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Distribution of Saccharide Residues on Membrane Fragments from a Myeloma-Cell Homogenate: Its Implications for Membrane Biogenesis

(ferritin conjugates/plant agglutinins/smooth- and rough-membrane elements)

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ABSTRACT **Ferritin conjugates of two plant agglutinins, concanavalin A and ricin, have been used as specific electron microscopic stains for covalently-bound saccharide residues on membrane fragments from a myeloma-cell homogenate. The results indicate that different saccharide residues are uniformly localized to a single surface of each membrane fragment. In particular, the ferritin-concanavalin A conjugate binds exclusively to the cisternal side of membrane fragments of the rough endoplasmic reticulum. If it is postulated that the biogenesis of eukaryotic plasma membranes involves an assembly-line process from precursor intracellular membranes, these observed asymmetric distributions of saccharides on cell membranes can be explained.**

Ferritin conjugates of plant agglutinins (proteins that are capable of binding to specific terminal saccharide residues on oligosaccharide chains) were recently introduced as specific electron microscopic stains for saccharide residues bound to biological structures (1). Investigations with these conjugates of the plasma membranes of different eukaryotic cells (2) have shown that the staining was localized exclusively to the outer surfaces of these membranes; no significant amounts of ferritin conjugates were found on the inner, cytoplasmic surfaces. This suggests, quite generally, that the saccharide residues of at least the glycoproteins, and perhaps also the glycolipids, of plasma membranes are confined to the outer membrane surface. This conclusion raises some important questions about plasma-membrane biogenesis that are elaborated in the *Discussion*. In order to investigate these questions further, we have undertaken similar studies with the intracellular membranes as well as the plasma membranes of eukaryotic cells. A preliminary phase of these studies, involving the membranes in a subcellular fraction of cells from a mouse myeloma tumor, is reported in this paper.

MATERIALS AND METHODS

A plasmacytoma tumor line designated P3K (3), derived from a Balb/c mouse, was used in these experiments. It produces a IgG1 myeloma protein with covalently-attached oligosaccharide chains. The preparation of cell suspensions and of cell homogenates, and a first separation of cell fractions by centrifugation of the homogenates in a convex exponential sucrose gradient, were performed essentially as described (4).

Abbreviation: con A, concanavalin A; ricin, *Ricinus communis* agglutinin.

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The fractions containing the rough-membrane elements were further purified by centrifugation in a stepwise sucrose gradient, collecting the material at the interface between the layers of 1.50 M and 2.00 M sucrose in 50 mM Tris·HCl (pH 7.60)–25 mM KCl–5 mM $MgCl_2$ buffer.

