THE DISTRIBUTION AND ASYMMETRY OF MAMMALIAN CELL SURFACE SACCHARIDES UTILIZING FERRITIN-CONJUGATED PLANT AGGLUTININS AS SPECIFIC SACCHARIDE STAINS

GARTH L. NICOLSON and S. J. SINGER

From the Cancer Council and Electron Microscopy Laboratories, Armand Hammer Cancer Center, The Salk Institute for Biological Studies, San Diego, California 92112 and the Department of Biology, University of California at San Diego, La Jolla, California 92037

ABSTRACT

The preparation, properties, and some applications of ferritin conjugates of two plant agglutinins, concanavalin A and *Ricinus communis* agglutinin, are reported. These conjugates serve as specific electron-dense stains for cell- and membrane-bound saccharide residues of the α-D-mannopyranosyl and β-D-galactopyranosyl configurations, respectively, and as examples of a wide range of ferritin-plant agglutinin conjugates useful as high resolution saccharide stains. By using a technique for preparing flattened membrane specimens, it was found with a variety of mammalian cell plasma membranes (lymphocyte, lymphoma, and myeloma and normal, spontaneously and virally transformed fibroblasts) that the ferritin conjugates were localized exclusively to the exterior face of the membrane, with essentially none found on the cytoplasmic face. On the exterior face the topographical distribution of ferritin conjugates appeared to be random. The asymmetrical distribution of saccharide residues to the outer membrane face can be explained by an "assembly line" process whereby new plasma membrane is made from intracellular precursor membranes. It also suggests that the saccharide-containing components of the plasma membrane do not rotate at any appreciable rate from one membrane surface to the other.

INTRODUCTION

Complex oligosaccharides represent a significant class of molecules in biological membranes. They are important constituents of cell surface antigens (Kabat, 1956; Pardoe et al., 1971), receptors (Cuatrecasas and Tell, 1973), and enzymes (Perrone and Blostein, 1973). The electron microscope localization of cell surface oligosaccharides has proceeded from the morphological identification of surface "fuzz" (Revel and Ito, 1967) to the development of histochemical techniques (Marinozzi, 1961; Movat, 1961; Van Heyningen, 1965) and the binding of colloidal iron (Gasic and Berwick, 1963; Gasic et al., 1968; Revel, 1964; Benedetti and Emmelot, 1967; Nicolson, 1973 *a*). A popular histochemical technique adapted from light microscopy uses periodic acid to oxidize saccharide 1,2-dihydroxy and α-amino alcohols to aldehydes which in turn reduce silver methen-

amine to an insoluble electron-dense product (Pahlke, 1954; Movat, 1961; Van Heyningen, 1965). These techniques are, however, limited in their specificity and spatial resolution for particular saccharide residues on biological structures.

We recently introduced methods to identify and localize specific cell surface saccharide residues using ferritin-conjugated plant agglutinins (Nicolson and Singer, 1971 *a*). Plant agglutinins are common proteins and glycoproteins that bind to specific sugar ligands (Mäkela, 1957; Sharon and Lis, 1972). Here we report in more detail the synthesis, purification, and properties of two ferritin-conjugated plant agglutinins: ferritin-conjugated concanavalin A (Fer-Con A)[1] specific for α-D-mannopyranosyl-like residues (Agrawal and Goldstein, 1967; So and Goldstein, 1967), and ferritin-conjugated *Ricinus communis* agglutinin (Fer-RCA) specific for β-D-galactopyranosyl-like residues (Drysdale et al., 1968; Nicolson and Blaustein, 1972). We also describe the use of these specific saccharide strains in determining the asymmetric distributions of α-D-mannopyranosyl-like and β-D-galactopyranosyl-like residues on several mammalian plasma membranes. Preliminary reports of this work have been published (Nicolson and Singer, 1971 *a*, 1971 *b*).

MATERIALS AND METHODS

Horse spleen ferritin (six times crystallized, cadmium free) was obtained in temperature controlled containers[2] from Miles Laboratories, Inc. (Kankakee, Ill.) and was further purified by cadmium sulfate crystallization (five additional times) and precipitation in 50% ammonium sulfate (twice). After extensive dialysis to remove ammonium ions, the ferritin was ultracentrifuged (35,000 rpm in the Beckman-Spinco [Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.] model L-2 using a model 40 rotor) for 2.5 h at 5°C. The pellet was carefully dissolved overnight, filtered through a 0.05-μm Millipore filter (Millipore Corp., Bedford, Mass.) and stored under sterile conditions. *Vibrio cholerae* neuraminidase was obtained from Sigma Chemical Co. (St. Louis. Mo.) as a chromatographically pure enzyme. Glutaraldehyde was obtained as a 50% solution (Fisher Scientific Co., Fair Lawn, N. J.) and was purified by fractional distillation. Biogel P-300 polyacrylamide (200 mesh) and Biogel A-1.5 m agarose (200–400 mesh) were obtained from Bio-Rad Laboratories (Richmond, Calif.). Sephadex G-75 and G-100 (fine) were obtained from Pharmacia Fine Chemicals (Piscataway, N. J.). Sucrose and β-lactose were products of J. T. Baker Chemical Co. (Phillipsburg, N. J.), and α-methyl-D-mannoside was obtained from Calbiochem (San Diego, Calif.).

