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Activities of Lectins and Their Immobilized Derivatives in Detergent Solutions. Implications on the Use of Lectin Affinity Chromatography for the Purification of Membrane Glycoproteins†

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ABSTRACT: The effects of several commonly used detergents on the saccharide-binding activities of lectins were investigated using lectin-mediated agglutination of formalin-fixed erythrocytes and affinity chromatography of glycoproteins on columns of lectins immobilized on polyacrylic hydrazide-Sepharose. In the hemagglutination assays, *Ricinus communis I* (RCA_I) and *II* (RCA_{II}), concanavalin A (Con A), and the agglutinins from peanut (PNA), soybean (SBA), wheat germ (WGA), and *Limulus polyphemus* (LPA) were tested with several concentrations of zwitterionic, cationic, anionic, and nonionic detergents. It was found that increasing detergent concentrations eventually affected hemagglutination titers in both test and control samples, and the highest detergent concentrations not affecting lectin hemagglutinating activities were determined. The effects of detergents on specific binding of [^{3}H]fetuin and asialo[^{3}H]fetuin to and elution from columns of immobilized lectins were less severe when compared with lectins in solution, suggesting that the lectins are stabilized by covalent attachment to agarose beads. Nonionic detergents did not affect the binding efficiency of the immobilized lectins tested at concentrations used for membrane solubilization while cationic and zwitterionic detergents caused significant inhibition of Con A- and SBA-Sepharose activities. In sodium deoxycholate (>1%) only RCA_I-Sepharose retained its activity, whereas the activities of the other lectins were reduced dramatically. Low concentrations of sodium dodecyl sulfate (0.05%) inhibited only the activity of immobilized SBA, but at higher concentration (0.1%) and prolonged periods of incubation (16 h, 23 °C) most of the lectins were inactivated. These data are compared with previous reports on the use of detergents in lectin affinity chromatography, and the conditions for the optimal use of detergents are detailed.

The study of cell surface membrane glycoproteins has been facilitated by the use of lectins which bind saccharides in a highly specific manner (Sharon and Lis, 1972; Nicolson, 1974). Thus, the number and distribution of surface glycoproteins have been studied by employing radioactive, fluorescent, or electron-dense lectin derivatives (Nicolson, 1974; Sharon and Lis, 1975). For the study of the chemical nature of membrane glycoproteins, it is desirable to obtain them in a pure form. This is a complicated endeavor, because in the cell membranes that have been well characterized, glycoproteins are classified as integral membrane components (Singer and Nicolson, 1972); that is, they are presumed to be stabilized by hydrophobic forces and are not easily solubilized and separated from membrane lipids into aqueous, low ionic strength, neutral solutions. The use of chaotropic agents or detergents (for review, see: Helenius and Simmons, 1975), such as sodium dodecyl sulfate (Akedo et al., 1972; Susz et al., 1973; Gombos et al., 1974), Triton X-100 (Cuatrecasas, 1972; Findlay, 1974; Schmidt-Ullrich et al., 1975), and sodium deoxycholate (Allan and Crumpton, 1971; Allan et al., 1972; Hayman and Crumpton, 1972; Gurd and Mahler, 1974; Berzins and Bolmberg, 1975; Nachbar et al., 1976; Pitlick, 1976), enables effective solubilization of cell membrane components by breaking hydrophobic and ionic bonds. Once solubilized and "stabilized" in solution, integral membrane glycoproteins can be purified by conventional methods or by affinity chromatography on immobilized lectins. The latter technique has been applied to the fractionation and purification of glycoproteins from membranes of lymphocytes (Allan et al., 1972; Hayman and Crumpton, 1972), leukemic cells (Jansons and Burger, 1973), erythrocytes (Adair and Kornfeld, 1974; Findlay, 1974), brain cells (Susz et al., 1973; Gombos et al., 1974; Gurd and Mahler, 1974; Zanetta et al., 1975; Pitlick, 1976), platelets (Nachman et al., 1973), and viruses (Hayman et al., 1973).

Since lectins are made of subunits which, in many cases, are held together by noncovalent forces, detergents and chaotropic agents used for the solubilization of membrane components may dissociate the native lectin molecules and/or change their active conformation. In spite of the increasing use of lectin affinity chromatography for the isolation of membrane glycoproteins in detergent solutions, no detailed study has been undertaken to investigate the effects of detergents on the activities of lectins.

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Here we describe the effects of several detergents (cationic, anionic, zwitterionic, and nonionic) on the hemagglutinating activities of concanavalin A (Con A), *Ricinus communis* agglutinins *I* and *II* (RCA_I and RCA_{II}),[1] wheat germ agglutinin (WGA), peanut agglutinin (PNA), soybean agglutinin (SBA), and *Limulus polyphemus* agglutinin (LPA) as well as on the ability of immobilized lectins to specifically bind glycoproteins in the presence of detergents.

Materials and Methods

Saccharides and Glycoproteins. Methyl α-D-mannopyranoside and lactose were obtained from Sigma Chemical Co. (St. Louis, Mo.); other monosaccharides were products of Calbiochem (San Diego, Calif.). Bovine glycoprotein (fraction VI, grade II) and rabbit γ-globulin were obtained from Miles Laboratories (Elkhart, Ind.). Fetal calf fetuin was obtained from Sigma (type I) or from Grand Island Biological Co. (Grand Island, N.Y.). Keyhole limpet (*Megathura crenulata*) hemocyanin was a generous gift of Dr. R. G. Painter (Syntex, Palo Alto, Calif.).

Detergents. Poly(oxyethylene glycol(9–10))-*p*-*tert*-octylphenol (Triton X-100) was obtained from Bass Chemical Co. (San Diego, Calif.); poly(oxyethylene glycol (20))-sorbitol monooleate (Tween 80), lot 1-5807 was from Baker Chemicals (Phillipsburg, N.J.); poly(oxyethylene glycol (9))-*p*-*tert*-octylphenol (NP40) was from Particle Data Laboratories (Elmhurst, Ill.); sodium dodecyl sulfate and sodium deoxycholate were from Matheson Coleman and Bell (Norwood, Ohio) and lauryl dimethyl aminooxide (LDAO), lot 11-21-73, was from Onyx Chemical Co. (Jersey City, N.J.); dodecyltrimethylammonium bromide (C_{12}TABr) and tridecyltrimethylammonium bromide (C_{13}TABr) were synthesized as described by Hong and Hubbell (1973).