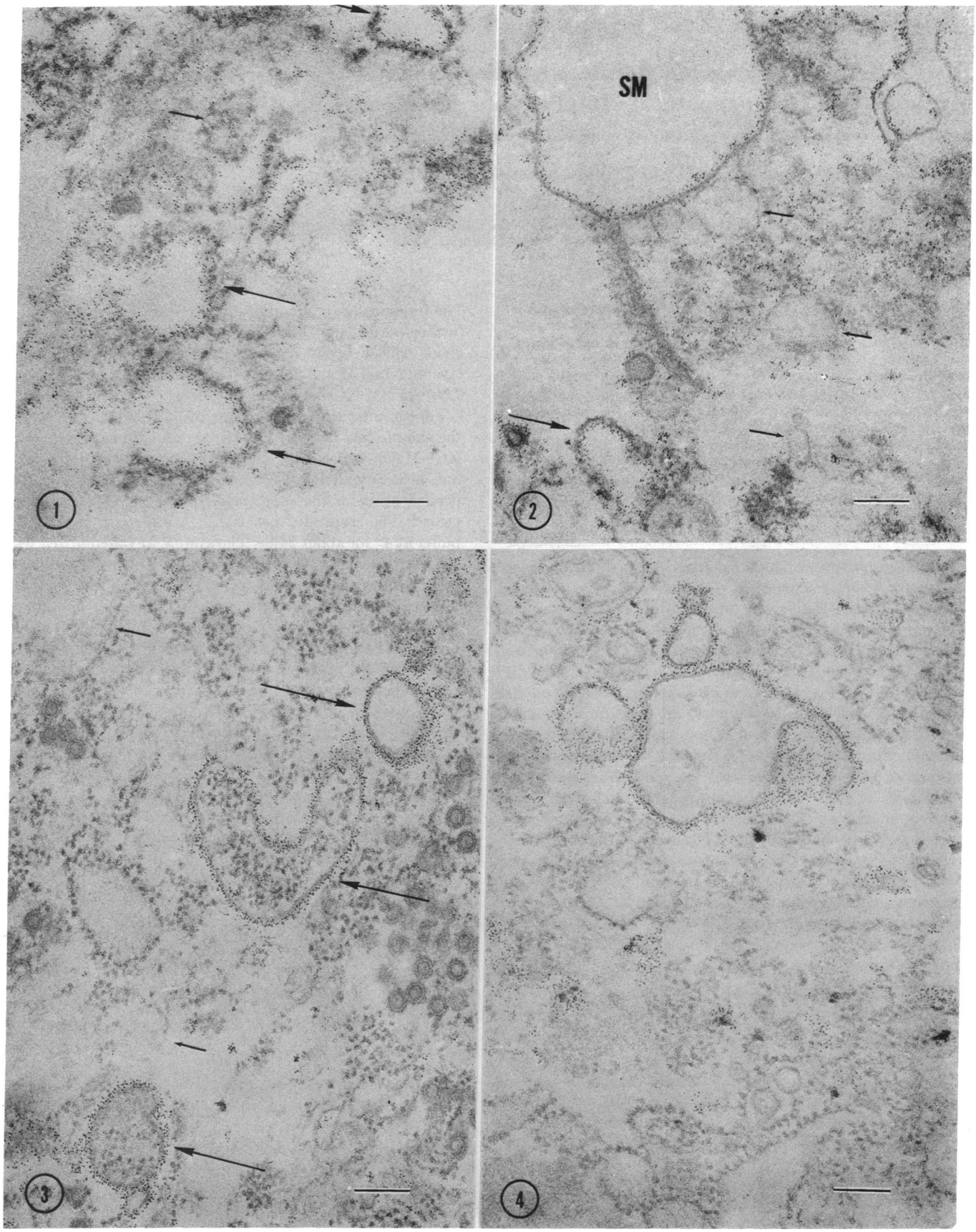
For staining with the ferritin conjugates, the sucrose in the sample was first removed by dilution 20-fold with Tris–KCl–$MgCl_2$ buffer and centrifugation at 10,000 rpm for 10 min in a Sorvall RC-2 centrifuge. The precipitate was suspended again in the same buffer and the process was repeated. The suspended pellet was then dialyzed against the buffer at 4° for about 24 hr. The sample was then mixed with the ferritin-conjugated agglutinin (at a concentration of 2–5 mg of protein per ml) for 20 min at room temperature. If the effect of an inhibitor of the staining reaction was to be determined, the inhibitor was most effective if it was first added to the ferritin conjugate before mixing with the sample. After 20 min, the mixture was washed twice by centrifugation as described above, and the final pellet was fixed in cold 2% OsO_4 in 0.1 M phosphate buffer (pH 7.2) for 1 hr. The sample was then embedded in Epon, and sections were prepared for electron microscopy by conventional methods.

The two plant agglutinins, concanavalin A (Con A) and ricin, were used. Con A was the twice crystallized product purchased from Miles–Yeda; ricin was isolated from the castor bean *Ricinus communis*, and purified by a procedure described elsewhere (5). Horse-spleen ferritin (6× crystallized, Miles–Pentex) was further purified (6). The ferritin conjugates of these agglutinins (ferritin–Con A and ferritin–ricin, respectively) were prepared by the glutaraldehyde coupling method of Avrameus (7), and were freed of unconjugated protein by gel filtration on columns of Biorad Agarose A-1.5m (ferritin–Con A) or Biogel P300 (ferritin–ricin) (1, 2, 6).

RESULTS

The subcellular fraction of the myeloma cells used in these experiments was markedly enriched in rough-membrane elements, compared to the original homogenate, but it contained some smooth-membrane fragments and vesicles as well. Therefore, comparison of the staining of rough- and smooth-membrane elements in the same specimen could be made.

