Agglutination and Fusion of Globoside GL-4 Containing Phospholipid Vesicles Mediated by Lectins and Calcium Ions†

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ABSTRACT: We have investigated the interaction of five *N*-acetylgalactosamine (GalNAc) specific lectins with the glycosphingolipid globoside GL-4, inserted into phospholipid vesicles composed of phosphatidylethanolamine and phosphatidic acid, with respect to their ability to induce vesicle agglutination, fusion, and destabilization. The following lectins were used: soybean agglutinin (SBA); *Sophora japonica* agglutinin (SJA); *Helix pomatia* agglutinin (HPA); *Ricinus communis* agglutinin II (RCA_{II}); and *Codium fragile* agglutinin (CFA). SBA and SJA caused rapid vesicle agglutination while HPA, CFA, and RCA_{II} were ineffective. However, in the presence of RCA_{II}, but not HPA and CFA, the addition of Ca^{2+} caused vesicle agglutination which was specifically inhibited by the haptenic sugar GalNAc, while ethylenediaminetetraacetic acid (EDTA) dissociated the vesicle complex. RCA_{II}/Ca^{2+}-induced vesicle agglutination was accomplished by binding of Ca^{2+} to RCA_{II} *after* the lectin/receptor interaction. The rate of SBA-induced vesicle agglutination was increased in the presence of Ca^{2+}, independent of the order of Ca^{2+} addition, and was not reversed by EDTA, indicating that the mechanism by which Ca^{2+} stimulated agglutination in this case was different from that observed in the presence of RCA_{II}. In contrast to RCA_{II}/Ca^{2+}, SBA/Ca^{2+} induced fusion of the vesicles, which occurred only when Ca^{2+} was added after lectin addition. Close approach of adjacent bilayers was accomplished by nonspecific interactions of SBA with the bilayer *after* lectin binding to the receptor as revealed by a limited extent of SBA-induced fusion and an enhanced membrane permeability upon lectin binding. The phenomena observed can be explained in terms of a Ca^{2+}-modulated reorientation of the carbohydrate head group, causing it to adopt a more perpendicular orientation with respect to the plane of the bilayer. The results suggested that Ca^{2+} exerted its effect indirectly, and the involvement of (local) bilayer dehydration in this process, as a result of the Ca^{2+}/phosphatidic acid interaction, is discussed.

Cell membranes are prone to fusion in such specialized events as mitosis, sperm/egg fusion during fertilization (Shapiro & Eddy, 1980), and upon formation of macrophage giant cells during certain types of immune response (Papadimitriou, 1978). Intracellularly, membrane fusion is involved in the translocation of membrane components between various subcellular organelles and in processes like exocytosis (Pollard et al., 1979) and certain types of viral infection (White et al., 1981). It seems reasonable to assume that membrane fusion is carefully regulated and requires specific receptor molecules which would function as sites for membrane recognition and contact and hence mediate the fusion process. Since glycoproteins and/or glycolipids play an important role as mobile membrane receptors (Neufeld & Ashwell, 1980; Hakomori, 1981; Olden et al., 1982), it is conceivable that the carbohydrate part of these molecules may provide the specific code for intermembrane recognition. Intermembrane contact would then require molecules which specifically recognize carbohydrate moieties, can act as a cross-linker and, hence, establish membrane/membrane contact. The properties of lectins or lectin-like molecules (Goldstein & Hayes, 1978) fully meet such criteria and, moreover, are present in wide abundance both at the cell surface and in the intracellular environment (Olden et al., 1982; Barondes, 1981). In this respect, it is noteworthy that lectins and glycolipids have been implicated in the process of myoblast fusion (Gartner & Podleski, 1976; Herman & Fernandes, 1982; Whatley et al., 1976). Recently, evidence has also been presented on the involvement of a cell surface lectin, bindin, in sperm/egg adhesion as an initial step toward sperm/egg fusion during fertilization (Glabe et al., 1982). In addition, the biological effects of lectin/glycolipid receptor interactions are of considerable interest because of similarities with receptor-mediated binding to membranes of hormones (Schechter et al., 1979; Fishman & Brady, 1976), toxins (Sandvig et al., 1976; Fishman, 1982), and viruses (Haywood & Boyer, 1982; Holmgren et al., 1980). Despite the vast knowledge about biological functions of glycolipids, the understanding of their effects on the local membrane structure, particularly during their interaction with specific ligands, and on the physical properties of membrane constituents is rather modest. However, insight into the molecular details concerning these aspects has greatly benefitted from the employment of artificial phospholipid vesicles containing glycolipids with carbohydrate moieties to which particular lectins specifically bind (Orr et al., 1979; Hampton et al., 1980; Peters et al., 1982; Mansson & Olofsson, 1983; Banerjee & Sen, 1983). In this investigation, we have followed a similar approach by studying the interaction of five lectins, recognizing terminal *N*-acetyl-D-galactosaminyl moieties (Sandvig et al., 1976; Hammarström et al., 1977; Sharon, 1980), with phospholipid vesicles comprised of phosphatidylethanolamine (PE),[1] phosphatidic acid (PA), and the major neutral glycosphingolipid of erythrocyte membranes (Sweeley & Siddiqui,

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1977), globoside GL-4 [GalNAc(β1 → 3)Gal(α1 → 4)Gal(β1 → 4)Glc(β1 → 1)Cer], as the carbohydrate receptor. The lectins were soybean agglutinin (SBA), *Sophora japonica* agglutinin (SJA), *Helix pomatia* agglutinin (HPA), *Codium fragile* agglutinin (CFA), and *Ricinus communis* agglutin II (RCA_{II}). In order to obtain an indication of how carbohydrate head-group behavior relates to surrounding lipids, we have compared the lectins with respect to their ability to agglutinate the vesicles and the effect of ligand/receptor interaction on membrane integrity. Furthermore, we examined the ability of the vesicles to undergo fusion in the presence of Ca^{2+}, in both the absence and presence of the lectins.

Experimental Procedures

Materials. Egg phosphatidylethanolamine (PE) and phosphatidic acid (PA, transphosphatidylated from egg phosphatidylcholine) were purchased from Avanti Polar Lipids (Birmingham, AL). Globoside GL-4, GalNac(β1 → 3)Gal(α1 → 4)Gal(β1 → 4)Glc(β1 → 1)Cer (98% pure), was obtained from Supelco Inc. and used without further purification. The source of the various lectins was as follows: soybean agglutinin (SBA) and *Sophora japonica* agglutinin (SJA) were from Vector Laboratories Inc., Burlingame, CA; *Codium fragile* agglutinin (CFA) and *Helix pomatia* agglutinin (HPA) were products from Sigma; *Ricinus communis* agglutinin II (RCA_{II}, molecular weight 60000) was obtained from Miles-Yeda Ltd., Rehovot, Israel). $TbCl_3 \cdot 6H_2O$ was obtained from Alfa (Danvers, MA); dipicolinic acid (DPA, sodium salt) and nitrilotriacetic acid (NTA, sodium salt) were from Sigma. All other chemicals used were of the highest purity available.