Plant Agglutinins

Plant agglutinins were purified using affinity chromatography. Concanavalin A (Con A), obtained as a twice crystallized product of Miles-Yeda (Miles Laboratories, Inc.), was further purified on an affinity column of Sephadex G-75 according to published procedures of Agrawal and Goldstein (1967). 100 mg of the protein was dialyzed into 0.5 M sodium chloride-0.05 M sodium phosphate, pH 6.5 (0.5PBS6.5) and applied to a 4 × 40-cm column of Sephadex G-75. After washing with 0.5PBS6.5, the Con A was specifically eluted with 0.2 M sucrose in 0.5PBS6.5. The Con A yield from the pooled protein-containing fractions eluted with sucrose was estimated to be about 70–75%. The agglutinin was filtered and stored in sucrose at 5°C until further use.

Ricinus communis agglutinins were isolated from local wild castor beans[3] and purified according to the procedures of Nicolson and Blaustein (1972). The pulverized beans were extracted at 5°C with 0.2 M sodium chloride-0.005 M sodium phosphate, pH 7.2 (0.2PBS7.2) and the agglutinins were precipitated in 60% ammonium sulfate after removing debris by filtration and centrifugation. The ammonium sulfate-fractionated *R. communis* agglutinin (120,000 mol wt) (RCA) was dialyzed against 0.2PBS7.2 and applied to 4 × 40-cm affinity column of Biogel A-1.5 m agarose. The protein bound to the gel was specifically

[1] *Abbreviations used in this paper are:* Con A, concanavalin A; Fer-Con A, ferritin-conjugated concanavalin A; Fer-Fer, ferritin conjugated to ferritin; Fer-RCA, ferritin-conjugated *Ricinus communis* agglutinin (120,000 mol wt *R. communis* agglutinin); P3, P3.6.2 murine myeloma cell line; RCA, *Ricinus communis* agglutinin of 120,000 mol wt; SDS, sodium dodecyl sulfate; S49, S49.1TB.2 murine lymphoma cell line; 300 mosmol PBS, phosphate buffered saline of 300 mosmol ionic strength; 0.5PBS6.5, 0.5 M sodium chloride-0.05 M sodium phosphate, pH 6.5; 0.2PBS7.2, 0.2 M sodium chloride-0.005 M sodium phosphate, pH 7.2.

[2] We have found that 6×-crystallized ferritin shipped in temperature controlled polyfoam containers alleviates exposure to extreme temperatures which can cause ferritin denaturation and increased background labeling with ferritin-conjugates.

[3] Great caution must be exercised when handling castor bean (*R. communis*) plants or extracts due to their extreme toxicity. In addition to its poisonous materials, dust from the dried plants or pulverized beans can cause severe allergic reactions in some individuals.

eluted with 0.2 M lactose in a single peak. It contained, however, two distinguishable agglutinins which were separated by chromatography on Sephadex G-100. The fractions containing the heavier RCA (approximately 120,000 mol wt by electrophoresis in 5% sodium dodecyl sulfate [SDS]-polyacrylamide gels [Fairbanks et al., 1971]) were pooled and filtered and stored at 5°C.

Cells

Cloned murine S49.1TB.2 lymphoma (S49) and P3.6.2 myeloma (P3) cell lines (Hyman et al., 1972) were grown in tissue culture in Dulbecco-modified Eagle's medium (Vogt and Dulbecco, 1963) containing 10% calf serum or 10% horse serum, respectively. The cells (furnished by Dr. Robert Hyman of the Salk Institute) were washed three times in 0.6% sodium chloride-0.05 M sodium phosphate, pH 7.2 (300 mosmol PBS) and stored at 0°C until use, usually not longer than 30–60 min.

Murine L cells and human HeLa cells were grown in Eagle's minimum essential medium containing 5% calf serum. The cells (furnished by Dr. John J. Holland of the University of California at San Diego) were washed and removed from monolayer with dilute trypsin as described (Merchant et al., 1960). After incubation in suspension with fresh medium for 4 h at 37°C to allow the cells to recover, they were washed and stored as described above.

Influenza Virus Labeling

Influenza virus (strain NWS) was grown in 12–14 day chicken embryos and isolated from chorioallantoic fluid. Virus was purified by absorption and elution from red blood cells, ammonium sulfate precipitation, and centrifugation in sodium tartrate gradients (Holland and Kiehn, 1970). The final titer was approximately 20,000 hemagglutination U/ml. Influenza virus (10,000 hemagglutination U/ml) was used to directly label lysed mounted rabbit erythrocyte membranes (5 min at 4°C) as described earlier (Nicolson, 1972 *b*). Control cells were treated with neuraminidase (1 unit/10^6 cells) for 60 min at 37°C in 300 mosmol PBS, pH 6.0 and then washed twice in 300 mosmol PBS, pH 7.4 (4°C).

Ferritin-Conjugated Plant Agglutinins

The affinity-purified plant agglutinins were conjugated to ferritin by a modification of the glutaraldehyde coupling procedure of Avrameas (1969). To a solution containing 4–5% ferritin and 1.5–2.0% plant agglutinin in the appropriate buffer containing a specific saccharide inhibitor to protect the active sites of the agglutinin (0.5PBS6.5–0.1 M sucrose for Con A; 0.2PBS7.2–0.1 M lactose for RCA) was carefully added, with stirring, 0.5–1.0% glutaraldehyde[4] to a final concentration of 0.02–0.04%. After 45 min at room temperature, one drop of 0.1 M ammonium chloride was added and the mixture was dialyzed for 3 h at 5°C against the appropriate buffer.