With ferritin-Con A, some (roughly 25–35%) rough- and smooth-membrane elements were densely stained with ferritin particles; the remainder were not stained at all. There was no correlation between staining and the appearance



of the element in the thin section as an open fragment or a closed vesicle. All rough-membrane elements that were stained showed ferritin particles *exclusively* on the side of the membrane opposite to that occupied by the ribosomes (Fig. 1). Smooth-membrane elements stained with ferritin–Con A also showed an exclusive localization of ferritin to *one* mem-

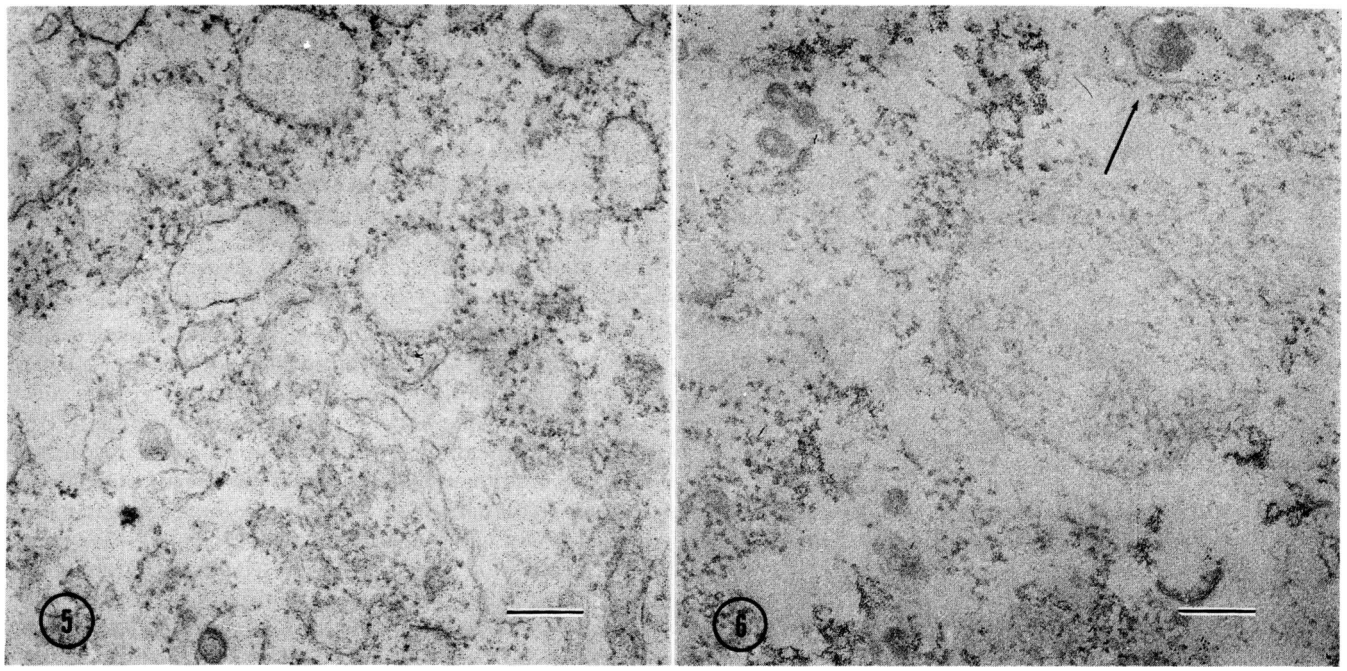


FIG. 1. A subcellular fraction enriched in fragments of rough endoplasmic reticulum, stained with a ferritin conjugate of concanavalin A. The *larger arrows* point to stained rough-membrane fragments with the ferritin molecules clearly localized to the side of the membrane opposite to the ribosomes. The *smaller arrow* points to a rough-membrane fragment that was not stained with ferritin. Post-stained with uranyl acetate. The *horizontal bar* represents 0.2 μm in all micrographs.

FIG. 2. A similar specimen as in Fig. 1. The ferritin–Con A is seen attached to the inner surface of a smooth-membrane (*SM*) vesicle and a fragment of rough-membrane (*larger arrow*). Other *SM* fragments (*smaller arrows*) were only lightly or not at all stained.

FIG. 3. A specimen similar to Fig. 1, but stained with a ferritin conjugate of ricin (ferritin–ricin). The *larger arrows* point to three fragments of the smooth membrane that show ferritin molecules exclusively on their outer surfaces. The *smaller arrows* designate smooth-membrane elements that were not stained. The field is full of rough-membrane fragments, none of them stained with ferritin–ricin.