Vesicle Preparation. Large unilamellar vesicles (LUV) were prepared by reverse-phase evaporation with some minor modifications (Düzgünes et al., 1983). The vesicles were sized to an average diameter of 0.1 μm by sequential extrusion through Unipore polycarbonate filters (Bio-Rad) of 0.2- and 0.1-μm diameter, respectively. Vesicles comprised of PE, PA, and GL-4 (molar ratio 3.5:1:0.45, respectively) were made in one of the following aqueous media: (i) Tb-containing vesicles, 2.5 mM $TbCl_3$/50 mM NTA (sodium salt)/5 mM TES, pH 7.4; (ii) DPA-containing vesicles, 50 mM DPA (sodium salt)/20 mM NaCl/5 mM TES, pH 7.4; (iii) Tb/DPA-containing vesicles, 1.25 mM $TbCl_3$/25 mM NTA/25 mM DPA/10 mM NaCl/5 mM TES, pH 7.4. Nonencapsulated material was removed by gel filtration on Sephadex G-75 (Pharmacia), using 100 mM NaCl/1 mM EDTA/5 mM TES, pH 7.4, as the elution buffer.

Vesicle Agglutination Measurements. Agglutination of the glycolipid-containing vesicles was determined by continuously monitoring the turbidity changes at 450 nm (Hoekstra et al., 1980) using a Beckman Model 34 spectrophotometer. The absorbance change obtained 2 min after induction of vesicle agglutination by adding the lectin was taken as a measure of the initial rate of agglutination.

Fusion Measurements. Fusion measurements were carried out with a 1:1 mixture of Tb- and DPA-containing vesicles in 100 mM NaCl/0.1 mM EDTA/5 mM TES (pH 7.4) at a final lipid concentration of 50 μM. The formation of the highly fluorescent $Tb(DPA)_3^{3-}$ complex, resulting from intermixing of the aqueous contents inside the fusing vesicles (Wilschut et al., 1980; 1983; Hoekstra, 1982a; Düzgünes et al., 1983), was monitored continuously with an SLM 4000 spectrofluorometer equipped with a chart recorder. Samples were excited at 276 nm and monitored at 545 nm, using a cutoff filter (>530 nm) between the sample and the emission monochromator. The sample chamber was equipped with a magnetic stirrer, and the temperature was controlled with a thermostated circulating water bath at 23 °C. Tb fluorescence calibration was done (using Tb-containing vesicles, freed from EDTA by gel filtration) by lysis of 25 μM Tb-containing vesicles with 0.5% (w/v) sodium cholate in the presence of excess free DPA (20 μM), followed by sonication to ensure complete release of contents. The rate of fusion was calculated as the percentage of the total Tb fluorescence obtained (percent maximum per minute).

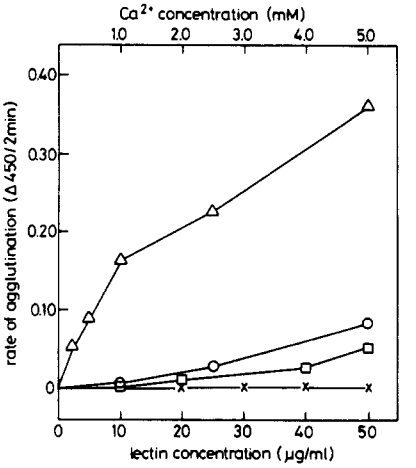

FIGURE 1: Rate of vesicle agglutination induced by various lectins as a function of the lectin concentration. PE/PA/GL-4 vesicles (molar ratio 3.5:1.0:0.45) were suspended in 100 mM NaCl/0.1 mM EDTA/5 mM TES, pH 7.4, at a final lipid concentration of 50 μM. The lectins, SBA (△), SJA (○), CFA (×), HPA (×), or RCA_{II} (×), were injected into the medium, and turbidity changes at 450 nm were monitored continuously. The rate of agglutination was determined as described under Experimental Procedures. For comparison, the rate of vesicle aggregation in the presence of various Ca^{2+} concentrations (□) is also shown. The experiments were performed at 23 °C.

Determination of Membrane Permeability. Membrane permeability changes, induced by lectin/glycolipid interaction or resulting from vesicle fusion, were determined by monitoring the release of the entrapped fluorescent Tb/DPA complex in parallel experiments. Leakage was revealed as a decrease in fluorescence intensity, caused by the instantaneous dissociation of the chelation complex by EDTA in the external medium as described elsewhere (Hoekstra et al., 1983).

Results

Ability of Various Lectins To Induce Vesicle Aggregation. Lectin-induced agglutination of glycolipid-containing vesicles results in an increase in the turbidity of the vesicle suspension which can be monitored continuously by measuring the absorbance at 450 nm (Hoekstra et al., 1980; Curatolo et al., 1978). As shown in Figure 1, the rate of agglutination of GL-4-containing PE/PA vesicles was dependent on the source of the lectin and increased with increasing lectin concentration. SBA readily induced agglutination of the vesicles, and an increase in turbidity was observed at a concentration as low as 2 μg/mL (the lowest lectin concentration tested). SJA also caused the vesicles to agglutinate although, relative to SBA, to a lesser extent, while the rate was also considerably slower. At the highest lectin concentration tested (50 μg/mL), the

[1] Abbreviations: PA, phosphatidic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; GL-4, globoside GL-4 [GalNAc(β1→3)Gal(α1→4)Gal(β1→4)Glc(β1→1)ceramide]; GalNAc, *N*-acetylgalactosamine; DPA, dipicolinic acid; NTA, nitrilotriacetic acid; SBA, soybean agglutinin; SJA, *Sophora japonica* agglutinin; CFA, *Codium fragile* agglutinin; RCA_{II}, *Ricinus communis* agglutinin, type II; HPA, *Helix pomatia* agglutinin; LUV, large unilamellar vesicle(s); TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; OD, optical density.

maximal absorbance reached was only 15% of that attained in the presence of SBA. Although quantitatively different, SBA-induced agglutination and SJA-induced agglutination of the vesicles were very similar with respect to their kinetics as revealed by first-order plots of the data (ln $[(A_\infty - A_t)/(A_\infty - A_0)]$ vs. time, A_∞ and A_t being the absorbances at $t = \infty$ and t, not shown). A typical bimodal agglutination curve was obtained, showing an initial rapid phase followed by a pseudo-first-order process. Similar kinetic behavior has been reported for lectin-induced agglutination of phospholipid vesicles containing various glycolipids (Maget-Dana et al., 1977, 1981) or a glycoprotein (Chicken & Sharom, 1983). Vesicle agglutination was not induced by HPA, CFA, or RCA_{II}, even at lectin concentrations as high as 150 μg/mL and after prolonged (30 min) times of incubation. (It should be noted that both HPA and CFA caused agglutination of human red blood cells at comparable lectin concentrations, while agglutination was inhibited by GalNAc; not shown.)