Fer-Con A and Fer-RCA were separated from unconjugated proteins by column chromatography. Fer-Con A (volume approximately 1 ml) was centrifuged at 15,000 *g* for 15–30 min before chromatography on a 1.5 × 120-cm Biogel A-1.5m column equilibrated with 0.5PBS6.5 (Fig. 1 *a*). Fractions (0.5 ml) were monitored for absorbance at 280 nm and for agglutinability of rabbit erythrocytes. Fer-RCA was similarly centrifuged and then chromatographed on a 1.5 × 150-cm column of Biogel P-300 equilibrated with 0.2PBS7.2 (Fig. 1 *b*). The conjugates located in the first peak behind fractions containing high molecular weight material, were concentrated to 1–5 mg/ml protein before use.

Ferritin-Plant Agglutinin Labeling

The labeling of various tissue culture cell membranes with Fer-Con A and Fer-RCA generally followed the procedures of Nicolson (1971, 1972 *a*, 1972 *b*). Cells were strengthened with 0.1–0.3% buffered (0.6% sodium chloride-0.5 M sodium phosphate, pH 7.2) formaldehyde for 2 min at room temperature to give mechanical strength to the plasma membranes without extensive fixation of the cytoplasmic components. After washing twice by centrifugation, the cells were suspended into buffer at a concentration of 30–50% by volume. A small volume of this suspension was dropped onto a surface of 20 mosmol sodium phosphate buffer, pH 7.2. Under these conditions a small fraction of the cells apparently ruptured at local sites in the plasma membrane, and remained at the air-buffer interface; the rest of the cells entered the buffer. The membranes of the cells remaining at the interface were spread flat by surface forces and could be picked up from above on collodion-coated carbon-strengthened electron microscope grids, and could be further fixed with formaldehyde, if necessary. The grids with the attached membranes were then conditioned with a 5% solution of bovine serum albumin in either 0.5PBS6.5 or 0.2PBS7.2 and, without drying, a large drop of Fer-Con A or Fer-RCA was applied to each grid. After 1–5 min incubation at 5°C or room temperature, the grids were washed by floating them face down on 6–8 consecutive fresh buffer surfaces, and finally on two distilled water surfaces. The specimens were not further stained and were dried in

[4] Glutaraldehyde was freshly purified by vacuum fractional distillation or by filtering through activated alkaline charcoal (Norite EX; Union Carbide Corp., New York) (Anderson, 1967).

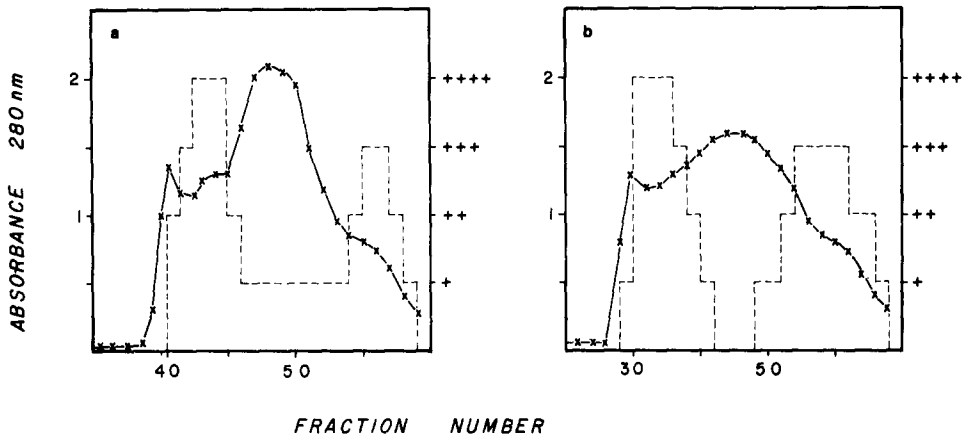


FIGURE 1 Gel chromatography of ferritin-conjugated plant agglutinins after coupling with glutaraldehyde: (*a*) an aliquot of ferritin-conjugated concanavalin A chromatographed on Biogel A-1.5m; (*b*) ferritin-conjugated *Ricinus communis* agglutinin chromatographed on Biogel P-300. Absorbance at 280 nm is indicated for various fractions (0.5 ml) by a solid line and hemagglutinating activity against rabbit erythrocytes (measured qualitatively from no agglutination, –, to complete agglutination, + + + +) is indicated by broken lines.

air or were dried in a critical-point drying apparatus (Ivan Sorvall, Inc., Newtown, Conn.). After drying, the specimens were observed by transmission electron microscopy in Philips model 300 or Hitachi model HU-12 electron microscopes.

