Biochimica et Biophysica Acta, 550 (1979) 318—327

BBA 78235

DIVALENT CATION ENHANCEMENT OF THE AGGLUTINABILITY BY SOYABEAN LECTIN OF LIPOSOMES PREPARED FROM TOTAL LIPID OF ERYTHROCYTES AND OF ERYTHROCYTE MEMBRANES

R. RENDI, A.E. VATTER and J.A. GORDON

Department of Pathology and Webb-Waring Lung Institute, University of Colorado Medical Center, Denver, CO 80262 (U.S.A.)

(Received June 12th, 1978)

Key words: Agglutination; Lectin; Liposome; Ca^{2+}; (Erythrocyte membrane)

Summary

$CaCl_2$ or $MgCl_2$ but not NaCl enhances the soyabean lectin-induced agglutination of liposomes prepared from total lipids of erythrocyte membranes. The addition of purified phosphatidylserine to the total lipids of erythrocyte membranes before the formation of liposomes inhibits lectin-induced agglutinability of the preparation in the absence of $CaCl_2$, but not in its presence. When preformed phosphatidylserine liposomes are added to liposomes of total lipids of erythrocyte ghosts, they do not inhibit agglutination, indicating that phosphatidylserine does not inhibit the lectin directly. $CaCl_2$ or $MgCl_2$ but not NaCl also stimulates the soyabean lectin-induced agglutination of human erythrocyte membranes.

Electron micrographs indicate that the liposome preparations are multilamellar and separate even in the presence of $CaCl_2$. When such liposomes are treated with lectin with or without $CaCl_2$, the electron micrographs show significant agglutination without apparent fusion. The reversal of the agglutination of liposomes by specific sugars followed by turbidimetric and electron microscopic techniques supports the conclusion that $CaCl_2$ stimulated lectin-induced agglutination is unaccompanied by fusion.

The stimulation by divalent cations of lectin-induced agglutination of erythrocyte ghosts or of our liposomes may be due to a decrease in apparent surface charge of these membrane systems.

Introduction

Plant lectins are popular tools to study membrane structure and function since they bind to specific saccharide structures on the cell surface [1]. Recent studies have shown that liposome model membrane-containing glycolipids will interact with lectins. Surolia et al. [2] found that *Ricinus* lectin binds to liposomes containing brain ganglioside with terminal galactose residues and that

treatment of such liposomes with the lectin causes their aggregation, as indicated by a turbidimetric assay. We have reported [3] that soyabean lectin but not concanavalin A agglutinates liposomes prepared from total lipids obtained from human erythrocyte membranes.

Nicolson [4], in a review on the factors affecting the agglutination reactions between cells, discusses 'cell charge repulsive forces'. Similar forces will be present in our liposomes prepared from total erythrocyte ghost lipid, since it is known that phosphatidylserine, a negatively charged phospholipid, represents 13—15 mol% of the total phospholipids [5]. Divalent cations interact quite strongly with liposomes prepared with phosphatidylserine to form neutral salts [6]. It is well known that calcium ions bind to the peripheral region of human erythrocytes and other cells, inducing a decrease in the negative surface charge as estimated by changes in electrophoretic mobilities of the cells [7]. We report here on the stimulatory effect of divalent cations on the agglutination by soyabean lectin of liposomes prepared from total lipids of human erythrocyte membranes and of these intact ghosts. Since, in the presence of divalent cations, the liposomes prepared from phosphatidylserine do fuse [6] we have complemented our turbidimetric analyses with the use of electron microscopy.