N,N-Dimethyl-*N*-dodecylglycine (DDGly) was prepared by the reaction of lithium bromoacetate (0.072 mol) and *N,N*-dimethyldodecylamine (0.072 mol) stirred in methanol (50 mL) at 4 °C. Following dissolution of the reactants, the solution was allowed to warm to room temperature and left to stand overnight. Methanol was removed in a rotary evaporator, and the thick liquid residue was triturated with dry ether. The resulting white solid suspension was collected by filtration, washed with ether, and dried under vacuum. Thin-layer chromatography on silica gel (developed with chloroform–methanol–acetic acid–water, 50:25:8:4) showed the product to be homogeneous, and it was used without further purification. This surfactant is also available commercially under the name Empigen BB (Allen and Humphries, 1975).

Lectins. All the lectins used were purified by affinity chromatography. Con A, obtained as a twice crystallized product (Calbiochem, San Diego, Calif.), was further purified on a column of Sephadex G-75 according to Agrawal and Goldstein (1967); *Ricinus communis* agglutinins were purified from castor beans by the procedure of Nicolson and Blaustein (1972). Further separation of the 120 000 molecular weight lectin (RCA_I) from the 60 000 molecular weight lectin (RCA_{II}) was achieved by gel filtration on a column of Sephadex G-100 as described (Nicolson et al., 1974). WGA was purified from wheat germ alkaline phosphatase (Worthington Biochemical Corp., Freehold, N.J.) on an ovomucoid-Sepharose column (Marchesi, 1972) or from commercial wheat germ on a column of 2-acetamido-*N*-(ε-aminocaproyl)-2-deoxy-β-D-glucopyranosylamine-Sepharose (Lotan et al., 1973). SBA and PNA were purified on a column of ε-aminocaproyl-β-D-galactopyranosylamine-Sepharose according to Gordon et al. (1972) and Lotan et al. (1975), respectively. *Limulus polyphemus* hemolymph agglutinin (LPA) was purified by the following procedures: *L. polyphemus* hemolymph (100 mL) was centrifuged at 90 000*g* for 2 h at 4 °C. The clear supernatant was concentrated to 15 mL (using Aquacide II, Calbiochem), applied to a column (2.5 × 90 cm) of Sephadex G-50, and eluted with 0.05 M Tris-HCl–0.1 M sodium chloride–0.01 M calcium chloride (pH 8.5) (buffer I). The fractions containing hemagglutinating activity (tested with horse erythrocytes) were pooled and applied to an affinity column of anti-LPA-Sepharose. Anti-LPA-Sepharose was prepared and used as follows: Antisera to LPA (purified by adsorption to and elution from formalin-fixed horse erythrocytes according to Nowak and Barondes (1975)) were raised in rabbits by footpad injection of 1 mg of purified LPA in 2 mL of complete Freund's adjuvant. Rabbits were boosted after the 3rd and 5th week with 0.4 mg each of LPA, and the rabbits were bled 1 week later. The immunoglobulin fraction containing anti-LPA was obtained by ammonium sulfate precipitation and the IgG fraction by DEAE-Sephadex chromatography (Winkelhake and Kasper, 1972). The latter anti-LPA was covalently coupled to Sepharose according to March et al. (1974). The partially purified LPA (pooled hemagglutinating fractions eluted from Sephadex G-50) was applied to an anti-LPA-Sepharose column (2.5 × 60 cm), and the column was washed with buffer I. LPA was eluted quantitatively with 2 M potassium iodide in buffer I concentrated by pressure dialysis and further purified on a column of Sephadex G-50. The latter gel filtration (conducted in 0.05 M Tris-HCl–0.1 M sodium chloride–0.01 M $CaCl_2$, pH 8.0) afforded two protein peaks of which the first contained all the hemagglutinating activity. This peak was concentrated and frozen at −20 °C.

Immobilization of Lectins. Lectin affinity columns were constructed using the high capacity, charge-free, and non-leaching polyacrylic hydrazide-Sepharose (Wilchek and Miron, 1974) as supporting matrix (available commercially, Miles Laboratories, Inc., Elkhart, Ind.). Lectins were coupled to glutaraldehyde-substituted polyacrylic hydrazide-Sepharose as follows: polyacrylic hydrazide-Sepharose (Wilchek and Miron, 1974; Wilchek et al., 1977) was suspended in distilled water (100 mL of packed gel in 150 mL of water), and the slurry was stirred while 50 mL of 50% glutaraldehyde was added. After an additional 4 h at 4 °C, the gel was washed with cold distilled water (1000 mL) until there was no detectable odor of glutaraldehyde. Lectins (WGA, SBA, PNA, or RCA_I) were dissolved in 0.1 M sodium bicarbonate–0.15 M sodium chloride (pH 8.5) containing 0.2 M of the appropriate saccharide inhibitor to give a protein concentration of 510 mg per mL. Con A (510 mg per mL) was dissolved in 1 M sodium chloride–0.1 M sodium acetate buffer, pH 6.8, containing 0.2 M methyl α-D-mannopyranoside. The lectin solutions were

[1] Abbreviations used: buffer I, 0.05 M Tris-HCl–0.1 M sodium chloride–0.01 M calcium chloride, pH 8.5; buffer II, 0.005 M sodium phosphate–0.1 M sodium chloride, pH 7.2; buffer III, 0.005 M sodium phosphate–0.15 M sodium chloride, pH 7.2; buffer IV, 0.01 M Tris-HCl–0.15 M sodium chloride, pH 7.2; Con A, concanavalin A; C_{12}TABr, dodecyltrimethylammonium bromide; C_{13}TABr, tridecyltrimethylammonium bromide; C_{16}TABr, hexadecyltrimethylammonium bromide; DDGly, dimethyldodecylglycine; DOC, sodium deoxycholate; GSPH-Sepharose, glutaraldehyde-substituted polyacrylic hydrazide-Sepharose; HA, hemagglutination; LDAO, lauryldimethylaminooxide; LPA, *Limulus polyphemus* hemolymph agglutinin; NP40, Nonidet P40; PNA, peanut agglutinin; RCA_I, *Ricinus communis I* agglutinin; RCA_{II}, *Ricinus communis II* agglutinin; SBA, soybean agglutinin; WGA, wheat germ agglutinin; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