RESULTS

Purification of Ferritin-Agglutinins

The conjugation mixtures after coupling with glutaraldehyde contain unconjugated ferritin and agglutinin and ferritin-ferritin (Fer-Fer), ferritin-agglutinin, and agglutinin-agglutinin conjugates. The unconjugated proteins are removed from the mixtures by column chromatography on Biogel A-1.5m (Fig. 1 *a*, Fer-Con A) or Biogel P-300 (Fig. 1 *b*, Fer-RCA). By the use of these procedures, the excluded volumes (after chromatography) contain the mixture of conjugated proteins. Electrophoretic analysis (immunoelectrophoresis) of chromatographed Fer-Con A conjugates indicates that most of the conjugated molecules are Fer-Fer or Fer-Con A. Fer-Con A or Fer-RCA can be further purified by utilizing their binding characteristics to Sephadex G-75 (Fig. 2 *a*, Fer-Con A) or Biogel A-1.5 m (Fig. 2 *b*, Fer-RCA) (see Materials and Methods on the purification of Con A and RCA). After elution of Sephadex bound Fer-Con A with 0.2 M sucrose (Fig. 2 *a*) or Biogel bound Fer-RCA with 0.2 M β-lactose (Fig. 2 *b*), the ratio of ferritin:agglutinin was estimated by iron (Bandemer and Schaible, 1944) and protein (Ma and Zuazaga, 1942) analyses to vary between 1:1 to 1:3 for different preparations. However, removal of Fer-Fer did not appreciably change the apparent extent of nonspecific staining so the chromatographed conjugate fraction was used routinely for labeling without further purification. Dilute samples of the conjugate fractions of Fer-Con A and Fer-RCA dried down on coated grids indicated that most of the ferritin in these solutions is present as small (1:1, 1:2, etc.) Fer-agglutinin or free ferritin molecular species (Fig. 3).

Occasionally the Fer-agglutinin conjugates were absorbed against intact formaldehyde-fixed cells before use to reduce further nonspecific binding (Nicolson, 1971). This was performed in the presence of 0.2 M saccharide inhibitor (sucrose for Fer-Con A; lactose for Fer-RCA), and the absorbed conjugates were then extensively dialyzed against their respective buffers to remove saccharide inhibitors.

Distribution of Virus Receptors on Erythrocyte Membranes

When erythrocytes are lysed on an air-water or air-hypotonic buffer interface, occasionally the cells break open leaving large holes in their plasma membranes (Nicolson and Singer, 1971 *a*). After mounting on collodion films, these membranes are

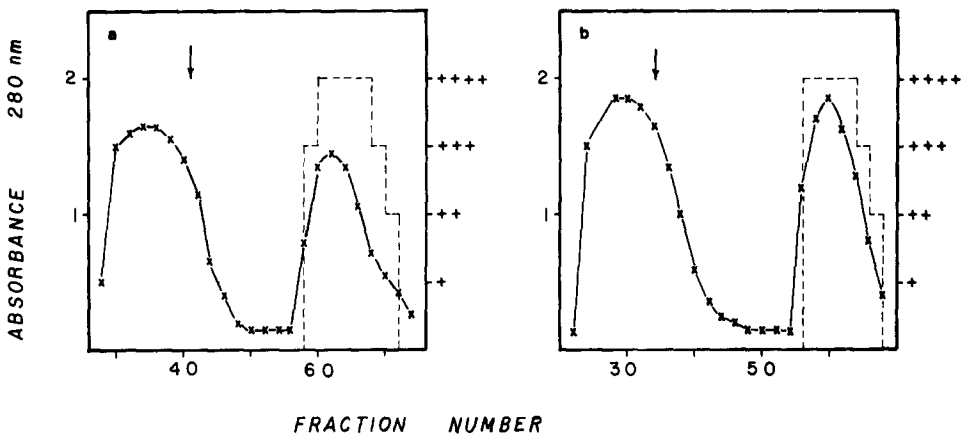


FIGURE 2 Affinity chromatography of ferritin-conjugated plant agglutinins: (*a*) ferritin-conjugated concanavalin A chromatographed on Sephadex G-75. Arrow indicates addition of 0.1 M sucrose to eluting buffer; (*b*) ferritin-conjugated *Ricinus communis* agglutinin chromatographed on Biogel A-1.5m. Arrow indicates addition of 0.1 M β-lactose to eluting buffer. For other figure symbols see Fig. 1.

flattened double-membrane-thick ghosts. When holes (broken in the membranes at lysis) are on the upper membrane layer, the exposed surface on the lower membrane layer (cytoplasmic inner side) is accessible to labeling. Thus, both the outer and inner (cytoplasmic) surfaces of the same cell membrane can be stained with specific labels under exactly the same microconditions. The outer and inner surfaces can be easily identified by their electron densities; the outer surface is identifiable as two membrane layers-thick regions and the inner surface is identifiable as only one membrane layer-thick regions (see Figs. 4 and 5 where *o* indicates outer and *i* indicates inner membrane surfaces).

When these mounted membranes are labeled with influenza virus at 4°C, the virions attach only to the surfaces identified as outer (Fig. 4). The virus attachment is specific, because neuraminidase-treated cells which have their influenza receptors destroyed do not bind virus particles to either surface (Fig. 5). In an earlier publication we demonstrated that the erythrocyte membrane surfaces identified as inner are accessible to labeling and could bind ferritin-conjugated antibodies