Materials and Methods

Soyabean agglutinin was prepared from saline-extracted soyabean flour (Central Soya, Chicago) by affinity chromatography as previously described [8]. Human erythrocytes were obtained from 3-week-old blood. Ghosts were prepared from human erythrocytes by the method of Dodge et al. [9]. Total lipid extractions from lyophilized ghosts, and preparation of liposomes by dialysis was carried out as previously described [3]. In some cases, exogenous phosphatidylserine was also added to the total lipid extract before dialysis. Thin layer chromatographic determination of the presence of phosphatidylserine was accomplished using Silica gel 60 plates developed with chloroform/methanol/water (65 : 25 : 4, v/v): and spraying with ninydrin reagent. For determining the concentration of phosphatidylserine, the liposomes were centrifuged for 30 min at 15 000 $\times g$, and suspended in chloroform/methanol (2 : 1, v/v). Aliquots of this solution and of pure phosphatidylserine standard (Sigma Chemical Co., St. Louis, Mo.) were pipetted into test tubes, dried and assayed according to the ninhydrin method of Spies [10]. For agglutination studies of erythrocyte membranes, ghosts were washed with 20 mM Tris-HCl buffer, pH 7.4, by centrifugation for 1 h at 15 000 $\times g$, to reduce the concentration of phosphate ions which complexed with Ca^{2+} or Mg^{2+}. Agglutination was measured turbidimetrically in 20 mM Tris-HCl (pH 7.4) at room temperature in a Gilford-Beckman spectrophotometer equipped with a recorder as previously described [3].

Electron microscopy

1. Negatively stained preparations. 5 μl liposome suspension was mixed with an equal volume of 0.5% sodium phosphotungstate, pH 7.0. This mixture was applied to the surface of a collodian-coated grid, blotted and immediately dried. All specimens were observed and photographed at the time of preparation.

2. Thin sections. 1—2 ml liposome preparation was preserved by mixing with an equal volume of tannic acid-glutaraldehyde fixative at room temperature. The composition of the fixative was 1% tannic acid (Mallinckrodt, Inc., St. Louis, Mo.) and 1% glutaraldehyde (Biological Grade, Fischer Scientific, Pittsburgh, Pa.) in 20 mM Tris-HCl buffer, pH 7.4. The specimen was pelleted after the addition of the fixative and left for 1—2 h after which it was given three 5-min rinses with buffer and refixed with 1% osmium tetraoxide buffered with Tris-HCl at pH 7.4 for 30 min and rinsed 3 times with buffer. Some specimens were then treated with 0.5% aqueous uranyl acetate for 60 min. The latter treatment did not appear to affect the structure of quality of the specimen. The specimens were dehydrated with cold acetone and embedded in Epon. Specimens were sectioned with a diamond knife in the silver to gold range and observed with and without lead staining.

Results

As shown in Fig. 1, the addition of soyabean lectin (200 μg/ml) to liposomes prepared from erythrocyte lipids elicits a continuous increase in agglutination as previously described [3]. When 1 mM $CaCl_2$ or 1 mM $MgCl_2$ but not 15 mM NaCl were added together with the soyabean lectin, the increased in absorbance is significantly higher than with the lectin alone. 2 mM $CaCl_2$ alone does not produce any change in the turbidity of liposome suspension. The reversibility of the increase in absorbancy by either lectin alone or lectin with Ca^{2+} after addition of acetylgalactosamine (50 μmol/ml) is shown in Fig. 1. This sugar

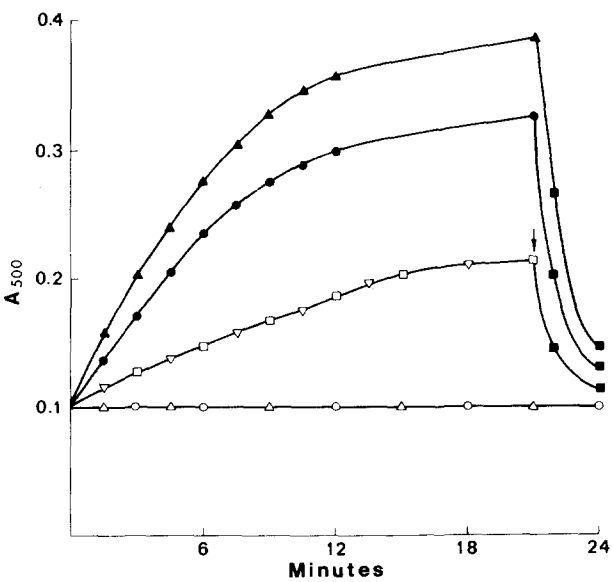

Fig. 1. Time course of agglutination of liposomes from total lipids of human erythrocytes by soyabean lectin in 20 mM Tris-HCl buffer (pH 7.4). Abscissa, time in min; ordinate, absorbance at 500 nm. ○, no lectin added; △, no lectin added but with 2 mM $CaCl_2$; □, 200 μg lectin; ▽, 200 μg lectin and 15 mM NaCl; ●, 200 μg lectin and 1 mM $MgCl_2$; ▲, 200 μg lectin and 1 mM $CaCl_2$. At 20 min, 50 μM of acetylgalactosamine (■).