TABLE I: Effect of Detergents on Agglutination of Formalin-Fixed Erythrocytes by Different Lectins.[a]

Lectin	Initial Lectin Concn (μg/mL)[b]	Highest Detergent Concn (%) Not Affecting Lectin-Mediated Agglutination								
		NP40	Triton X-100	Tween 80	DDGly	C_{12}TABr	C_{13}TABr	LDAO	DOC	$NaDodSO_4$
RCA_I[c]	1100	0.01	2.0	2.0	2.0	1.0	0.25	0.1	0.25	0.1
RCA_{II}[c]	1000	0.01	0.25	0.1	2.0	0.25	0.25	0.1	0.1	0.1
PNA[d]	1000	1.0	1.0	0.1	1.0	1.0	1.0	1.0	1.0	0.1
SBA[d]	1000	0.1	0.1	1.0	1.0	0.1	0.1	0.1	0.1	0.1
Con A[c]	750	0.5	2.0	1.0	1.0	1.0	0.5	0.25	0.25	0.2
WGA[c]	750	0.1	0.25	0.5	2.0	0.5	0.25	0.25	0.5	0.1
LPA[e]	380	0.01	2.0	1.0	0.5	2.0	0.5	0.1	0.25	0.1

[a] Lectin agglutination of formalin fixed erythrocytes in PBS2 (0.005 M sodium phosphate–0.15 M sodium chloride buffer, pH 7.2) containing various detergent concentrations was measured after 45 min at 23 °C (see Materials and Methods for additional details). Values represent an average of three or four determinations. [b] Lectin concentrations (giving titers of 512–1024 in PBS) were pretreated with indicated detergent concentrations for 60 min at 23 °C before serial dilution. [c] Agglutination performed with formalin-fixed rabbit erythrocytes. [d] Agglutination performed with neuraminidase-treated, formalin-fixed rabbit erythrocytes. [e] Agglutination performed with formalin-fixed horse erythrocytes in Tris–calcium chloride buffer instead of PBS2.

mixed overnight at 4 °C with the glutaraldehyde-substituted polyacrylic hydrazide-Sepharose (GSPH-Sepharose) at a ratio of 5 mg of lectin per mL of packed gel in 2 volumes of solvent. The gels were washed on a sintered-glass funnel, and the protein concentration in the wash solutions was determined from their absorbance at 280 nm. The lectin–GSPH-Sepharose conjugates were suspended in 0.005 M sodium phosphate–0.1 M sodium chloride, pH 7.2 (buffer II) (3 volumes of buffer per volume of packed gel) and solid sodium borohydride was added to a final concentration of 0.5 mg per mL. Reduction was allowed to proceed for 3 h at 4 °C, and the gels were washed extensively with buffer II. The efficiency of coupling was more than 85% with all the lectins used. Con A–GSPH-Sepharose was stored in 0.1 M sodium acetate–1.0 M sodium chloride, pH 6.0, containing 0.2 M methyl α-D-mannopyranoside; WGA–GSPH-Sepharose was stored in 0.1 M Tris-HCl–0.15 M sodium chloride, pH 7.2, containing 0.2 M *N*-acetyl-D-glucosamine, and the other lectin–GSPH-Sepharose gels were kept in PBS1 containing 0.2 M of the appropriate saccharide inhibitor.

Hemagglutination. Analysis of lectin-mediated hemagglutination (HA) in detergent solutions was performed with formalin-fixed rabbit or horse erythrocytes using microtiter techniques (Nicolson and Blaustein, 1972). Lectins (at concentrations giving titers of 512 to 1024 in 0.005 M sodium phosphate–0.15 M sodium chloride, pH 7.2 (buffer III)) were incubated in detergent solutions in buffer III for 60 min at 23 °C and then diluted serially in microtiter test trays (Cooke Engineering Co., Alexandria, Va.) containing the various detergents in a final volume of 25 μL. After 15-min incubation at 23 °C, a 25-μL aliquot containing 2×10^8 cells/mL of formalin-fixed rabbit (fixed in 2% formaldehyde for 4 h at 23 °C) or horse (fixed in 3% formaldehyde for 16 h at 37 °C) erythrocytes was added to each well. In assays of the agglutination by LPA, additional calcium chloride (0.03 M) was present in buffer I. Agglutination by SBA and PNA was performed with rabbit erythrocytes treated with neuraminidase (50 U/10^8 cells in 1 mL of buffer II, pH 6.0, 60 min at 37 °C) and fixed with formaldehyde as above. Agglutination end points and titers were assessed after 45 min incubation at 23 °C. Controls contained 0.1 M of the appropriate saccharide inhibitor.

Binding of Glycoproteins to Lectin–Sepharose Columns. The glycoproteins used for this assay were fetuin and its desialated derivative. Fetuin was radioactively labeled with KB^3H_4 by reductive alkylation according to Gregoriadis and Ryman (1972) and desialylated by heating at 80 °C for 1 h in 0.1 N HCl followed by neutralization and dialysis against 0.01 M Tris-HCl–0.15 M sodium chloride, pH 7.2 (buffer IV). Lectin–GSPH-Sepharose columns (1 mL of packed gel in Pasteur pipets) were washed with detergents in buffer IV containing 0.2 M of a saccharide inhibitor and then with buffered detergent alone at 23 °C unless otherwise indicated. Asialo-[^{3}H]fetuin ($2–5 \times 10^4$ cpm, 200–500 μg in 0.1 mL of buffer IV or detergent–buffer IV) was applied to the lectin–Sepharose columns at a flow rate of 0.1 mL per min. Binding was allowed to proceed for 10 min after which the columns were washed with buffer IV (or detergent–buffer IV) at a rate of 0.3 mL per min. When no radioactivity could be detected in the effluent after washing with 10 mL, the columns were eluted with the appropriate saccharide (0.2 M in buffer IV or detergent–buffer IV). The radioactivity in the eluted fractions was measured in toluene–Triton scintillation liquid using a Beckman Model LS200 liquid scintillation spectrometer. The total radioactivity recovered from the columns was usually more than 85% of the applied radioactivity ($2–5 \times 10^4$ cpm). The glycoprotein binding efficiency of each lectin–GSPH-Sepharose column was defined as: [(cpm eluted with saccharide)/(cpm washed without saccharide + cpm eluted with saccharide)] × 100%. Since the amount of asialo[^{3}H]fetuin or [^{3}H]fetuin applied to each column was less than half the saturating amount, the cpm washed off without saccharide inhibitors represents unbound glycoprotein which is a function of the affinity of the lectin to the glycoprotein. The effects of detergents on the binding of glycoprotein to the lectin–GSPH-Sepharose were expressed as percent binding efficiency measured in the presence of detergent compared with that measured in the absence of detergent.