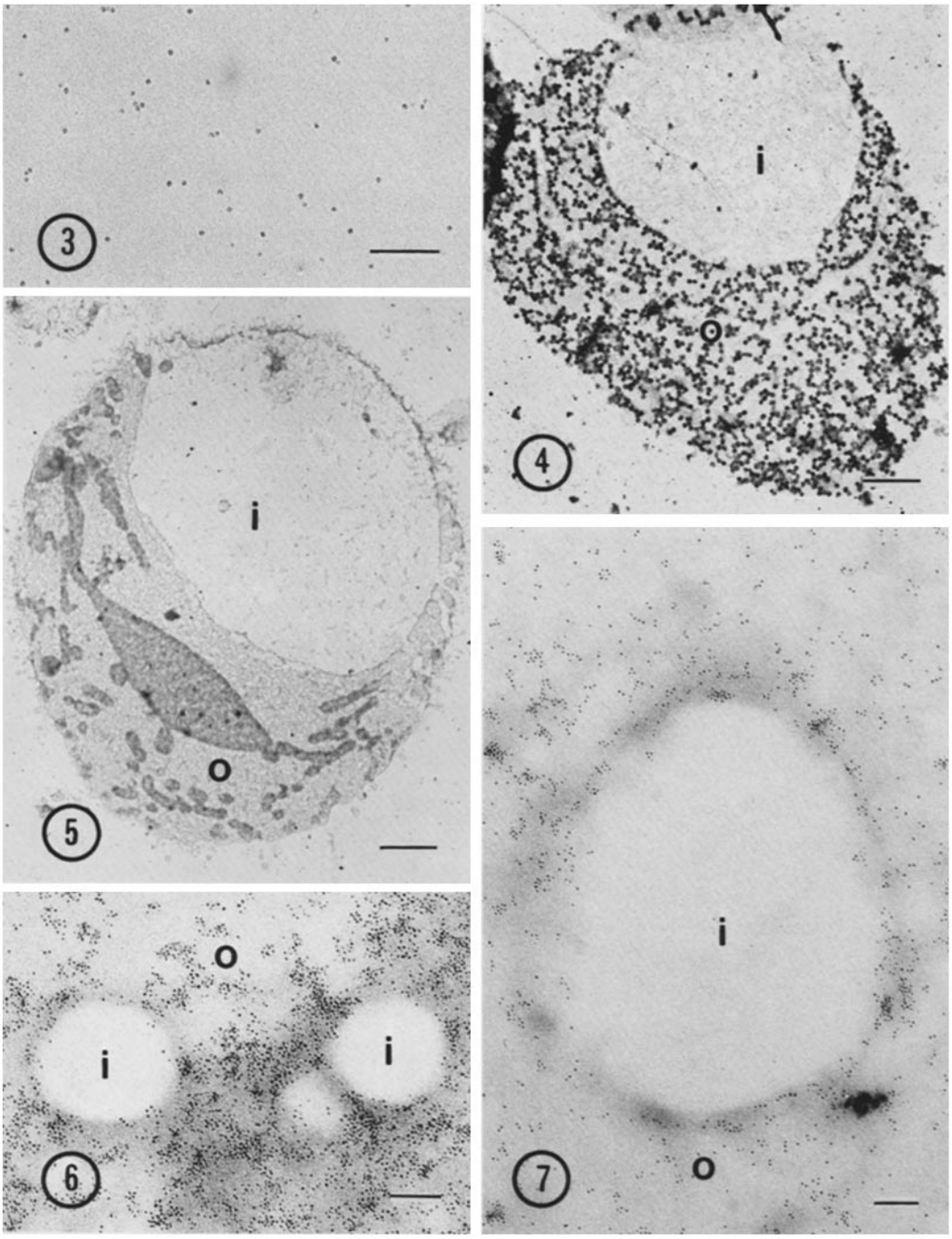
FIGURE 3 Ferritin-conjugated concanavalin A dried down on a carbon-collodion film. Bar, 0.1 μm. × 100,000.

FIGURE 4 Erythrocyte ghost labeled with influenza virus. A rabbit erythrocyte was lysed at an air-buffer interface and its membrane-ghost mounted on a carbon-collodion film such that its outer surface (*o*) is distinctly identified from its inner surface (*i*) by the difference in electron density between the two regions. The mounted ghost was labeled with purified influenza virus and post stained with osmium tetroxide. Bar, 1μm. × 8,200.

FIGURE 5 Neuraminidase control. Legend is the same as in Fig. 4 except that the cells were treated with neuraminidase (see Materials and Methods) before lysis. Bar, 1 μm. × 8,600.

FIGURE 6 A P3 myeloma cell was strengthened with 0.1% formaldehyde for 2 min, washed and then lysed at an air-buffer interface. The plasma membrane ghost formed during lysis was picked up on a carbon-collodion film and stained with ferritin-conjugated concanavalin A. The outer plasma membrane surface (*o*) can be distinctly identified from the inner surface (*i*). Bar, 0.1 μm. × 73,800.

FIG. 7 The legend is the same as in Fig. 6 except that a P3 myeloma cell membrane was mounted and stained with ferritin-conjugated *Ricinus communis* agglutinin. Bar, 0.1 μm. × 61,500.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directed against an inner membrane surface component, spectrin (Nicolson et al., 1971 *b*).

Distribution of Ferritin-Agglutinins on Plasma Membranes

Fer-Con A does not bind to regions identified as inner plasma membrane surfaces which form during lysis of formaldehyde-strengthened P3 myeloma cells (Fig. 6). Although this cell line has cell surface immunoglobulin molecules that can be labeled with anti-IgG_{2a} (Knopf et al., 1973), it is doubtful that the observed Fer-Con A staining is to a significant extent attributable to these IgG determinants. This was demonstrated by Fer-Con A labeling of a P3 variant (P3.6.2.8.4/Fab 3×) selected for absence of surface IgG (Knopf et al., 1973). There was no significant difference in the amount of Fer-Con A found on the variant cell surface. P3 cells also have RCA-binding sites on their surfaces, and Fer-RCA could not be found on regions identified as inner plasma membrane surface (Fig. 7). Similar results (not shown) with Fer-Con A and Fer-RCA were found with other lymphoid cells such as murine lymphomas (S49) and rabbit thoracic duct lymphocytes.