PE/PA vesicles aggregate extensively in the presence of Ca^{2+}, and aggregation is virtually completed within 2 min after addition of the cation (Sundler et al., 1981). By contrast, Ca^{2+}-induced aggregation of GL-4-containing PE/PA vesicles (Figure 1) was only minimal, and, compared to lectin-induced agglutination of the vesicles, the absorbance level obtained was less than 10% of the maximal absorbance reached in the presence of SBA (not shown). Apparently, the protrusion of the globoside carbohydrate structure at the vesicle surface profoundly influenced the ability of Ca^{2+} to induce vesicle aggregation.

Effect of Ca^{2+} on Lectin-Induced Vesicle Agglutination. As indicated in Figure 1, both RCA_{II} (≤150 μg/mL) and low Ca^{2+} concentrations (≤1.0 mM) did not agglutinate the globoside-containing vesicles. However, agglutination was observed when 1 mM Ca^{2+} was added to the vesicles after their preincubation with various lectin concentrations (Figure 2A). The combined lectin/cation effect on vesicle agglutination became apparent at a threshold RCA_{II} concentration of approximately 50 μg/mL, indicating that a threshold number of liposomal receptor sites had to be occupied before agglutination occurred. The extent of agglutination was only slightly enhanced (approximately 10%) when the Ca^{2+} concentration was further increased at a constant RCA_{II} concentration (not shown). Furthermore, the combined action of the lectin and Ca^{2+} was critically dependent on the order of addition. Thus, vesicle agglutination was not accomplished when (i) Ca^{2+} was added prior to the addition of RCA_{II} or when (ii) Ca^{2+} and RCA_{II} were preincubated, followed by the addition of the vesicles to the incubation mixture. One millimolar Ca^{2+} did not modulate the properties of the other lectins, with respect to the rate or extent of agglutination. However, higher Ca^{2+} concentrations greatly stimulated SBA-induced agglutination as shown in figure 2B, whereas no effect was seen on HPA and CFA activity (not shown). The Ca^{2+}-facilitated increase of SBA-induced agglutination could not be accounted for by a cumulative effect of the lectin and Ca^{2+} when added separately, since Ca^{2+} per se induced very little agglutination (Figure 1). Furthermore, the stimulating effect of Ca^{2+} was most pronounced at lower lectin concentrations (≤30 μg/mL) and became progressively less when the lectin concentration was raised. At 50 μg/mL SBA, an effect of Ca^{2+} was no longer observed, even at cation concentrations as high as 5 mM. These results suggested that Ca^{2+} did not interfere with SBA activity per se, as in the case of RCA_{II}-facilitated agglutination, but instead indicated that agglutination was accelerated by virtue of Ca^{2+} binding to the phospholipid bilayer,

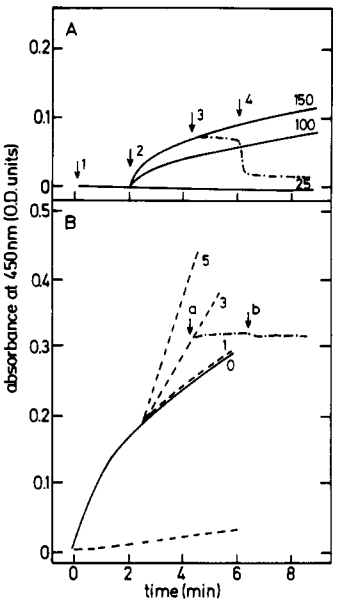

FIGURE 2: Effect of Ca^{2+} on lectin-induced agglutination. (A) At $t = 0$ (arrow 1), various concentrations of RCA_{II} (25, 100, or 150 μg/mL) were added to the vesicle suspension. After 2 min, 1 mM Ca^{2+} (arrow 2) was injected into the incubation mixture. In a parallel experiment, 200 μM GalNAc (arrow 3) and, subsequently, 2 mM EDTA (arrow 4) were added during the time course of vesicle agglutination in the presence of 150 μg/mL RCA (–·–). (B) Agglutination was induced by adding 10 μg/mL SBA to the vesicles at $t = 0$. The kinetics of agglutination (solid curve, 0) were followed continuously by monitoring A_{450}. Various Ca^{2+} concentrations (1, 3, and 5 mM) were added during the time course of agglutination (dashed curves). In addition, the effect of GalNAc (200 μM, arrow a) and EDTA (6 mM, arrow b) on SBA-induced agglutination in the presence of 3 mM Ca^{2+} is shown (–·–). The figure is a composite of five separate experiments. The lower dashed curve indicates the kinetics of vesicle aggregation induced by 5 mM Ca^{2+} in the absence of SBA.

presumably involving the negatively charged PA molecules. This suggestion would imply that, in contrast to the combined action of Ca^{2+} and RCA_{II}, an increase in the rate of SBA-induced agglutination should be independent of the order of addition of Ca^{2+} and SBA. Indeed, this proved to be the case. The enhancement in the rate of SBA-induced agglutination (up to 30 μg/mL) by Ca^{2+}, relative to the rate obtained in the presence of SBA alone, appeared to be very similar (approximately 2–3-fold), irrespective of the order of Ca^{2+} addition. The effect of Ca^{2+} on SBA-induced agglutination not only resulted in an enhancement of the rate of agglutination but also increased the extent of agglutination. As revealed by a double-reciprocal plot of absorbance vs. time (not shown), a nearly 2.7-fold increase in the maximal absorbance was obtained, when comparing the absorbance level obtained in the presence of Ca^{2+}/SBA to that observed with SBA alone.

Effect of GalNAc and EDTA on Lectin/Ca^{2+}-Induced Vesicle Agglutination. Addition of the haptenic sugar GalNAc prior to SBA, SJA, or RCA_{II}/Ca^{2+} (Figure 2A) completely prevented vesicle agglutination, whereas lactose and galactose were ineffective. These results indicated that lectin-induced agglutination was mediated by the specific interaction of the lectins with the terminal GalNAc residue of the carbohydrate structure of the glycolipid receptor. Upon addition of GalNAc (up to 1.5 mM) during the course of lectin-induced agglutination (Figure 2A, arrow 3), vesicle cross-linking was arrested instantaneously, but no reversal in turbidity was observed. These results indicated that the lectin/globoside complex did not dissociate and that GalNAc primarily interacted with nonbound lectin still present in bulk solution, thus preventing

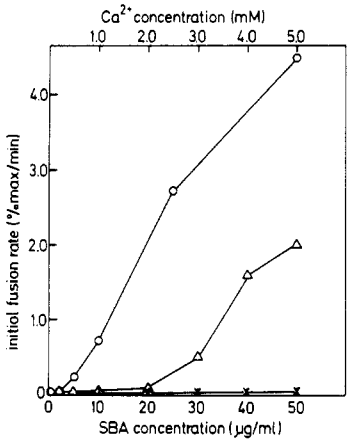

FIGURE 3: SBA-induced fusion of globoside-containing PE/PA vesicles. Various amounts of SBA were added to an equimolar mixture of Tb- and DPA-containing vesicles, and the formation of the fluorescent Tb/DPA complex was monitored. Experimental conditions were as described under Experimental Procedures. The initial fusion rates were determined and plotted as a function of the SBA concentration (○). For comparison, the data for Ca^{2+}-induced fusion (in the absence of lectin) were included in the figure (△, upper scale). Fusion was not observed upon addition of various concentrations of SJA, or upon preincubation of the vesicles with RCA_{II} followed by 1 mM Ca^{2+} (×). Lectin concentrations in the latter experiments were the same as those used for SBA.