Results

Effects of Detergents on Lectin Hemagglutinating Activity. The effects of nine detergents on the agglutination of formalin-fixed erythrocytes by lectins are shown in Table I. Since, in some cases, increasing concentrations of detergents caused nonspecific aggregation of erythrocytes (see below), the values presented in Table I are the highest detergent concentrations that neither decreased nor increased lectin titers. Although each of the lectins was affected in a distinct pattern by the

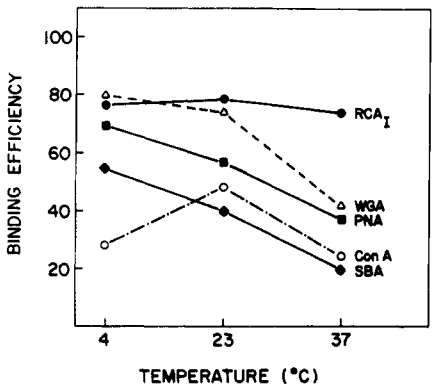

FIGURE 1: Effect of temperature on glycoprotein-binding efficiencies of lectin-GSPH-Sepharose columns. Lectin-GSPH-Sepharose columns (1 mL of gel in Pasteur pipets) were washed with 0.15 M sodium chloride-0.01 M Tris-HCl buffer, pH 7.2 (5 mL), and allowed to equilibrate at the indicated temperatures for 16 h. The columns were then washed with the above buffer at the appropriate temperature (5 mL), and 0.1 mL of buffer IV containing 200 μg of asialo[^{3}H]fetuin (2×10^4 cpm) was applied to each column except for the WGA-GSPH-Sepharose column which received 250 μg of [^{3}H]fetuin (5×10^4 cpm). Glycoprotein binding was allowed to proceed for 10 min, and the columns were washed with 10 mL of buffer IV followed by 10 mL of 0.2 M of the appropriate saccharide inhibitors in buffer IV. Radioactivity in each fraction collected during the wash and elution was measured, and the binding efficiencies were calculated for each column according to the equation: (cpm eluted with saccharide)/(cpm washed without saccharide + cpm eluted with saccharide) $\times$ 100%. Standard deviations calculated from triplicate experiments were in the range of $\pm$5%.

different detergents, it appears that the anionic detergents (DOC and $NaDodSO_4$) caused more deleterious effects than did the cationic or nonionic detergents, while the zwitterionic detergent DDGly had little, if any, effect on the activities of most lectins. Of the lectins tested, RCA_{II} and SBA were more sensitive to detergent effects than the other lectins. It is noteworthy that marked differences were found between the activities of RCA_I and RCA_{II} in several detergents. Interestingly, increasing concentrations of DOC enhanced the titers of certain lectins (e.g., RCA_I, Con A); this could, however, be explained by increased nonspecific aggregation of erythrocytes since controls containing lectin and its specific saccharide inhibitor exhibited increased titers in the presence of DOC concentrations at and above 0.5%. Since saccharide inhibitors had little effect on these increased titers with increasing DOC concentrations, the specific lectin activities were actually decreased in DOC solutions, indicating detergent inhibition of lectin activities. It is noteworthy that the nonspecific aggregation of erythrocytes in DOC was not observed when the lectins were omitted, suggesting that it was mediated by the lectin molecules at sites other than saccharide-binding sites.

The lectin HA titers (fixed erythrocytes) in detergents were sensitive to pH and ionic strength but not to temperature. While the HA titers were unaffected in the pH range 7-9, nonspecific aggregation of the erythrocytes occurred at pH 4-6 (data not shown). Increasing ionic strength up to 1.25 M sodium chloride in buffers containing detergent resulted in two- to fourfold higher titers than at near physiological ionic strength, while the controls remained unaffected. Increasing ionic strength above 0.2 M sodium chloride in DOC solutions caused gelling. Temperatures in the range 0-50 °C had little or no effect on the HA titers in buffer III-detergent solutions.

The effects of detergents on HA titers were not due to extraction of lectin receptors from the fixed erythrocytes. This was shown by labeling formalin-fixed erythrocytes by lactoperoxidase-catalyzed ^{125}I iodination of surface proteins (for review, see Juliano, 1973) and incubating the cells (5×10^8 erythrocytes, ~1-2 cpm/cell) in buffered detergent solutions (1% of Triton X-100, C_{12}TABr, NP40, or DDGly) for 15 min at 37 °C. The cell suspensions were centrifuged and the supernatants treated with cold 10% trichloroacetic acid to precipitate proteins. No detectable radioactivity was found in the trichloroacetic precipitates. In another experiment, cells (5×10^9 fixed erythrocytes per mL) were treated with the above-mentioned detergents and the supernatants extracted with chloroform-methanol (2:1). The extracts were dried in a warm stream of nitrogen and suspended in (0.1 volume) buffer III by sonication. Such solutions did not inhibit the HA of the lectins measured with fixed erythrocytes, indicating that the detergents do not extract glycolipids possessing significant lectin inhibitory activity.