FIG. 4. A specimen similar to that in Fig. 3; the ferritin–ricin conjugate used for staining was at $^1/_{16}$ of its usual concentration. As in Fig. 3, some smooth-membrane elements were stained exclusively on their outer surfaces, other smooth-membrane elements were not stained, and no rough-membrane elements were stained.

FIG. 5. A specificity control for ferritin–Con A staining. The presence of 0.25 M α-methyl-D-mannoside during the ferritin–Con A treatment of the fraction inhibited the ferritin–Con A binding to both rough- and smooth-membrane elements.

FIG. 6. A specificity control for ferritin–ricin staining. The present of 1 M β-lactose inhibited the binding of ferritin–ricin used at $^1/_{12}$ of its usual concentration (compare Fig. 4). Some faint reaction persisted, however (*arrow*).

brane surface of the element. When the smooth-membrane element appeared to be a closed vesicle, it was most often stained on its outer surface. Occasionally, however, the ferritin was localized to the inner surface of the vesicle (Fig. 2). The ferritin–Con A staining was essentially eliminated in the presence of 0.25 M α-methyl-D-mannoside (Fig. 5) or 0.25 M D-mannose (not shown), both competitive inhibitors of Con-A binding, but 0.25 M β-D-galactose, not an inhibitor, had no effect. Under the same staining conditions, 0.25 M sucrose did not effectively inhibit ferritin–Con A staining, but 1.0 M sucrose eliminated it.

With ferritin–ricin, no staining of rough-membrane elements was observed (Figs. 3 and 4). A significant fraction (roughly 30%) of the smooth-membrane elements in the same fields, however, showed dense ferritin-staining, the remainder none at all (Figs. 3 and 4). The ferritin was again localized to one surface of the smooth-membrane element, and if the element appeared vesiculated, ferritin was usually found on the outer surface of the membrane. Occasionally, however, the ferritin–ricin was localized to the apparent inner surface of the membrance (not shown). The ferritin–ricin staining was somewhat difficult to inhibit specifically (5). The competitive inhibitors of ricin binding, β-D-galactose and β-lactose, at concentrations up to 0.5 M were not effective in completely abolishing all ferritin–ricin staining, unless the ferritin–ricin in the mixture was substantially diluted (Fig. 6). If the ferritin–ricin was used at 1/5 to 1/7 the usual concentration, it still exhibited intense staining of smooth-membrane elements which, however, was now nearly eliminated in the presence of 0.5 M β-lactose.

DISCUSSION

Ferritin-conjugated plant agglutinins (1) have several distinct advantages over other reagents (for review, see ref. 8) for the electron microscopic localization of oligosaccharides and polysaccharides in biological specimens. Among these advantages are their relatively high spatial resolving power and their saccharide specificity. Both of these advantages were used in this study. The high resolving power made it feasible to show unambiguously that only one surface of a membrane element was stained. The use of agglutinins with different saccharide-binding specificities permitted differential staining of rough- and smooth-membrane elements, as is discussed more fully below. Concanavalin A binds specifically

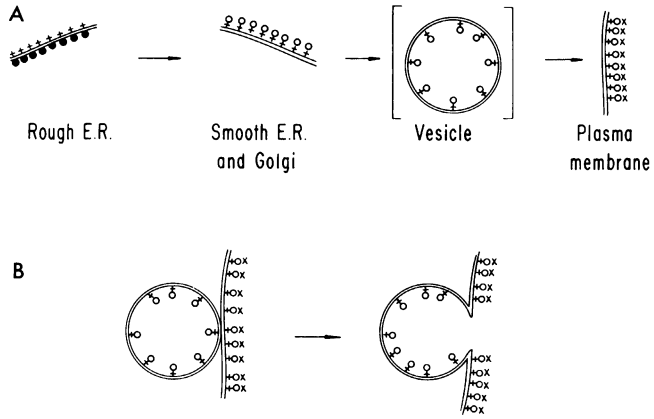


FIG. 7. Schematic mechanism for the biogenesis of plasma membranes of eukaryotic cells (16). An intracellular membrane "assembly line" (*A*) leads penultimately to the formation of vesicles, and new plasma membrane is generated by the fusion of such vesicles with previously existing plasma membrane (*B*). The large *filled circles* represent ribosomes. At different stages along the assembly line, saccharide units (+, O, ×) are added successively to growing oligosaccharide chains on membrane-bound glycoproteins (and glycolipids). This mechanism can explain the observations (1, 2) that oligosaccharides of plasma membranes are exclusively localized to the outer surface of the membrane (see text). E. R., endoplasmic reticulum.