interacts specifically with soyabean agglutinin. The time course experiments (Fig. 1) were repeated at different concentrations of $MgCl_2$ or $CaCl_2$, keeping the soyabean agglutinin constant at 200 μg/ml. Fig. 2 is a summary of these experimental data, where the increase of absorbance is plotted against the concentration of divalent cations used in the assay. It can be seen that at all concentrations of either Mg^{2+} or Ca^{2+} there is a significant increase in the rate of agglutination. The stimulation of soyabean lectin-induced agglutination by Ca^{2+} is significantly inhibited by the presence of ethylenediaminetetraacetic acid, a Ca^{2+} chelator (data not shown).

When the liposome suspension prepared from the total lipid extract of erythrocytes is centrifuged for 30 min at 15 000 $\times g$, a tight pellet is formed which can be dissolved in chloroform/methanol. Thin layer chromatography of the liposomes and of chloroform/methanol (2 : 1) lipid extracted from erythrocyte ghosts revealed identical patterns when visualized by iodine vapor. Analyzed by a ninhydrin spray, only phosphatidylserine and phosphatidylethanolamine in the approximate proportion of 1:2 or 3 was seen, which has been reported for human erythrocyte lipids [5]. The approximate concentration of phosphatidylserine in the lipid extract of erythrocyte membranes was determined by the ninhydrin method using phosphatidylserine as standard and dividing the determined amount by 3, because phosphatidylethanolamine was present at approximately twice the concentration of phosphatidylserine. Liposomes were prepared as usual from the total lipid of erythrocytes or with added phosphatidylserine dissolved in chloroform/methanol and added to the lipid extract before making the liposomes. As seen in Fig. 3, the agglutination of the liposomes prepared with phosphatidylserine by soyabean lectin in the absence

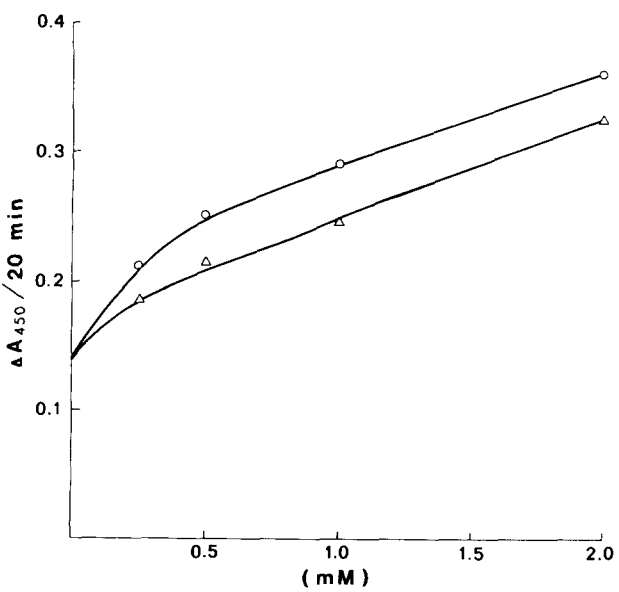

Fig. 2. Relationship between divalent cation concentration and agglutinability of liposomes by soyabean lectin. Ordinate, divalent cation concentration; abscissa, increase of absorbance after 20 min. All experiments were performed with 200 μg lectin. △, $MgCl_2$; ○, $CaCl_2$.