Fibroblast cell lines were also examined for asymmetry of their plasma membrane lectin-binding sites. When formaldehyde-strengthened murine L cells were labeled with Fer-Con A (Fig. 8) or Fer-RCA (Fig. 9), the labeling was only found on outer plasma membrane surfaces. In these and other preparations stained with ferritin-agglutinins specificity of binding was checked on control cell membranes stained in the presence of excess saccharide inhibitor. For example, when sucrose was present during Fer-Con A staining, Fer-Con A labeling of the mounted membranes was blocked (Fig. 10).

DISCUSSION

These studies and others (Nicolson, 1971, 1972 *a*, 1972 *b*; Tillack et al., 1972; Klein and Adams, 1972; Williams and Voaker, 1972) indicate the usefulness of ferritin-conjugated plant agglutinins as specific electron microscope stains for oligosaccharides attached to biological structures. Using direct labeling methods, the spatial resolution of these techniques is limited by the dimension of the ferritin-agglutinin molecular complex (approximately 150 Å), and the specificity is that of the plant agglutinin. Plant agglutinins can be chosen either with broad binding specificities for general saccharide labeling or with very restricted specificities for labeling unique sugar residues. An example of the former broad spectrum type of agglutinin is the *Lens culinaris* lectin (inhibited by α-linked *N*-acetyl-D-glucosamine- or D-glucose-like saccharides [Toyoshima et al., 1970; Howard and Sage, 1969; Young et al., 1971]) and an example of the more restricted type is the *Dolichos biflorus* agglutinin (inhibited by α-linked *N*-acetyl-D-galactosamine-like saccharides [Etzler and Kabat, 1970]). By choosing the proper agglutinin, a variety of different saccharide residues can be specifically localized. The specificity of the ferritin-agglutinin conjugates also allows simple controls to be performed (in parallel) by including the appropriate saccharide inhibitors in the control staining solutions. Another advantage of the direct ferritin-conjugated plant agglutinin staining compared to other recently developed indirect agglutinin-staining techniques is that the agglutinin molecule is covalently linked to its marker. This is in contrast to the methods of Bernhard and Avrameas (1971) and Smith and Revel (1972) where the attachment of the marker (horseradish peroxidase or keyhole limpit hemocyanin, respectively) is dependent on the binding of saccharide residues present on the marker molecules to the remaining saccharide sites on the target-bound agglutinins.

In the present study Fer-agglutinins such as Fer-Con A (specific for α-D-mannopyranosyl-like residues [Agrawal and Goldstein, 1967; So and Goldstein, 1967]) and Fer-RCA (specific for β-D-galactopyranosyl-like residues [Drysdale et al., 1968; Nicolson and Blaustein, 1972]) were used to investigate the asymmetry of plasma membrane oligosaccharides. We originally developed a method of spreading lysed erythrocyte membranes flat at an air-water interface, exposing large two-dimensional sections of the membrane for staining with ferritin conjugates (Nicolson and Singer, 1971 *a*). Subsequently we found that other types of mammalian cells have plasma membranes with mechanical properties that are markedly different from those of erythrocyte membranes. When these untreated cells were subjected to rupture at the air-water interface, the plasma membranes completely fragmented, unlike the situation with erythrocytes. Therefore, these cells were first mildly treated with formaldehyde, which strengthens their plasma membranes (Nicolson 1971, 1972 *a*, 1972 *b*). When the strengthened cells were ruptured and the flattened membranes mounted