α-D-mannopyranosyl, α-D-glucopyranosyl, and sterically related terminal sugar residues (9), whereas ricin is specific for β-D-galactopyranosyl and sterically related structures (5, 10).

The specificity of the observed ferritin–agglutinin staining reactions for saccharide structures was established by the observations that: (*a*) some membrane elements in the same preparation were intensely stained, while neighboring elements were not stained at all, and (*b*) small-molecule competitive inhibitors eliminated or markedly reduced the staining reactions (Figs. 5 and 6), whereas similar compounds known *not* to be competitive inhibitors of the particular agglutinin were without effect.

Before discussing the conclusions derived from the results described, the limitations of this preliminary study should be appreciated. With respect to the discrimination of different membrane elements in the subcellular fraction, only the two classes of rough- and smooth-membrane elements were distinguished. The rough-membrane elements, derived from the rough endoplasmic reticulum, were clearly identified by their attached ribosomes. The specific cellular site of orgin of a particular smooth-membrane element, however, was not ascertainable in these experiments; it might have been plasma, Golgi, mitochondria, or other smooth membranes. Furthermore, although it is most probable that the oligosaccharides that were detected were attached to glycoproteins, to what extent the myeloma immunoglobulin or the membrane glycoproteins were involved in the staining reactions was not determined. These limitations, however, are taken into account in the conclusions that follow.

The most important finding of the present study is that all the clearly recognizable membrane elements that were stained with either ferritin–Con A or ferritin–ricin were exclusively stained on only one of the two surfaces of the membrane element. This generalizes the unsymmetrical distribution of saccharide residues previously found for plasma membranes (1, 2) to rough endoplasmic reticulum membranes, and probably to other intracellular smooth membranes as well. We suggest the last point because it seems unlikely that all of the hundreds of stained smooth-membrane elements we have observed originated exclusively from the plasma membrane; and none from intracellular smooth membranes. Nevertheless, to prove unambiguously that the intracellular smooth membranes are also unsymmetrically stained, some suitable ultrastructural markers would have to be used to distinguish intracellularly derived smooth-membrane elements from plasma membrane fragments in the subcellular fraction.

A corollary of this finding is that the molecules in these membranes to which the saccharide groups are attached (presumably mainly glycoproteins, although glycolipids may also be stained) cannot rotate at any significant rate from one surface in the intact membrane to the other under physiological conditions (11). Otherwise, at least a significant fraction of the ferritin particles should have been found on both surfaces of most of the stained membrane elements. This inability of glycoproteins associated with membranes to rotate about an axis parallel to the membrane may be a general phenomenon: e.g., the Na^+-K^+-dependent and Mg^{++}-dependent ATPase activities of the erythrocyte membrane are exclusively localized to the inner membrane surface (12); and anionic phospholipid molecules undergo little or no such rotations in synthetic phospholipid vesicles (13). The absence of such rotational motions is particularly interesting in view of the likelihood that at least some membrane proteins (14) and lipids (15) undergo translational movements rather freely in the plane of the membrane under physiological conditions.

Our earlier finding (1, 2) that the oligosaccharides of the plasma membranes of several eukaryotic cells are exclusively located on the outer surfaces of the membranes raised the problem of the origin of this unsymmetrical distribution. Is it a reflection of secondary effects, such as unsymmetrical glycolytic cleavage reactions, acting on a membrane that is initially symmetrical as synthesized? Or is it the direct consequence of the mechanisms of plasma-membrane biogenesis in eukaryotic cells? Concerning the latter possibility, it was suggested first by Palade (16) that the plasma membranes of eukaryotic cells are not formed *de novo*, but instead, by analogy to protein secretory mechanisms, by an "assembly-line" process from intracellular precursor membranes. It is proposed that membrane elements are assembled starting from the rough endoplasmic reticulum; they are then transmitted and converted into smooth membrane and Golgi elements, where they are packaged into vesicles. These vesicles, it is suggested, then fuse with already-existing plasma membrane to generate new plasma membrane. Presumably, this fusion process is followed by a mixing of the newly inserted and the previously present membrane components by translational diffusion within the membrane (14). Although possible precursor vesicles have been observed under electron microscopy (see refs. 17–20), their functional role has not positively been established. On the other hand, several kinds of indirect experimental evidence are at least consistent with this precursor membrane hypothesis. Among these is the finding (21) that while cycloheximide treatment stops further incorporation of a previously applied pulse of [^{3}H] leucine label into the cytoplasmic proteins of rat-liver cells, it does not markedly affect the continued incorporation of the label into their plasma membranes.