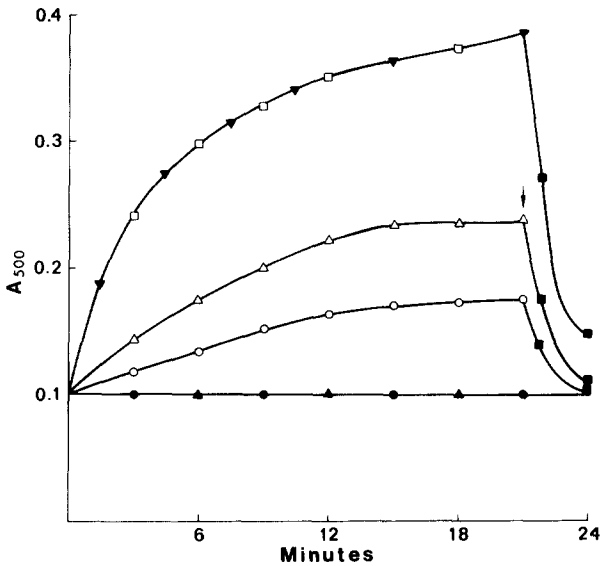

Fig. 3. Agglutination of liposomes prepared from total lipids of human erythrocytes or of total lipids with added phosphatidylserine by soyabean lectin in 20 mM Tris-HCl buffer (pH 7.4). Abscissa, time in min; ordinate, absorbance at 500 nm. △, 200 μg lectin with no $CaCl_2$, and liposomes from total lipids; ○, 200 μg lectin with no $CaCl_2$ and liposomes from total lipids with phosphatidylserine; ▼, 200 μg lectin with 1 mM $CaCl_2$ and liposomes from total lipid; □, 200 μg lectin with 1 mM $CaCl_2$ and liposomes from total lipid with phosphatidylserine; ●, no lectin with 1 mM $CaCl_2$ and liposomes from total lipid; ▲, no lectin with 1 mM $CaCl_2$ and liposomes from total lipid with phosphatidylserine. At 20 min 50 μM acetylgalactosamine (■).

of Ca^{2+} was inhibited when compared to the liposomes prepared from the total lipid extract alone. However, when the agglutination of the two liposome preparations was tested in the presence of both lectin and Ca^{2+}, no difference was observed. The lack of change is presumably due to the low level of phosphatidylserine. This compound is present at concentrations that would inhibit the agglutination in the absence of calcium ions, but not high enough to affect the agglutination in the presence of divalent cations. Note also that in the presence of Ca^{2+} alone, no increase in absorbance is seen with either preparation. The two different liposome preparations were centrifuged and analyzed by thin layer chromatography to determine the incorporation of phosphatidylserine in the liposomes. It was estimated visually that the total erythrocyte lipid liposomes prepared with added phosphatidylserine contained at least 3 times as much phosphatidylserine as the other liposome preparation. These experiments suggest that the charge of phosphatidylserine when inserted into liposomes inhibits the soyabean lectin-induced agglutination of liposomes in the absence of calcium. However, it is also possible that phosphatidylserine does inhibit the lectin directly. To test this suggestion, we prepared phosphatidylserine liposomes and tested them on the lectin-induced agglutinability of liposomes prepared from total lipid extract only. As seen in Fig. 4, the addition of twice as much phosphatidylserine (as liposomes) does not affect the agglutinability of liposomes prepared from total lipid extract. The data presented in Fig. 4 also indicate that the liposomes prepared with only phos-