Binding of Glycoproteins to Lectin-GSPH-Sepharose Columns. Prior to investigating the effects of detergents on the binding of glycoproteins by immobilized lectin columns, we have characterized the binding in the absence of detergent. WGA-GSPH-Sepharose bound fetuin preferentially, while the other lectin columns exhibited higher affinities for the asialo derivative (data not shown). Therefore the binding activity of WGA-GSPH-Sepharose was assayed with [^{3}H]fetuin and that of the other immobilized lectins was assayed with asialo[^{3}H]fetuin. Binding of these glycoproteins to the lectin-GSPH-Sepharose columns was affected by salt concentration and by temperature. In the absence of sodium chloride in the buffer (0.01 M Tris-HCl or 0.01 M sodium phosphate buffer, pH 7.2), the binding efficiency of PNA- and SBA-GSPH-Sepharose decreased by 70 and 80%, respectively, and that of Con A- and WGA-GSPH-Sepharose by 60% as compared with the values obtained in buffer IV. The binding efficiency of RCA_I-GSPH-Sepharose was not changed when salt was deleted from the assay buffer.

Glycoprotein binding at 4 °C improved for PNA- and SBA-GSPH-Sepharose, remained unchanged for RCA_I- and WGA-GSPH-Sepharose, and decreased for Con A-GSPH-Sepharose as compared with 23 °C. The binding at 37 °C decreased for all immobilized lectins (Figure 1). The standard assay conditions were chosen to be buffer IV at 23 °C for the sake of convenience. The recovery of radioactivity ([^{3}H]fetuin or asialo[^{3}H]fetuin) was at least 85%, and the binding efficiency was reproducible ($\pm$5%) when triplicates of each lectin-GSPH-Sepharose column were compared. Even different preparations of lectin-GSPH-Sepharose columns exhibited similar binding efficiencies provided that they were operated below saturation conditions.

Effects of Detergents on the Glycoprotein Binding Efficiency of Lectin-GSPH-Sepharose Columns. The effects of various concentrations of five detergents on the binding efficiency of immobilized lectin columns are summarized in Figure 2. The nonionic detergents (NP40 and Triton X-100) had the smallest inhibitory effect on the binding efficiency of the lectin columns, and even at 2.5% these detergents did not decrease the binding of fetuin or asialofetuin by more than 25% (Figure 2A,B). DDGly and C_{12}TABr did not affect the binding activity of RCA_I-, PNA-, or WGA-GSPH-Sepharose at any of the concentrations tested (Figure 2C,E). The activity of SBA- and of Con A-GSPH-Sepharose was decreased by 50 and 35%, respectively, in the presence of 0.1% DDGly. Higher DDGly concentrations (e.g., 2.5%) enhanced somewhat its deleterious effect on both of these immobilized lectins (Figure 2C). C_{12}TABr also affected the activity of the SBA- and Con A-GSPH-Sepharose columns; this detergent caused greater

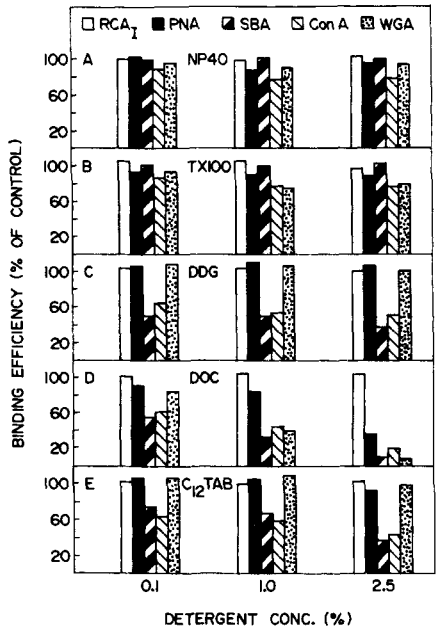

FIGURE 2: Effect of increasing concentrations of detergents on the glycoprotein binding efficiencies of immobilized lectins. Lectin-GSPH-Sepharose columns (1 mL of gel packed in Pasteur pipets) were washed with 0.15 M sodium chloride-0.01 M Tris-HCl, pH 7.2 (10 mL), followed by detergent solutions at the indicated concentrations in the same buffer (5 mL). The columns were allowed to stand for 30 min and were then further washed with additional 5 mL of the buffered detergent solution. [^{3}H]Fetuin (200 or 500 μg; 2-5 $\times$ 10^4 cpm) in 0.1 mL of the appropriate buffered detergent solution was preincubated for 30 min and applied to the WGA-GSPH-Sepharose columns. Asialo[^{3}H]fetuin (200-400 μg/2-4 $\times$ 10^4 cpm) in 0.1 mL of buffered detergent solution was applied to the other lectin columns. After 10-min incubation the columns were washed with buffered detergent solution (10 mL) and then with 0.2 M of the appropriate saccharide in buffered detergent solution (10 mL). The binding efficiencies of the lectin-GSPH-Sepharose columns at each detergent concentration were calculated as in the legend to Figure 1 and expressed as relative efficiencies: (binding efficiency in detergent)/(binding efficiency in absence of detergent) $\times$ 100%. The results represent the average of at least two separate determinations with different batches of lectin-GSPH-Sepharose and detergents. Standard deviations were approximately $\pm$5%. In the absence of detergent, the recovery of radioactivity from the column was not complete (80-90%), but in the presence of any of the detergents the recoveries were more than 95%. For this reason some columns yielded greater than 100% binding efficiencies. All procedures were carried out at 23 °C, except the experiment testing the effect of 2.5% DOC in 0.15 M sodium chloride-0.01 M Tris-HCl, pH 7.2, which was performed at 37 °C in order to prevent gelling of the solution.

decreases in binding efficiency with increasing detergent concentrations (Figure 2E). DOC caused the most pronounced deleterious effects on the activities of most lectin-GSPH-Sepharose columns with the exception of RCA$_I$-GSPH-Sepharose which was not affected even at 2.5% DOC (Figure 2D). PNA- and WGA-GSPH-Sepharose columns were only slightly affected at the lower DOC concentration (0.1%); however, higher concentrations markedly decreased the binding efficiency of these lectins (Figure 2D). The binding of glycoproteins in the presence of 2.5% DOC was carried out at 37 °C since at room temperature the solution formed a gel unless sodium chloride was eliminated from the buffer. When the binding in 1 or 2.5% DOC was performed in a buffer depleted of sodium chloride at 23 °C, the efficiencies of the immobilized lectins were similar to that shown in Figure 2D for the binding in 2.5% DOC (buffer containing 0.15 M sodium chloride at 37 °C).