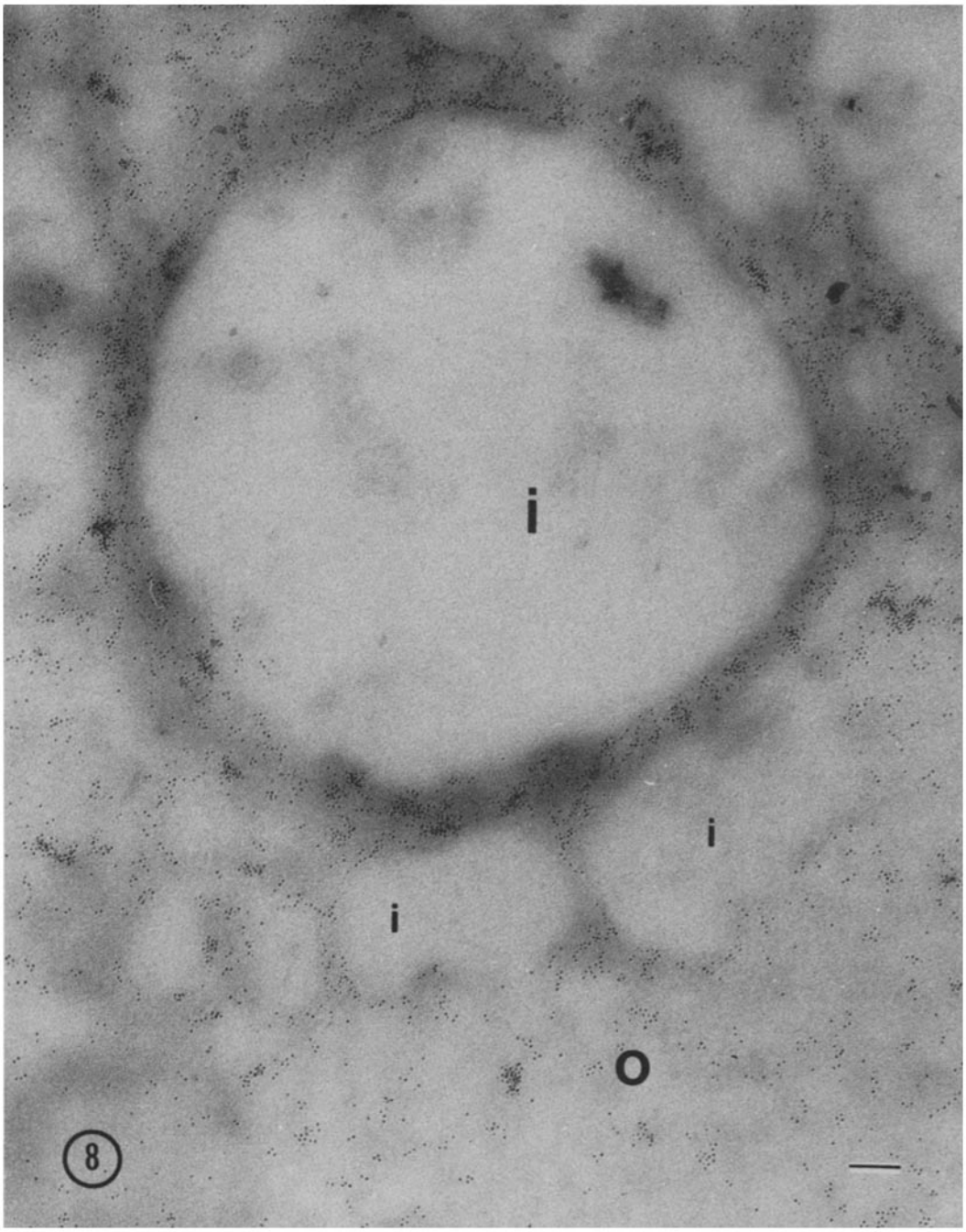

FIGURE 8 The legend is the same as in Fig. 6 except that a murine L fibroblast cell was labeled with ferritin-conjugated concanavalin A. Bar, 0.1 μm. × 71,800.

on support films, they were found to contain holes sparsely scattered over the surface. In these holes the inner, cytoplasmic surface of the lower membrane was revealed and could be recognized by its position and density on the electron micrograph, distinct from the densities of the double membrane regions and of the coated grid itself (cf. Fig. 10).

The long-range topographical distributions of

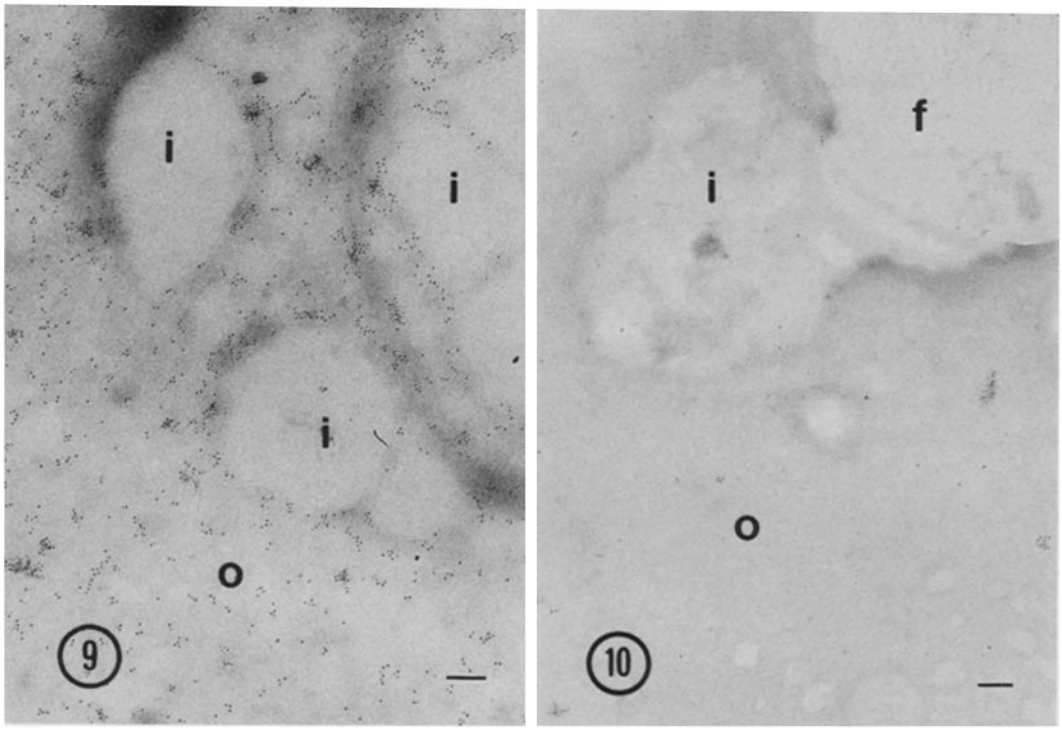

FIGURE 9 The legend is the same as in Fig. 6 except that an L cell was labeled with ferritin-conjugated *Ricinus communis* agglutinin. Bar, 0.1 μm. × 53,300.