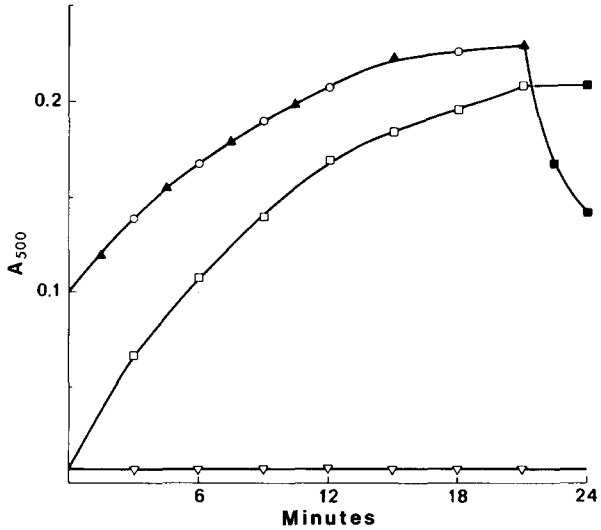

Fig. 4. The effect of adding phosphatidylserine liposomes to the soyabean lectin-induced agglutination of liposomes prepared from total lipids of human erythrocytes in 20 mM Tris-HCl (pH 7.4). Abscissa, time in min; ordinate, absorbance at 500 nm. ▽, 200 μg lectin with no $CaCl_2$ and liposomes from only phosphatidylserine; □, no lectin with 1 mM $CaCl_2$ and liposomes from only phosphatidylserine; ▲, 200 μg lectin with no $CaCl_2$ and liposomes from total lipids and phosphatidylserine; ○, 200 μg lectin with no $CaCl_2$ and liposomes from total lipids. At 20 min 50 μM acetylgalactosamine (■).

phatidylserine are not agglutinated by soyabean lectin but are aggregated by calcium ions, as previously reported [6]. The mechanism of aggregation of phosphatidylserine liposomes by Ca^{2+} is different from the lectin-dependent

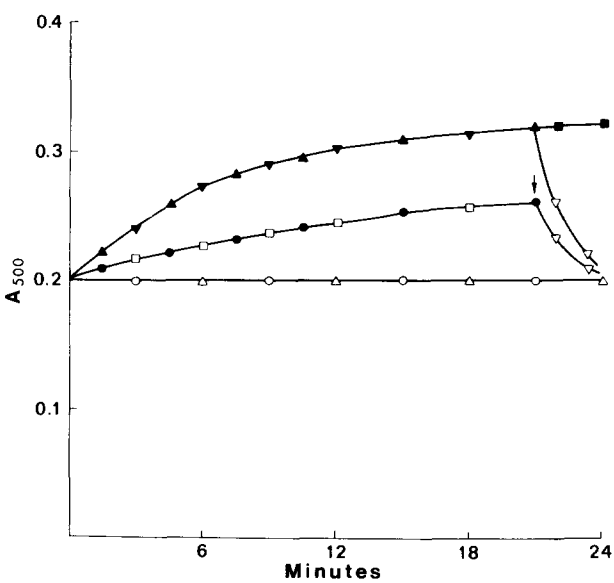

Fig. 5. Time course of agglutination of human erythrocyte ghosts by soyabean lectin in 20 mM Tris-HCl (pH 7.4). Abscissa, time in min; ordinate, absorbance at 500 nm. ○, no lectin; △, no lectin but with 2 mM $CaCl_2$; □, 200 μg lectin; ●, 200 μg lectin and 15 mM NaCl; ▼, 200 μg lectin and 2 mM $CaCl_2$, ▲, 200 μg lectin and 2 mM $MgCl_2$. At 20 min, 50 μM of acetylgalactosamine (▽) or mannopryanoside (■) were added.