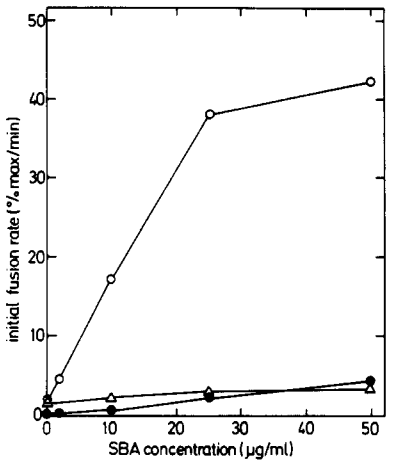

FIGURE 4: Effect of SBA concentration and order of SBA and Ca^{2+} addition on Ca^{2+}-induced fusion. Globoside-containing PE/PA vesicles were incubated with various amounts of SBA during a time interval of 1.5 min. Then, 5 mM Ca^{2+} was injected into the medium, and fusion was monitored with time. The initial fusion rate (○) was determined and plotted as a function of the SBA concentration. Alternatively, vesicles were incubated with 5 mM Ca^{2+} prior to the addition of SBA (△). For comparison, the initial rates of SBA-induced fusion (in the absence of Ca^{2+}) are included (●).

its time-dependent binding (Ketis & Grant, 1983) to the receptor site and subsequent vesicle agglutination. However, in the case of RCA_{II}/Ca^{2+}-induced agglutination, a virtually complete reversal in turbidity was accomplished upon addition of EDTA (Figure 2A, arrow 4); an essentially similar drop in A_{450} was observed when EDTA was injected into the medium prior to GalNAc (not shown). The effect of EDTA on Ca^{2+}-facilitated agglutination induced by SBA was distinctly different. In this case, EDTA reduced the rate of Ca^{2+}/SBA-induced agglutination to the rate obtained by the lectin alone. In a typical experiment, vesicles were agglutinated by 25 μg/mL SBA. During the first minute, the turbidity increased to 0.10 OD unit. Then 5 mM Ca^{2+} was added, and the agglutination rate increased to 0.16 OD unit/min. Subsequently, 7.5 mM EDTA was added, and the rate flattened off to 0.11 OD unit/min, which was essentially similar to the rate obtained prior to addition of Ca^{2+}. Interestingly, EDTA did *not* reverse vesicle agglutination (Figure 2B, arrow b). Thus, in contrast to RCA_{II}/Ca^{2+}, SBA/Ca^{2+}-induced vesicle agglutination apparently caused the formation of larger, obviously more stable, vesicle structures. A possible explanation for the lack of EDTA-induced dissociation of the vesicle complex in the latter case could be the occurrence of vesicle fusion under these conditions.

Fusion of GL-4-Containing PE/PA Vesicles. The presence of GL-4 in PE/PA bilayers strongly inhibited (approximately 90%, not shown) Ca^{2+}-induced fusion of such vesicles, relative to fusion of PE/PA vesicles devoid of the globoside. Presumably, the bulky oligosaccharide protrusions on the vesicle surface prevented the bilayers from coming into close proximity, a necessary, though not sufficient, requirement for vesicle fusion to occur (see below). Interestingly, when vesicles were agglutinated by SBA in the absence of Ca^{2+}, this process was accompanied by a limited, though significant, fusion event, as shown in Figure 3. On a molar basis, the fusion-inducing capacity of the lectin (0.04–0.4 μM) proved to be even more effective than that of the cation (2–5 mM). The fusogenic capacity of SBA did not appear to reflect a general, intrinsic characteristic of lectins with respect to their interaction with the vesicles used in this study. Neither SJA nor the combined action of RCA and Ca^{2+}, although inducing vesicle agglutination, caused vesicle fusion (Figure 3). The inability of RCA_{II}/Ca^{2+} to induce fusion was thus consistent with the observation (Figure 2A) that EDTA caused the dissociation of the agglutinated vesicle complex.

Relative to fusion induced by Ca^{2+} alone, a 6–8-fold increase in the initial fusion rate was observed when Ca^{2+} was added after preaggregation of the vesicles by 10 μg/mL SBA. For example, at 2 and 5 mM Ca^{2+}, the initial rates of vesicle fusion were 0.2% and 2.3% max/min, respectively, while addition of Ca^{2+} to vesicles, which had been aggregated by 10 μg/mL SBA, showed initial fusion rates of 1.3% and 17.6% max/min at 2 and 5 mM Ca^{2+}, respectively (not shown). Clearly, these results were in accordance with the above suggestion that the combined action of SBA and Ca^{2+} had resulted in substantial fusion as inferred from the inability of EDTA to reverse vesicle agglutination (Figure 2B). A further enhancement in the initial fusion rate was accomplished when the lectin concentration was increased to 50 μg/mL (Figure 4) at a constant Ca^{2+} concentration of 5 mM.

As demonstrated above, vesicle agglutination induced by SBA was facilitated by Ca^{2+} independent of the order of additions. Remarkably, the enhancement of fusion required the addition of Ca^{2+} *after* addition of the lectin. Thus, as shown in Figure 4, prior addition of 5 mM Ca^{2+} followed by the lectin (△) did not result in an enhancement of the initial fusion rate. Rather, the vesicles fused with initial rates very similar to those found in the absence of Ca^{2+} (●). From these results, it can be concluded that agglutination by itself did not suffice to induce fusion but, presumably, had to be accompanied by a lectin-induced modulation of the bilayer taking place before the addition of Ca^{2+}. Such a modulation, most likely involving a lectin/glycolipid receptor interaction, would thus represent the rate-limiting step for the occurrence of fusion.