FIGURE 10 Saccharide control. A murine L cell was lysed, mounted, and stained with ferritin-conjugated concanavalin A in the presence of its saccharide inhibitor, 0.1 M α-methyl-D-mannoside. The outer plasma membrane surface can be distinctly distinguished from the inner surface (*i*) and the carbon-collodion film (*f*). Bar, 0.1 μm. × 46,100.

membrane-bound Fer-agglutinins appeared to be essentially random, similar to Fer-antibody-binding sites (Nicolson et al., 1971 *a*, 1971 *c*), although on most of the membranes examined the Fer-agglutinin molecules were found in non-uniform, random clusters, unless the membranes were further strengthened with 2% formaldehyde to prevent the polyvalent agglutinins from aggregating the agglutinin-binding sites (Inbar and Sachs, 1973; Nicolson, 1973 *b*; de Petris et al., 1973; Rosenblith et al., 1973). Antigenic sites on lymphocytes (Taylor et al., 1971; Loor et al., 1972; Unanue et al., 1972) and fibroblasts (Edidin and Weis, 1972) are also mobile in the plane of the membrane, consistent with a fluid structure of biological membranes (Singer and Nicolson, 1972). Agglutinin-binding sites on membrane surfaces appear to be more free to move laterally in the membrane plane after treatment with proteolytic (Nicolson, 1972 *a*) or lipolytic enzymes (Nicolson, 1972 *b*), and this change in distribution of agglutinin-binding sites can be stopped by formaldehyde fixation. Formaldehyde fixation does not destroy agglutinin-binding sites (Inbar et al., 1973; Rosenblith et al., 1973; Nicolson, unpublished), so prior formaldehyde-fixation (up to 10%; Inbar et al., 1973) can be used to stop agglutinin-induced distributional modifications.

Fer-Con A and Fer-RCA were specifically and exclusively bound to the outer surfaces of a variety of cell types. To rule out the possibility that the brief formaldehyde fixation might cross-link cytoplasmic proteins to the plasma membrane inner surface, thereby blocking Fer-agglutinin binding, cells were also directly lysed on buffer surfaces (250 mosmol) without prior fixation. Labeling mounted unfixed membranes with Fer-Con A or Fer-RCA gave exactly the same results as labeling membranes that had been briefly fixed

with formaldehyde. The conditions of formaldehyde fixation were chosen on the criteria that the lysed cells yielded intact plasma membrane ghosts without inactivating specific cell surface antigens (such as the murine H-2 and θ antigens) as determined by direct cytoxic and quantitative absorption assays using specific anti-H-2 and anti-θ alloantibodies (R. Hyman and G. L. Nicolson, unpublished results). In addition, Fer-agglutinin labeling of purified plasma membrane fragments shows exclusive binding of the Fer-agglutinins to one of the membrane surfaces.

We have also shown that influenza virus particles are exclusively localized to the outer surface of human erythrocyte membranes (Fig. 4 and 5). The receptors for this virus are known to contain terminal sialic acid residues (Klenk and Uhlenbruck, 1958). Our morphological studies are in agreement with earlier chemical data that virtually all of the sialic acid can be cleared by neuraminidase treatment from the intact erythrocytes, indicating that the sialic acid residues are only on the outer surface of the red cell (Eylar et al., 1962). Using galactose oxidase-[^{3}H]borohydride techniques on "normal" and "inverted" erythrocyte vesicles, Steck (1972) has shown that the terminal galactose residues are only present on the exterior surface.

Our results suggest that plasma membrane oligosaccharides of all mammalian cells, and perhaps of all eukaryotic cells, could be confined to the outer membrane surface. We have dealt with the consequences of this proposal elsewhere and will only briefly discuss them here. First, the biosynthetic origin of this saccharide asymmetry can be explained by Palade's proposal (1959), that the plasma membrane is not synthesized *de novo*, but via an "assembly-line" process involving precursor intracellular membranes. If new plasma membrane arises from the fusion of existing plasma membrane with intracellular precursor membrane vesicles containing saccharides exclusively on their *inner* surfaces, then the plasma membrane saccharides would appear solely on the *outer* plasma membrane surface. This requires that the first saccharides attached in the rough endoplasmic reticulum be exclusively transferred to the cisternal side of the membrane. Using ferritin-conjugated plant agglutinins, Hirano et al. (1972) recently confirmed this prediction using intracellular membranes from homogenates of myeloma cells. Second, the asymmetry of membrane saccharides suggests that membrane components containing oligosaccharide chains (glycoproteins and glycolipids) do not rotate at any appreciable rate from one membrane surface to the other. Otherwise, the saccharides would have been found on both membrane surfaces. We have suggested that the absence of *trans*-membrane rotation may be a general phenomenon of amphipathic molecules (Singer and Nicolson, 1972). Moving the hydrophilic portions of these molecules through the hydrophobic interior of the membrane would require such large energies of activation (Singer, 1971) that such processes would occur only at negligible rates. These considerations are relevant to the recent proposal of Burger (1970) and Borek et al. (1973) that such *trans*-membrane rotations or "swivels" could account for the differential agglutinability of transformed cells by the inversion of certain agglutinin sites with the simultaneous appearance of another class of agglutinin sites. We have investigated the distribution of Fer-Con A and Fer-RCA on a variety of human, mouse, and hamster normal, virally and spontaneously transformed cells and found no experimental evidence for this proposal. The agglutinin-binding sites remained on the plasma membrane exterior after transformation and were not inverted to the inner membrane surface.

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