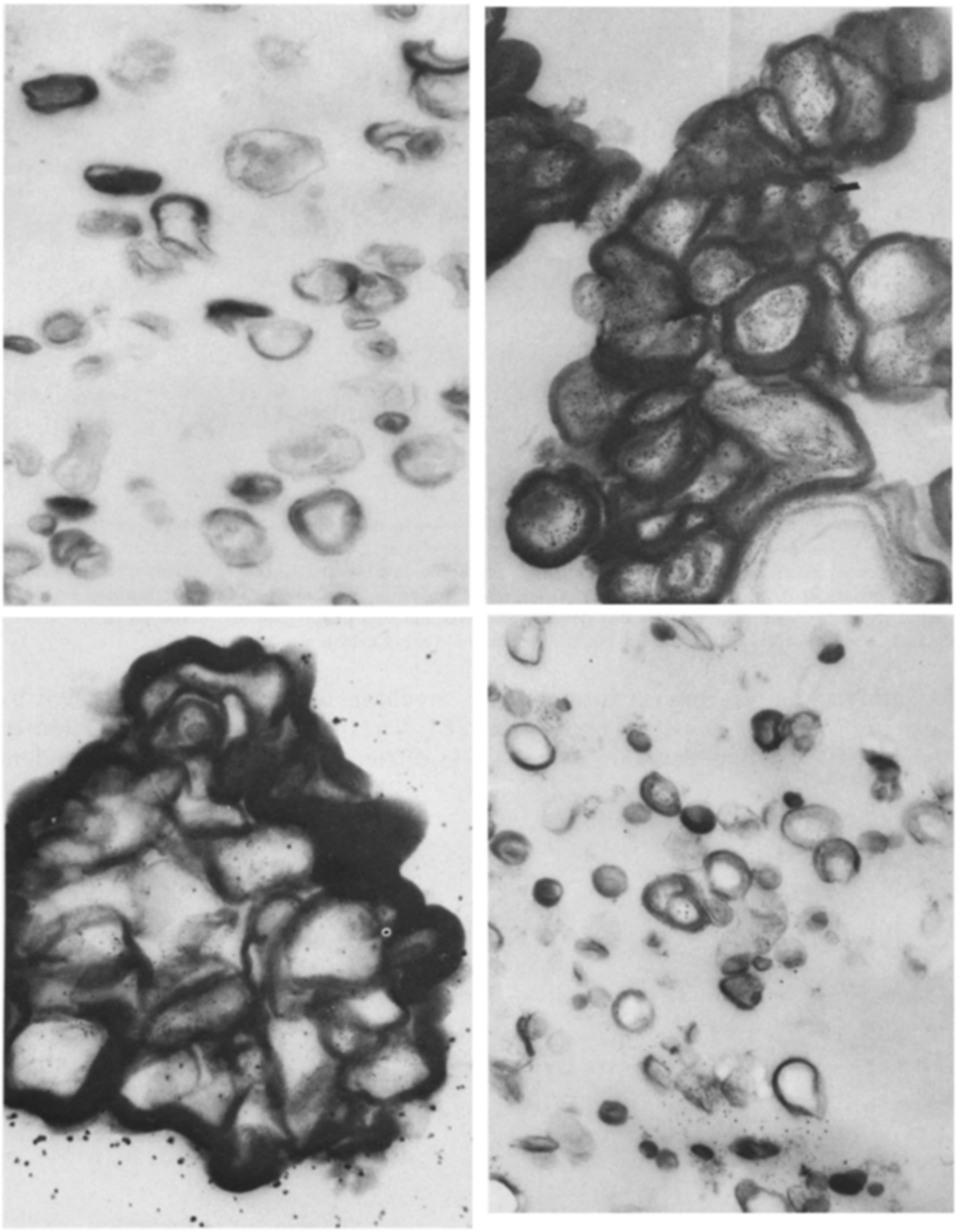

Fig. 6. Electron micrograph of liposomes prepared from total lipids of human erythrocytes incubated in 20 mM Tris-HCl (pH 7.4) showing that the material is multilamellar and separate. Magnification, ×30 000.

Fig. 7. Electron micrograph of liposomes incubated with soyabean lectin (200 μg/ml) showing that the material is agglutinated. Magnification, ×50 000.

Fig. 8. Electron micrograph of liposomes incubated with soyabean lectin and $CaCl_2$ showing that the material is agglutinated but not fused. The concentration of the lectin was 200 μg/ml and $CaCl_2$, 2 mM. Magnification, ×50 000.

Fig. 9. Electron micrograph of liposomes preincubated with soyabean lectin (200 μg/ml) and then incubated with acetylgalactosamine (50 μM/ml) showing that the material is separate. Magnification, ×25 000.

agglutination of liposomes prepared with total lipids of erythrocytes ghosts. This is demonstrated by the irreversibility of agglutination on addition of a specific sugar (cf. Figs. 4 and 3).

As previously described, this turbidimetric assay for agglutination can be used not only with liposomes prepared from lipids, but also to monitor the agglutination of erythrocyte ghosts [11]. Fig. 5 shows the agglutinability of human erythrocyte ghosts by soyabean lectin and its stimulation by 2 mM $CaCl_2$ and by 2 mM $MgCl_2$. Shown in the figure is the reversibility of the agglutination by acetylgalactosamine but not by mannose.