Effect of Inhibitors on Vesicle Fusion Induced by the Combined Action of SBA and Ca^{2+}. As shown in Figure 5, addition of GalNAc before SBA and Ca^{2+} prevented the fusion of the vesicles. Interestingly, when GalNAc was added during the course of the fusion process, *no* inhibition was observed, whereas agglutination was arrested instantaneously (Figure

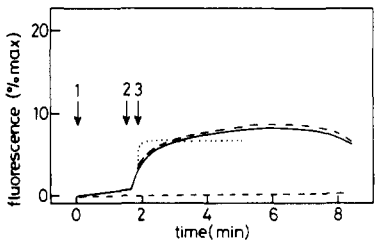

FIGURE 5: Effect of EDTA and GalNAc on SBA/Ca^{2+}-induced fusion of globoside-containing vesicles. The time course of fluorescent Tb/DPA complex formation (solid curve) was monitored as a measure of fusion induced by addition of 10 μg/mL SBA (arrow 1) followed by 5 mM Ca^{2+} (arrow 2). In separate experiments, 400 μM GalNAc (arrow 3, dashed curve) or 10 mM EDTA (arrow 3, dotted curve) was subsequently added to the incubation mixture. The lower dashed curve was obtained when 200 μM GalNAc and 10 mM EDTA were added to the vesicles prior to addition of SBA and Ca^{2+}.

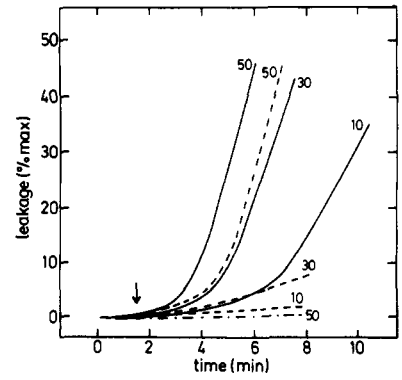

FIGURE 6: Perturbation of membrane permeability upon lectin/globoside interaction in the presence and absence of Ca^{2+}. PE/PA/GL-4 vesicles were prepared containing the fluorescent Tb/DPA complex. Various amounts of SBA (micrograms per milliliter, as indicated on the curves) were added to the vesicle suspension at $t = 0$. After 1.5 min (arrow), 5 mM Ca^{2+} (solid curves) or the same volume of buffer (dashed curves) was injected into the medium. Membrane permeability changes were recorded as a function of time by monitoring the quenching of Tb/DPA fluorescence as a result of complex dissociation by EDTA and, if present, Ca^{2+}, as described under Experimental Procedures. The curve, designated by (–·–), was obtained when the same experiment was done, using 50 μg/mL SJA, in either the presence or the absence of 5 mM Ca^{2+}.

2B). This observation provided further support for the above conclusion, that fusion represented the rate-limiting step in the overall process of agglutination and fusion, induced by the combined action of SBA and Ca^{2+}.

EDTA inhibited the fusion reaction, when added both prior (Figure 5, arrow 3) or after (not shown) addition of GalNAc, indicating that Ca^{2+} was the driving force in the fusion event. Rather intriguing was the observation that upon addition of EDTA the Tb/DPA fluorescence level increased instantaneously before leveling off. At present, we have no explanation for this peculiar effect. A similar effect was observed upon EDTA-induced inhibition of fusion of vesicles consisting of PE, PA, and disialoganglioside induced by the combined action of wheat germ agglutinin and Ca^{2+} (N. Düzgünes, unpublished results), and calcium phosphate induced fusion of erythrocyte ghosts (Hoekstra et al., 1983). Since the fluorescence level obtained after addition of the chelator did not change over a time interval of at least 1 h, the Tb/DPA complex was evidently separated from the external EDTA-containing medium, thus excluding the possibility that the observed increase was due to leakage of vesicle contents (see below).

The transient nature of Tb fluorescence in the absence of EDTA (solid curve) probably reflected the leakage of vesicle contents and possibly the entry of Ca^{2+} and/or EDTA into the vesicles, two events which would result in dissociation of the fluorescent Tb/DPA complex. This increase in membrane permeability could have resulted from the instability of the final fusion product but could also have been caused by membrane modulations resulting from lectin/receptor interactions (see above). To examine these possibilities, the fluorescent Tb/DPA complex was encapsulated into the vesicles, and the kinetics of leakage, as revealed by fluorescence quenching (see Experimental Procedures), were monitored upon addition of the lectins and/or Ca^{2+}.

Effect of Lectin/Glycolipid Interaction on Vesicle Bilayer Integrity. Leakage of vesicle contents was induced during the course of SBA-mediated vesicle agglutination, as shown in Figure 6 (dashed curves). During the early stages of agglutination, release of contents was virtually negligible. However, after approximately 1.5 min, membrane integrity became progressively perturbed with increasing lectin concentration. The apparent initial rates of leakage, which were calculated from the tangents to the curves at $t = 1.5$ min, were <0.3% max/min, 0.9% max/min, and 1.2% max/min at 10, 30, and 50 μg/mL SBA, respectively. Subsequently, an upward inflection in these curves became apparent, which was most pronounced at the higher lectin concentrations. Thus, 5–6 min after addition of 30 μg/mL SBA, the rate of leakage further increased to 2.8% max/min (calculated from the tangent to the steepest part of the curve), while in the presence of 50 μg/mL the rate of this secondary phase increased to 18.1% max/min. In contrast to SBA, both SJA (Figure 6) and the combined action of RCA_{II} and Ca^{2+} (not shown), although causing vesicle agglutination, did not disturb the stability of the vesicle membrane. In line with the stimulating effect of Ca^{2+} on SBA-induced vesicle agglutination and fusion, a similar effect was seen on membrane destabilization (Figure 6, solid curves). Prior incubation of the vesicles with SBA followed by the addition of 5 mM Ca^{2+} (arrow) significantly enhanced the apparent initial rate of leakage. The combined effect of SBA and Ca^{2+} on leakage was most pronounced at the lower lectin concentrations, since at the higher concentrations the lectin itself induced leakage. Relative to the rates obtained in the presence of SBA alone, subsequent addition of 5 mM Ca^{2+} resulted in a 2-fold increase of the initial rates (0.5%, 1.8%, and 2.3% max/min at 10, 30, and 50 μg/mL SBA, respectively). Similarly as observed in the absence of Ca^{2+}, after the initial slow release a rapid leakage phase became apparent, its induction time being dependent on the lectin concentration (Figure 6). The leakage rates obtained were 8.5%, 15%, and 19.1% max/min at 10, 30, and 50 μg/mL SBA, respectively. Interestingly, when similar experiments were performed with SJA, we observed also that the combined action of SJA and Ca^{2+} did not induce vesicle leakage. Finally, the order of addition did not appear to be a critical factor in Ca^{2+}/SBA-induced leakage of vesicle contents; i.e., the kinetics of leakage were essentially identical, irrespective of the order of Ca^{2+} and lectin addition.

