol. Biol. 71, 523—528
24 Verkleij, A.J., Zwaal, R.F.A., Roelofsen, B., Comfurius, P., Kastelijn, D. and van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 323, 178—193