When the liposomes are incubated in the presence of buffer alone, the material formed is of multilamellar structure with the individual liposomes quite separate from each other (see Fig. 6). When $CaCl_2$ is added to such a suspension of liposomes in the absence of the lectin, the structure of the liposomes is unaffected similar to Fig. 6 (data not shown). The liposome suspension, following incubation with soyabean lectin and sectioning, shows aggregation of the individual multilamellar components into clusters of vesicles without any apparent fusion of the individual particles (see Fig. 7). When an aliquot of liposome preparation is incubated in the present of both $CaCl_2$ and lectin, the appearance of the aggregate (Fig. 8) is strikingly similar to that treated with soyabean lectin in the absence of $CaCl_2$. Reversal of aggregation by the addition of *N*-acetylgalactosamine to the lectin-induced agglutination of liposomes, both in the presence and absence of $CaCl_2$, is shown in Fig. 9; one can easily see that the multilamellar structures have separated from each other. Another saccharide, mannose, was used as a control substance to determine the effect of sugars on the agglutination. Mannose had no effect upon the reversal of agglutination. The agglutinated structures appear as in the absence of the sugar as in Fig. 8 (data not shown). All experimental preparations have been studied using negatively stained and fixed, pelleted specimens. The conclusions

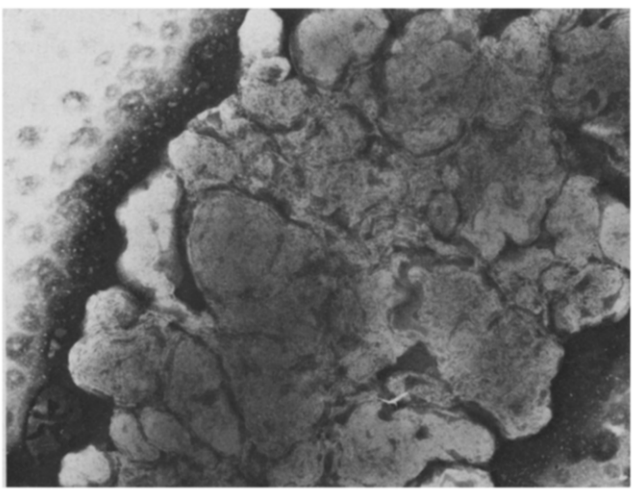

Fig. 10. Negative staining electron micrograph of liposomes incubated with soyabean lectin and $CaCl_2$ showing that the material is agglutinated. The concentration of the lectin was 200 μg/ml and $CaCl_2$, 2 mM. Magnification, ×50 000.

obtained from the observations of both types of specimen were the same. Fig. 10 shows a negative staining of liposomes agglutinated in the presence of both soyabean lectin and calcium chloride, and is a typical image using this technique.

Discussion

The experiments presented here indicate that divalent cations, but not monovalent cations, stimulate the agglutinability induced by soyabean lectin in both erythrocyte ghosts and liposomes prepared from total lipids of human red blood cells. It is well known that Ca^{2+} binds to the peripheral region of human erythrocytes and other cells, producing a decrease in the negative surface charge as estimated by changes in electrophoretic mobilities of the cells. Seaman et al. [7] have reported that in human erythrocytes, calcium binding sites on the membrane are comprised of neuraminate ions and other sites. It has been reported by Papahadjopoulas [6] that liposomes containing phosphatidylserine show an electrokinetic potential as measured by electrophoretic mobility; and that this potential is decreased by the addition of calcium ions. It is well known that the lipids of human erythrocyte membranes contain a significant proportion of phosphatidylserine [5]. We suggest that the simplest explanation of Ca^{2+} enhancement of soyabean lectin-induced agglutination of erythrocyte ghosts and of liposomes prepared from total lipids of human erythrocytes is due to a decrease in the surface potential characteristic of these two membrane systems. Another plausible explanation for the effects of divalent cations on the lectin-induced agglutination of liposomes is the fact that these ions can induce phase changes in pure phosphatidylserine liposomes [12]. However, we favor the first explanation since the divalent cations also stimulate the lectin-induced agglutination of erythrocyte ghosts, a system where the divalent cations most likely interact preferentially with the neuraminic acid of the glycoproteins [7], and where phosphatidylserine is not exposed [13]. We have been unable to find in the literature any indications that the interactions of divalent cations with erythrocyte ghosts produces differences in the phase changes of the plasma membrane.