The kinetics of SBA/Ca^{2+}-induced leakage as a function of the Ca^{2+} concentration are shown in Figure 7. The lectin concentration was 10 μg/mL. It should be noted that both 10 μg/mL SBA (Figure 6) and Ca^{2+} concentrations up to 5 mM (not shown), when incubated separately with the vesicles, did not induce significant leakage. Interestingly, the haptenic sugar GalNAc did not inhibit the release of contents (Figure 7, dashed line), suggesting that the release of contents was not associated with the process of vesicle agglutination, since agglutination was arrested by GalNAc (cf. Figure 2B). Addition of EDTA, however, immediately inhibited the release (Figure 7), indicating that Ca^{2+} played a prominent role in the actual

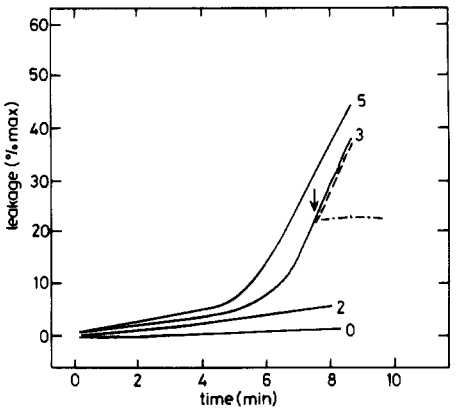

FIGURE 7: Alteration of membrane permeability after SBA/globoside interaction induced by various Ca^{2+} concentrations and the effect of inhibitors. Vesicles, containing the fluorescent Tb/DPA complex, were incubated with 10 μg/mL SBA (incubation time was 1.5 min). At $t = 0$, various Ca^{2+} concentrations were injected into the medium (0, 2, 3, and 5 mM Ca^{2+}), and Tb/DPA fluorescence quenching was monitored as a function of time. In parallel experiments, the effects of 6 mM EDTA (–·–) and 400 μM GalNAc (dashed curve) were examined on SBA/Ca^{2+} (3 mM)-induced release of contents.

cause of leakage of vesicle contents in this case.

Discussion

Dependence of Vesicle Agglutination on the Lectin Source. All lectins used in the present study display GalNAc binding specificity (Goldstein & Hayes, 1978; Sandvig et al., 1976; Hammarström et al., 1977; Sharon, 1980) and hence may interact with globoside GL-4. Therefore, the observation that multivalent SBA and SJA agglutinated PE/PA/GL-4 vesicles was expected. Due to its monovalency (Goldstein & Hays, 1978; Baenziger & Fiete, 1979), the inability of RCA_{II} to induce vesicle agglutination by itself is readily explained. However, the inability of both the multivalent HPA and CFA to cause vesicle agglutination was not anticipated. Differences in the reactivities of SBA and SJA on the one hand and CFA and HPA on the other, although containing specific interaction sites for GalNAc, could be explained in terms of a further specificity with respect to the stereochemical orientation of the terminal sugar moiety and, in addition, may include the size of the carbohydrate binding site, which need not necessarily be restricted to the size of a single monosaccharide (Goldstein & Hayes, 1978; Momoi et al., 1982). A detailed analysis by Hammarström et al. (1977) has shown that the HPA carbohydrate binding site corresponds to the size of a single monosaccharide, i.e., GalNAc. Furthermore, although HPA displays some anomeric specificity, binding to both α-anomers and β-anomers has been demonstrated (Hammarström et al., 1977). However, Hammarström et al. used soluble, carbohydrate-containing biopolymers and protein conjugates as lectin receptors, whereas in the present study the lectin receptor was incorporated in a lipid bilayer. It seems possible, therefore, that the interaction of certain lectins with specific receptors might not only be determined by factors as described above but also could be modulated by the macroscopic environment of the receptor, such as the bilayer in which the receptor is incorporated, which would be consistent with observations reported by others (Hampton et al., 1980; Mansson & Olofsson, 1983; Sundler, 1982; Wang & Edelman, 1978).

Effect of Ca^{2+} on Lectin-Induced Agglutination of Vesicles. RCA_{II}-mediated vesicle agglutination was only observed in the presence of Ca^{2+}, provided that the cation was added *after* the lectin (Figure 2A). On the other hand, Ca^{2+} stimulated SBA-induced vesicle agglutination, irrespective of the order of Ca^{2+} and lectin addition. These results indicated that the stimulating effect of Ca^{2+} on these lectins was accomplished by different mechanisms. The critical dependence of RCA_{II}-mediated agglutination on the order of addition revealed that (i) Ca^{2+} did not activate RCA_{II} in bulk solution by imparting a pseudomultivalent binding character to the lectin prior to its interaction with the receptor and (ii) Ca^{2+} was not required for RCA_{II} binding to the glycolipid molecule. Accordingly, it appears to be rather unlikely that Ca^{2+}, by virtue of its binding to the negatively charged PA molecule, alters the properties of the vesicles by enhancing the carbohydrate head-group accessibility to or reactivity with RCA_{II}. Moreover, even if an enhanced binding of RCA_{II} would have been accomplished in this manner, it would still be difficult to comprehend vesicle agglutination by a monovalent agglutinin. Rather, the results indicated that by binding to the RCA_{II} molecule *after* lectin/receptor binding, Ca^{2+} conveyed a pseudomultivalent binding character to the glycolipid-bound lectin molecule, which would suffice to cause vesicle agglutination. The presence of Ca^{2+} binding sites on the agglutinin can be inferred from the observation that preincubation of Ca^{2+} and RCA_{II} completely abolished the agglutination process, presumably as a result of a modification of the conformational structure of the lectin, thus explaining the required order of addition. The validity of this proposal, implicating that both RCA_{II} (by providing the Ca^{2+} binding site) and Ca^{2+} (by establishing intervesicular cross-links via adjacent RCA_{II} molecules) were involved in the actual agglutination process, was further supported by the observations that the haptenic sugar GalNAc inhibited vesicle agglutination, while EDTA dissociated the aggregated vesicle complex.

As to SBA-induced vesicle agglutination, addition of Ca^{2+} not only caused an enhancement in agglutination but also resulted in vesicle fusion, suggesting that the stimulating effect of Ca^{2+} could partly be explained by cation binding to the negatively charged bilayer. Screening of negative surface charges, causing a decrease in interbilayer electrostatic repulsion, has been suggested to increase SBA-induced agglutination of liposomes prepared from total lipids of erythrocyte membranes (Rendi et al., 1979). Alternatively, Ca^{2+}/PA complexation may induce changes in the physical properties of the bilayer which could subsequently affect the local environment of the glycolipid receptor and hence alter glycolipid/receptor interactions (Hampton et al., 1980; Sundler, 1982). It is possible that the addition of Ca^{2+} facilitated the binding of the lectin since the rate and extent of agglutination increased (similarly as observed when increasing the lectin concentration per se), while EDTA did not reverse vesicle agglutination but rather diminished the rate of SBA-induced agglutination to the rate observed in the presence of SBA alone. This indicated that Ca^{2+} was not directly involved in establishing cross-links between adjacent vesicles, as observed in the case of RCA_{II}.