The results presented in Figure 2 demonstrate that RCA$_I$, when covalently bound to GSPH-Sepharose, is the most stable lectin among those tested in the present study. Immobilized PNA and WGA are also unaffected by most detergents except DOC ($\geq$1%). Con A, and in particular SBA, are sensitive to DDGly, DOC and C_{12}TABr even at 0.1% detergent.

In an attempt to find out whether the effects of the detergents were reversible, columns of SBA- and Con A-GSPH-Sepharose were tested for binding efficiency in buffer alone, in buffer containing 2.5% of DDGly, DOC or C_{12}TABr, and then after washing the columns with 20 mL of buffer without detergent. We found that after treatment with 2.5% DDGly both SBA- and Con A-GSPH-Sepharose could be "reactivated" to 80% and 90% of the original activity, respectively. After C_{12}TABr (2.5%) the recovery was only 25% and 50% for SBA- and Con A-GSPH-Sepharose, respectively. The effect of 2.5% DOC was irreversible under the recovery conditions described above. None of the five detergents in this study caused dissociation of material absorbing at 280 nm from any of the lectin-GSPH-Sepharose columns used.

Effect of Sodium Dodecyl Sulfate on the Integrity and Binding Efficiency of Lectin-GSPH-Sepharose Columns. Sodium dodecyl sulfate is a very potent anionic detergent which binds to most soluble proteins and usually dissociates multimeric proteins into subunits. The lectins used in this study were comprised of between two (WGA) and four (RCA$_I$, Con A, PNA, and SBA) subunits. The binding of sodium dodecyl sulfate to proteins frequently causes denaturation and loss of activity. Therefore, we investigated the ability of low concentrations of sodium dodecyl sulfate to dissociate and release noncovalently bound lectin subunits and to affect the binding efficiency of the immobilized lectins (Table II). Exposure to 0.05% NaDodSO$_4$ for 1 h released differing amounts of lectin subunits from each of the immobilized lectin columns, ranging from 5.4% of the originally coupled lectin (Con A) to 26% (PNA). The binding efficiencies of such treated columns either remained unchanged (RCA$_I$), decreased slightly (WGA), or decreased significantly (PNA and Con A). The activity of SBA-GSPH-Sepharose was markedly reduced (by 78%). The resulting reduction in activity could not be reversed by extensive washing of the columns with buffer. Prolonged incubation of the lectin columns in 0.05% NaDodSO$_4$ (16 h at 23 °C) resulted in a higher release of lectin subunits from the SBA- and the Con A-GSPH-Sepharose columns, whereas the amount released from the other columns was similar to that released by 0.05% NaDodSO$_4$ after 1 h of incubation. Nevertheless, the binding efficiencies of all lectin-GSPH-Sepharose columns decreased more markedly after long-term exposure than by short-term exposure to the same NaDodSO$_4$ concentration. When analogous experiments were carried out with 0.1% NaDodSO$_4$, it was found that short-term exposure to 0.1% NaDodSO$_4$ released as much lectin subunits as did the prolonged exposure to 0.05% NaDodSO$_4$, except for Con A-GSPH-Sepharose where twice as much lectin was released by the higher NaDodSO$_4$ concentration. The activities of the immobilized lectins in 0.1% NaDodSO$_4$ after 1 h at 23 °C were similar to those observed after 16 h exposure to 0.05% NaDodSO$_4$, except for the binding efficiency of the WGA-GSPH-Sepharose column which was markedly reduced. Prolonged (16 h) incubation in 0.1% NaDodSO$_4$ at 23 °C released more lectin subunits and caused a more dramatic reduction in the binding efficiency than any of the other treatments. Control experiments using a 1-mL fetuin-GSPH-Sepharose (8.3 mg of fetuin per mL of gel) column indicated that, even under the latter conditions (16 h, 0.1% NaDodSO$_4$ at 23 °C), not more than 0.27 mg of protein (highest of triplicate measurements) was released from the column. Since

TABLE II: Effect of Sodium Dodecyl Sulfate on Lectin-GSPH-Sepharose Columns.[a]

Sepharose-Bound Lectin (mg/mL gel)		Lectin (mg/mL gel) Dissociated and Eluted by $NaDodSO_4$				Glycoprotein Binding Efficiency (% of control) in the Presence of $NaDodSO_4$			
		0.05%		0.1%		0.05%		0.1%	
		1 h	16 h	1 h	16 h	1 h	16 h	1 h	16 h
RCA_I	4.4	0.47	0.57	0.42	0.68	100	76	70	23
PNA	4.5	1.17	1.05	0.98	1.1	82	52	64	40
SBA	4.4	0.22	0.41	0.33	0.82	22	18	11	7
Con A	7.2	0.39	0.74	1.3	1.68	70	32	26	18
WGA	6.7	0.65	0.73	0.65	0.95	89	78	13	5

[a] Lectin-GSPH-Sepharose columns (1 mL gel) were washed with 0.15 M sodium chloride-0.01 M Tris-HCl, pH 7.2 (10 mL), followed by $NaDodSO_4$ solution in the same buffer (at either 0.05% or 0.1%). After passing 5 mL of $NaDodSO_4$ through the column (1-mL fractions), the flow was stopped and the column was allowed to stand at 23 °C for 1 h or for 16 h. The columns were then further washed with $NaDodSO_4$ solution and fractions (1 mL) were collected. The absorbance at 280 nm of all fractions eluted with $NaDodSO_4$ was measured. When no more UV-absorbing material was detected in the effluent, the columns were tested for their efficiency in binding [^{3}H]fetuin or asialo[^{3}H]fetuin as described in Materials and Methods except that all buffer solutions contained the indicated $NaDodSO_4$ concentration.

fetuin is composed of a single polypeptide chain, this control suggests that the $NaDodSO_4$ released noncovalently bound lectin subunits rather than uncoupled lectin.