It is well known that liposomes prepared from pure phosphatidylserine fuse upon the addition of Ca^{2+}, and this fusion process is followed by an increase of absorbance [14]. However, in the presence of 2 mM $CaCl_2$, a fusogenic concentration for pure phosphatidylserine [14], our liposomes do not show an increase of turbidity (see Fig. 1). A plausible explanation for this difference is the fact that our liposomes contain only around 10—15% phosphatidylserine, or that they contain other lipid components such as cholesterol, glycolipids and sphingomyelin. It is of some interest that liposomes prepared from gangliosides and phosphatidylcholine, which can be fused by the addition of wheat-germ agglutinin, are not fused by calcium ions [15]. Not only are the liposomes prepared from total lipids of human erythrocytes not affected by calcium ions alone (Fig. 1), but neither are the ghosts of erythrocytes (Fig. 5). This observation is in keeping with the findings of Zakai et al. [16] that the agglutinability and fusibility of human erythrocyte ghosts by calcium ions requires phosphate which is not present in our assay system. Moreover, magnesium ions which do

stimulate the lectin-induced aglutinability of erythrocyte ghosts (see Fig. 5) do not fuse them even in the presence of phosphate ion [16]. Redwood and Polefka [15] have recently reported that liposomes prepared with phosphatidylcholine and ganglioside can be fused in the presence of wheat-germ agglutinin. However, Redwood et al. [17] has previously shown that this lectin agglutinates and does not fuse liposomes prepared with phosphatidylcholine and glycophorin, a glycoprotein extracted from erythrocyte membranes. Our system of liposomes prepared from total lipids of human erythrocytes do, in fact, agglutinate and do not fuse. This is supported by the electron micrograph of the liposomes reversed by the addition of acetylgalactosamine to the lectin-agglutinated system (Fig. 9) and the reversal by the appropriate sugar of the absorbance of the suspension (Fig. 1). Our results show that our multilamellar liposome system can be used for agglutination studies in a manner similar to unilamellar vesicle preparations [17].

Acknowledgements

This work was supported by grants CA-15823 and CA-14313 from the National Institutes of Health.

References

1 Lis, H. and Sharon, N. (1973) Annu. Rev. Biochem. 42, 541—574
2 Surolia, A., Bachhawat, B.K. and Podder, S.K. (1975) Nature 257, 802—804
3 Rendi, R., Kuettner, C.A. and Gordon, J.A. (1976) Biochem. Biophys. Res. Commun. 72, 1071—1076
4 Nicolson, G.L. (1974) Int. Rev. Cytol. 39, 89—190
5 Van Deenen, L.L.M. and de Gier, J. (1974) in The Red Blood Cell (Surgenor, D.M., ed.), Vol. 1, edn. pp. 150—156, Academic Press, New York
6 Papahadjoupoulos, D. (1968) Biochim. Biophys. Acta 163, 240—254
7 Seaman, G.V.F., Vassar, P.S. and Kendall, M.J. (1969) Arch. Biochem. Biophys. 135, 356—362
8 Gordon, J.A., Blumberg, S., Lis, H. and Sharon, N. (1972) FEBS Lett. 24, 193
9 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119
10 Spies, J.R. (1957) Methods Enzymol. 3, 468
11 Rendi, R. and Gordon, J.A. (1977) Exp. Cell Res. 110, 263—266
12 Papahadjoupoulos, D., Vail, W.J., Newton, C., Nir, S., Jacobson, K., Poste, G. and Lazo, R. (1977) Biochim. Biophys. Acta 465, 579
13 Rothman, J.E. and Lenard, J. (1977) Science 195, 743—753
14 Day, E.P., Ho, J.T., Kunze, Jr., R.K. and Sun, S.T. (1977) Biochim. Biophys. Acta 470, 503—508
15 Redwood, W.R. and Polefka, T.G. (1976) Biochim. Biophys. Acta 455, 631—643
16 Zakai, N., Kulka, R.G. and Loyter, A. (1976) Nature 263, 696—699
17 Redwood, W.R., Jansons, V.K. and Patel, B.L. (1975) Biochim. Biophys. Acta 406, 347—361