Role of SBA and Ca^{2+} in the Fusion of GL-4-Containing Vesicles. For vesicle fusion to occur, a close intermembrane contact of apposing surface regions is required in order to overcome the strong repulsive hydration forces (Cowley et al., 1978; Portis et al., 1979; Hoekstra, 1982b). In the case of PS vesicles, the formation of an anhydrous Ca^{2+}/PS complex of specific stoichiometry, possibly serving as the trigger for membrane fusion, has been suggested (Ekerdt & Papahadjopoulos, 1982), while a similar proposal has been described for PA-containing vesicles (Sundler et al., 1981). Interestingly, the insertion of GL-4 into PS bilayers inhibits both the for-

mation of the specific Ca^{2+}/PS complex (Ekerdt & Papahadjopoulos, 1982) and fusion (Düzgünes et al., 1984). The observation that Ca^{2+} (in the absence of SBA) caused very little fusion of GL-4-containing PE/PA vesicles (Figure 3) suggests that, analogous to the PS system, the presence of the globoside prevented the formation of the specific and fusion-triggering Ca^{2+}/PA complex. This effect of the globoside can be explained in terms of an interference of the bulky and strongly hydrated carbohydrate head group (Pasher, 1976) with the process of close approach.

Addition of SBA *prior to* Ca^{2+} induced an interbilayer contact sufficiently close to allow for the immediate formation of a trans Ca^{2+}/PA complex, as revealed by the occurrence of fusion (Figure 4). Remarkably, when the order of SBA and Ca^{2+} addition was reversed, the stimulating effect on fusion was abolished. On the other hand, SBA-induced agglutination was stimulated when Ca^{2+} was added either prior to or after the addition of the lectin. These results indicate that (i) the carbohydrate moiety played a regulatory role by determining the interbilayer distance of apposing membranes and that (ii) Ca^{2+} facilitated a spatial rearrangement of the carbohydrate chain of the globoside, affecting the interbilayer distance within the SBA-induced vesicle aggregate. Two observations in particular provided support for the latter suggestion. First, SBA by itself caused some membrane fusion (Figure 3), suggesting that the lectin, after binding to the receptor, interacted with the lipid bilayer (see below) such that it caused the bilayers to come into close, i.e., fusion-susceptible, proximity. Second, when Ca^{2+} was added to the vesicles that had been agglutinated by SBA (Figure 5), fusion started rapidly but levelled off rather abruptly, which could not be explained by the occurrence of vesicle leakage (cf. Figure 5 vs. Figure 7). It would thus appear that Ca^{2+} not only induced fusion but also, paradoxically, inhibited this process, most likely by an indirect effect on the spatial orientation of the carbohydrate/lectin complex which resulted in an increase of the intervesicular distance within the agglutinated vesicle system. This process may be envisaged as follows. The oligosaccharide chain in the aqueous phase can be displaced as much as 80° from its perpendicular position, relative to the plane of the bilayer (Maggio et al., 1981). In principle, such a displacement would permit a direct lectin/bilayer interaction as noted above, which moreover, was only accomplished *after* lectin/receptor interaction, since fusion of vesicles that did not contain GL-4 was not observed (not shown). This suggested that changes in the tertiary structure of the lectin were required for this interaction to occur. Indeed, as revealed by circular dichroism (Jirgensons, 1978), conformational changes in SBA are induced upon lectin/receptor interaction. As observed in the case of other lectins (Hampton et al., 1980; Wang & Edelman, 1978; Boldt et al., 1977; Bosch & McConnell, 1975; Grollman et al., 1977), nonspecific interactions could thus occur between the lectin and phospholipid bilayers, causing membrane perturbations which resulted in (some) fusion of vesicles of appropriate lipid composition (Figure 3; Bosch & McConnell, 1975) and/or leakage of vesicle contents (Figure 6; Grollman et al., 1977). Subsequent addition of Ca^{2+} would then induce fusion at the points of contact formed by the lectin in a manner as outlined above. Clearly, only a fraction of the vesicles fused, notwithstanding the fact that agglutination not only continued but also was considerably stimulated when Ca^{2+} was added. These considerations lead us to conclude that an increase in the interbilayer distance was induced upon addition of Ca^{2+}, which could be achieved by a more perpendicular reorientation of the carbohydrate head group (see below). Such a reorientation may increase the rate of lectin binding (Ketis & Grant, 1983) and thus would explain the observation that the stimulating effect of Ca^{2+} on SBA-induced agglutination was independent of the order of addition. With increasing interbilayer distance, the possibility of trans Ca^{2+}/PA complex formation and concomitant membrane fusion would be abolished. Therefore, fusion will only be expected when Ca^{2+} is added after the lectin, while the fraction of vesicles that become fused will depend on the extent of vesicle agglutination, before the addition of Ca^{2+}. The observation that GalNAc, when added after the lectin and Ca^{2+}, inhibited agglutination but not fusion is consistent with this notion.

A more perpendicular position of the oligosaccharide chains can be induced upon closer packing of adjacent glycolipid molecules (Maggio et al., 1981), as a result of either lectin-induced clustering of glycolipid molecules (Peters et al., 1982; Edelman, 1976; Suzuki et al., 1983) or Ca^{2+}-induced lipid phase separation (Hoekstra, 1982c). Alternatively, it seems possible that partial dehydration of the bilayer upon binding of Ca^{2+} to PA may induce a reorientation of the strongly hydrophilic carbohydrate head group, forcing it to tilt toward a more aqueous environment. The observation that SBA/Ca^{2+}-induced leakage of vesicle contents (Figure 7) was inhibited only upon addition of EDTA, but not by GalNAc, is consistent with such Ca^{2+}-induced membrane modulations.

In conclusion, the Ca^{2+}-facilitated steric reorientation of the carbohydrate head group would appear to be an important factor in determining the interbilayer distance and hence the degree of fusion between adjacent glycolipid-containing bilayers.

Registry No. Ca, 7440-70-2; GalNAc, 1811-31-0; globoside GL-4, 11034-93-8.

REFERENCES

Baenziger, J. V., & Fiete, D. (1979) *J. Biol. Chem. 254*, 9795–9799.

Banerjee, K. K., & Sen, A. (1983) *FEBS Lett. 162*, 248–251.

Barondes, S. H. (1981) *Annu. Rev. Biochem. 50*, 207–231.

Boldt, D. H., Speckart, S. F., Richards, R. L., & Alving, C. R. (1977) *Biochem. Biophys. Res. Commun. 74*, 208–214.

Bosch, V. d. J., & McConnell, H. M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4409–4413.

Chicken, C. A., & Sharom, F. J. (1983) *Biochim. Biophys. Acta 729*, 200–208.