Discussion

Affinity chromatography using lectin–Sepharose columns is an effective procedure for fractionation and isolation of soluble glycoproteins and glycopeptides (Bessler and Goldstein, 1973; Nordman and O'Brien, 1974; Rush et al., 1974; Surolia et al., 1975; Davey et al., 1976; Slayter and Coligan, 1976; Krusius, 1976). Since most membrane glycoproteins are for all practical purposes almost insoluble in neutral aqueous solutions, their solubilization and subsequent purification by lectin affinity chromatography requires the use of buffered detergent solutions. Indeed, a variety of detergents have been used for the purification of cell membrane lectin receptors (see introductory section). The large selection of detergents available is reflected in these studies, and the types of detergents used in different laboratories is vast. Yet, the choice of detergent systems seems, in many reports, to have been random or in the best examples, empirical. Although in one study the harmful effects of a detergent ($NaDodSO_4$) were observed (Gombos et al., 1974) and recently studied in greater detail (Kahane et al., 1976), to our knowledge there has been no systematic study on the effects of different types of detergents on a variety of soluble or immobilized lectins.

The data reported here represent our investigations on the effects of several detergents on the hemagglutinating activities of lectins in solution and on glycoprotein binding by immobilized lectins. We chose detergents that are commonly used for membrane solubilization at concentrations well above their critical micellar concentrations.

The use of formalin-fixed erythrocytes enabled us to screen the effects of several detergents on the hemagglutinating activities of several lectins. In this assay DOC and $NaDodSO_4$ proved to be more harmful to most of the lectins than did the cationic and nonionic detergents. The zwitterionic detergent DDGly had a significant inhibitory effect only on the activity of LPA. The mechanism of detergent inhibition of lectin-mediated hemagglutination may be complex, involving modification of cell surface potentials (in the case of ionic detergents), in addition to possible subunit dissociation or denaturation induced by conformational changes in the lectin molecules.

The effects of detergents on soluble lectins may not be directly extrapolated to lectins immobilized onto an insoluble matrix since the covalent attachment to an inert polymer may modify lectin properties. Immobilized enzymes have been found to be "stabilized", and they exhibit increased resistance toward denaturing agents (Mosbach, 1975; Schnapp and Shalitin, 1976). We therefore used our data on the effects of detergents on soluble lectins as a guideline for eliminating certain detergent–lectin combinations or conditions and proceeded to repeat the investigation using immobilized lectins. Since previous reports indicated the occurrence of possible hydrophobic interactions between lectins immobilized on CNBr-activated Sepharose and water soluble or detergent solubilized glycoproteins (Davey et al., 1974; Nachbar et al., 1976), we avoided these complications by using hydrophilic, charge-free, and nonleaching polyacrylic hydrazide-Sepharose (Wilchek and Miron, 1974) which was activated with glutaraldehyde. As expected, the lectin-GSPH-Sepharose columns exhibited very low nonspecific adsorption of glycoproteins, and recoveries of the applied material were high (80–90%). Fetuin and its desialated derivative were used as model glycoproteins to assess the effects of detergents on immobilized lectins. This choice was made because fetuin contains two types of carbohydrate side chains: the "serum glycoprotein type" which is N-glycosidically bound to asparagine and is comprised of sialic acid (*N*-acetylneuraminic acid), galactose, *N*-acetyl-D-glucosamine, and mannose; and the "mucin type" oligosaccharide which is O-glycosidically bound to serine or threonine and contains sialic acid, galactose, and *N*-acetyl-D-galactosamine (Spiro, 1973; Spiro and Bohyroo, 1974). Similar structures have been identified on cell membrane glycoproteins and glycopeptides from erythrocytes (Thomas and Winzler, 1969; Kornfeld and Kornfeld, 1971; Tomita and Marchesi, 1975), lymphocytes (Newman et al., 1976), brain cells (Finne, 1975), and ascites hepatoma (Funakoshi et al., 1974; Nakada et al., 1975). An additional advantage of fetuin is its ability to bind a variety of lectins such as Con A, WGA, LPA, and *Phaseolus vulgaris* phytohemagglutinin (Sela et al., 1975). We tested the ability of the lectin-GSPH-Sepharose columns to bind [^{3}H]fetuin, and we found that only the WGA column bound the glycoprotein efficiently, whereas the other immobilized lectins bound very little (RCA_I and Con A) or none at all (SBA and PNA). When we tested the binding of asialo[^{3}H]fetuin, the results were reversed; WGA did not bind asialo[^{3}H]fetuin well, while the other lectin columns bound it efficiently. Surolia et al. (1975) reported that RCA_I-Sepharose bound only 12% of applied commercial fetuin and that this fraction contained much less sialic acid than the unbound material. They sug-

gested that the partially desialated fetuin was either present in vivo or arose as a result of modification during preparation and purification. We have confirmed this finding with fetuin obtained from Sigma or Gibco (Spiro method) both by affinity chromatography and by isoelectric focusing in polyacrylamide slab gels.