Cowley, A. C., Fuller, N. L., Rand, R. P., & Parsegian, V. A. (1978) *Biochemistry 17*, 3163–3168.

Curatolo, W., Yau, A. O., Small, D. M., & Sears, B. (1978) *Biochemistry 17*, 5740–5744.

Düzgünes, N., Wilschut, J., Fraley, R., & Papahadjopoulos, D. (1981) *Biochim. Biophys. Acta 642*, 182–195.

Düzgünes, N., Wilschut, J., Hong, K., Fraley, R., Perry, C., Friend, D. S., James, T. L., & Papahadjopoulos, D. (1983) *Biochim. Biophys. Acta 732*, 289–299.

Düzgünes, N., Hoekstra, D., Hong, K., & Papahadjopoulos, D. (1984) *FEBS Lett. 173*, 80–86.

Edelman, G. M. (1976) *Science (Washington, D.C.) 192*, 218–226.

Ekerdt, R., & Papahadjopoulos, D. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2273–2277.

Fishman, P. H. (1982) *J. Membr. Biol. 69*, 85–97.

Fishman, P. H., & Brady, R. O. (1976) *Science (Washington, D.C.) 194*, 906–915.

Gartner, T. K., & Podleski, T. R. (1976) *Biochem. Biophys. Res. Commun. 67*, 972–978.

Glabe, C. G., Grabel, L. B., Vacquier, V. D., & Rosen, S. D. (1982) *J. Cell Biol. 94*, 123–128.

Goldstein, I. J., & Hayes, C. E. (1978) *Adv. Carbohydr. Chem. Biochem. 35*, 127–340.
Grollman, E. F., Lee, G., Ambesi-Impiombato, F. S., Meldosi, M. F., Aloj, S. M., Coon, H. G., Kaback, H. R., & Kohn, L. D. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2352–2356.
Hakomori, S. (1981) *Annu. Rev. Biochem. 50*, 733–764.
Hammarström, S., Murphy, L. A., Goldstein, I. J., & Etzler, M. E. (1977) *Biochemistry 16*, 2750–2755.
Hampton, R. Y., Holz, R. W., & Goldstein, I. J. (1980) *J. Biol. Chem. 255*, 6766–6771.
Haywood, A. M., & Boyer, B. P. (1982) *Biochemistry 21*, 6041–6046.
Herman, B. A., & Fernandes, S. M. (1982) *Biochemistry 21*, 3275–3283.
Hoekstra, D. (1982a) *Biochim. Biophys. Acta 692*, 171–175.
Hoekstra, D. (1982b) *Biochemistry 21*, 2833–2840.
Hoekstra, D. (1982c) *Biochemistry 21*, 1055–1061.
Hoekstra, D., Tomasini, R., & Scherphof, G. (1980) *Biochim. Biophys. Acta 603*, 336–346.
Hoekstra, D., Wilschut, J., & Scherphof, G. (1983) *Biochim. Biophys. Acta 732*, 327–331.
Holmgren, J., Svennerholm, L., Elwing, H., Fredman, P., & Strannegard, O. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1947–1950.
Jirgensons, B. (1978) *Biochim. Biophys. Acta 536*, 205–211.
Ketis, N. V., & Grant, C. W. M. (1983) *Biochim. Biophys. Acta 730*, 359–368.
Maget-Dana, R., Roche, A., & Monsigny, M. (1977) *FEBS Lett. 79*, 305–309.
Maget-Dana, R., Veh, R. W., Sander, M., Roche, A., Schauer, R., & Monsigny, M. (1981) *Eur. J. Biochem. 114*, 11–16.
Maggio, B., Cumar, F. A., & Caputto, R. (1981) *Biochim. Biophys. Acta 650*, 69–87.
Mansson, J., & Olofsson, S. (1983) *FEBS Lett. 156*, 249–252.
Momoi, T., Tokunaga, T., & Nagai, Y. (1982) *FEBS Lett. 141*, 6–10.
Neufeld, E. F., & Ashwell, G. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W. J., Ed.) pp 241–262, Plenum Press, New York.
Olden, K., Parent, J. B., & White, S. L. (1982) *Biochim. Biophys. Acta 650*, 209–232.
Orr, G. A., Rando, R. R., & Bangerter, F. W. (1979) *J. Biol. Chem. 254*, 4721–4725.
Papadimitriou, J. M. (1978) *Cell Surf. Rev. 5*, 181–218.
Pasher, I. (1976) *Biochim. Biophys. Acta 455*, 433–451.
Peters, M. W., Barber, K. R., & Grant, C. W. M. (1982) *Biochim. Biophys. Acta 693*, 417–424.
Pollard, H. B., Pazoles, C. J., Creutz, C. E., & Zinder, O. (1979) *Int. Rev. Cytol. 58*, 160–198.
Portis, A., Newton, C., Pangborn, W., & Papahadjopoulos, D. (1979) *Biochemistry 18*, 780–790.
Rendi, R., Vatter, A. E., & Gordon, J. A. (1979) *Biochim. Biophys. Acta 550*, 318–327.
Sandvig, K., Olsnes, S. S., & Phil, A. (1976) *J. Biol. Chem. 251*, 3977–3984.
Shapiro, B. M., & Eddy, E. M. (1980) *Int. Rev. Cytol. 66*, 257–302.
Sharon, N. (1980) in *Progress in Immunology* (Fougereau, M., & Dausset, J., Eds.) Vol. 1, pp 255–278, Academic Press, New York.
Shechter, Y., Chang, K., Jacobs, S., & Cuatrecasas, P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2720–2724.
Sundler, R. (1982) *FEBS Lett. 141*, 11–13.
Sundler, R., Düzgünes, N., & Papahadjopoulos, D. (1981) *Biochim. Biophys. Acta 649*, 751–758.
Suzuki, T., Inoue, K., Nojima, S., & Wiegandt, H. (1983) *J. Biochem. (Tokyo) 94*, 373–377.
Sweeley, C. C., & Siddiqui, B. (1977) in *The Glycoconjugates I* (Horowitz, A., & Pigman, W., Eds.) pp 475–506, Academic Press, New York.
Wang, J. L., & Edelman, G. M. (1978) *J. Biol. Chem. 253*, 3000–3007.
Whatley, R., Ng, S. K.-C., Rogers, J., McMurray, W. C., & Sanwal, B. D. (1976) *Biochem. Biophys. Res. Commun. 70*, 180–185.
White, J., Matlin, K., & Helenius, A. (1981) *J. Cell Biol. 89*, 674–679.
Wilschut, J., Düzgünes, N., Fraley, R., & Papahadjopoulos, D. (1980) *Biochemistry 19*, 6011–6021.
Wilschut, J., Düzgünes, N., Hong, K., Hoekstra, D., & Papahadjopoulos, D. (1983) *Biochim. Biophys. Acta 734*, 309–318.