Our data on the effects of various detergents on the glycoprotein binding efficiency of the lectin-GSPH-Sepharose columns indicate that the nonionic detergents (TX100 and NP40) are the most suitable for the lectin affinity chromatography, because their effects on the lectins were negligible. These findings are not surprising since studies on the binding of [^{3}H]TX100 to water soluble proteins indicate that it does not bind in detectable amounts (Helenius and Simons, 1972) and that it is very inefficient in breaking noncovalent bonds which are responsible for the quaternary structure of multimeric proteins (Helenius and Simons, 1975). We found that the zwitterionic detergent DDGly, as well as the cationic detergent C_{12}TABr, are both suitable for use with immobilized RCA_I, PNA, and WGA, even at 2.5%. Con A- and SBA-GSPH-Sepharose columns were less stable to these latter detergents, and their binding capacity was appreciably reduced in 0.1% detergent. DOC has been used in lectin affinity chromatography of membrane glycoproteins by several groups (Allan and Crumpton, 1971; Allan et al., 1972; Gurd and Mahler, 1974; Nachbar et al., 1976); yet in our assay system DOC caused the most deleterious effects on the activities of the lectin columns with the exception of RCA_I-GSPH-Sepharose. Most of the reports on the use of DOC for lectin affinity chromatography actually demonstrate the inhibitory effect of this detergent. For example, Allan et al. (1972) used 1% DOC with Con A-Sepharose and reported a 5% yield of the applied glycoproteins from pig lymphocyte plasma membranes, while Gurd and Mahler (1974), using the same DOC concentrations but with WGA-Sepharose, obtained a 20% yield of synaptic membrane glycoproteins. A binding efficiency of 14% was calculated from the data of Winqvist et al. (1976) on the binding of labeled liver microsomal glycoproteins solubilized with 1% cholate. The mechanism by which DOC inhibits glycoprotein binding by the lectins is unknown. DOC has rigid and bulky apolar moieties which do not allow easy penetration into crevices on protein surfaces, and this has been proposed to be the main reason why DOC does not usually denature proteins even at high concentrations (Helenius and Simons, 1975). The binding of DOC to fetuin has not been studied; however, a somewhat similar glycoprotein (thyroglobulin) has been found to bind 200 mol of DOC per mol of glycoprotein (Helenius and Simons, 1972). If fetuin binds DOC in a similar manner, such binding could change lectin conformation and decrease carbohydrate availability to the lectin-binding sites. This possibility does not explain why RCA_I is relatively unaffected by DOC. In addition, the lectin from *Lens culinaris* (lentil) also seems to be more stable in DOC solutions than Con A or WGA, as the former lectin had binding efficiencies of 40% (Gurd and Mahler, 1974) to 83% (Hayman and Crumpton, 1972). Most studies with DOC have used buffers without sodium chloride present mainly because DOC ($\geq$1%) gels at room temperature in solutions containing 0.15 M or more sodium chloride. We found that all the lectins tested here, with the exception of RCA_I, were much less efficient in glycoprotein binding in buffers depleted of sodium chloride, and this could also explain the low efficiencies reported for lectin-affinity chromatography in DOC. Absence of sodium chloride in the Triton X-100 solution used by Adair and Kornfeld (1974) to solubilize and chromatograph human erythrocyte membrane glycoproteins may explain the low yield (6%) of WGA receptors compared with a yield of 59% for the RCA_I receptors. This is consistent with our findings that WGA was less efficient in salt-free solutions, whereas RCA_I retained its full activity. Recently Kahane et al. (1976) pointed out the importance of using relatively high (0.25 M) sodium chloride concentrations in the detergent ($NaDodSO_4$) solution used to isolate in high yield the major human erythrocyte sialoglycoprotein on WGA-Sepharose columns.

Zanetta et al. (1975) and Kahane et al. (1976) have used low concentrations of $NaDodSO_4$ (0.08 and 0.05%, respectively) for affinity chromatography on Con A- and WGA-Sepharose columns. We have investigated the effect of this potent protein denaturant on our lectin-GSPH-Sepharose columns and observed that $NaDodSO_4$ released lectin subunits from the columns, but under mild conditions (0.05% $NaDodSO_4$ for 1 h at 23 °C), the lectins (except SBA) remained active, and even longer exposure (0.05% $NaDodSO_4$ for 16 h at 23 °C) did not dramatically decrease their activities. A higher $NaDodSO_4$ concentration (0.1%) was more harmful, especially after prolonged incubation.

Kahane et al. (1976) tested the effects of several detergents on the binding of radioactively labeled glycophorin to WGA-Sepharose and to other insolubilized lectins. Some of the results obtained in our model system do not agree with their observations; e.g., we did not find any inhibition of the binding of [^{3}H]fetuin by WGA-Sepharose in the presence of C_{12}TABr, C_{13}TABr, Triton X-100, or NP40 up to a detergent concentration of 2.5%—both in our buffer (0.01 M Tris-HCl-0.15 M sodium chloride, pH 7.2) or in the buffers used by Kahane et al. (1976) (0.05 M sodium phosphate-0.1 or 0.25 M sodium chloride, pH 7.2). In their system inhibition (30–40%) of glycophorin binding was observed at 0.06% Triton X-100 and C_{16}TABr and at 0.5% C_{12}TABr. The reason for these discrepancies could be due to the fact that we used fetuin, a hydrophilic, nonmembranous glycoprotein. Fetuin probably binds very little detergent, while glycophorin is an amphiphilic glycoprotein containing a "hydrophobic" segment of more than 30 nonpolar residues (Tomita and Marchesi, 1975) which could bind large amounts of detergents. Helenius and Simons (1972) found that delipidated erythrocyte membrane proteins bind from 0.29 to 0.64 mg of DOC per mg of protein, and the human erythrocyte MN membrane glycoprotein (glycophorin) binds $NaDodSO_4$ at ratios as high as 5–7 g of $NaDodSO_4$ per g of protein (Grefrath and Reynolds, 1974a) which are much higher than the 1–2 g per g of ratios found with nonmembrane proteins and glycoproteins (Reynolds and Tanford, 1970). Triton X-100 has been found to bind to viral membrane glycoproteins and to increase their tendency to aggregate (Simons et al., 1973). If glycophorin, which aggregates in neutral aqueous solutions, even in the presence of 6 M guanidine hydrochloride (Grefrath and Reynolds, 1974b), also aggregates in Triton X-100 or C_{16}TABr, then many of the glycophorin carbohydrate side chains could be inaccessible to lectin binding.

We believe that by using a hydrophilic glycoprotein in our model system we are observing mainly the effects of the detergents on the lectin molecules. Comparison of the effects of detergents on soluble lectins with their effects on immobilized lectins indicates that in most cases covalent attachment to the polymeric matrix stabilizes lectin molecules and decreases their susceptibility to the detergent inactivation. These data indicate the types and concentrations of detergents that are most suitable for each immobilized lectin and point out unfavorable conditions for lectin affinity chromatography such as low salt,

high temperature, etc. Since the use of immobilized lectins for the fractionation of isolated membrane glycoproteins has gained popularity, it is hoped that, based on the results presented here, the choice of detergents will be less random and yields will improve significantly.

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