From other investigations (for reviews, see refs. 22 and 23), it is known that the covalent attachment of saccharide residues to glycoproteins proceeds in a stepwise manner, with certain residues attached in different cellular compartments. For example, it has been suggested from experiments with puromycin that those residues most proximal to the polypeptide chain become attached to the nascent chains in the rough endoplasmic reticulum (24, 25), but other residues that are attached further along the oligosaccharide chains, such as D-galactose (26–28) and L-fucose (18), are bound only subsequently in smooth-membrane elements, probably in the Golgi zone.

These facts and hypotheses are summarized in Fig. 7. If the mechanism of a fusion of precursor vesicle membranes with existing plasma membrane is adopted, the exclusive localization of oligosaccharides to the outer surface of the plasma membrane can readily be accounted for, *if we further postulate that the oligosaccharides present on the precursor vesicles are exclusively localized to the inner surface of the vesicle membranes* (Fig. 7).

The experimental results described in this paper are consistent with the formulation in Fig. 7, in the following respects:

(*a*) Ferritin–ricin stained only smooth- and not rough-membrane elements. This result shows directly that terminal β-D-galactopyranosyl (or sterically related) residues are not attached to membranes in the rough endoplasmic reticulum, but only to smooth-membrane elements, as had been suggested previously by other methods (26–28). That, on the contrary, ferritin-Con A did stain rough-membrane elements is consistent with earlier observations (see ref. 29) that the radioactivity of labeled mannose is incorporated into rough-membrane elements. The ferritin–Con A staining of smooth- as well as rough-membrane elements may have one or more explanations, but probably reflects the attachment of mannose or sterically related sugar residues to the growing oligosaccharide chains, not only at early stages, but also after the galactose residues are attached. The fact that the majority of membrane elements are completely unstained is possibly due to their inaccessibility to the stain, but this seems unlikely. It is more likely an indication of the heterogeneity of the rough- and smooth-membrane elements in the preparation that could be due to differences among different cells in the population, or to *intracellular* heterogeneity (30) such as might be expected from the assembly-line process depicted in Fig. 7.

(*b*) The oligosaccharide chains are always localized to only a single surface of a membrane element, as is required by the mechanism of Fig. 7. Although it is not known in our experiments whether the oligosaccharides being stained are attached to the myeloma immunoglobulin or membrane glycoproteins, or both, the results are nevertheless relevant since the mechanisms of synthesis of the oligosaccharide chains of different mammalian plasma-type glycoproteins appear to be closely parallel (22, 23). On rough-membrane elements, the oligosaccharide chains appear to be confined to the side of the membrane opposite to the side to which the ribosomes are attached (Fig. 1), i.e., the cisternal side of the rough endoplasmic reticulum. It is proposed, by analogy to pancreatic zymogen secretory processes, that the interior space of the postulated precursor vesicles corresponds to the cisternal space of the rough endoplasmic reticulum. The observed orientation of the saccharides on the rough-membrane elements is therefore consistent with a localization of oligosaccharide chains to the inner surface of the precursor vesicles, as is postulated in Fig. 7. It is of further interest that some of the smooth-membrane elements that appeared to be vesiculated (see Fig. 2) showed ferritin–agglutinin staining on their inner surfaces, whereas most of them were stained on their outer surfaces. However, as discussed above, without additional ultrastructural markers to distinguish specific smooth-membrane elements and their two surfaces, the significance of the apparent staining of the inner surface is not yet clear.

1. Nicolson, G. L. & Singer, S. J. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 942–945.
2. Nicolson, G. L. & Singer, S. J., submitted to *J. Cell Biol.*
3. Horibata, K. & Harris, A. W. (1970) *Exp. Cell Res.* **60**, 61–77.
4. Choi, Y. S., Knopf, P. M. & Lennox, E. S. (1971) *Biochemistry* **10**, 659–667.
5. Nicolson, G. L. & Blaustein, J. C. (1972) *Biochim. Biophys. Acta* **266**, 543–547.
6. Nicolson, G. L., Marchesi, V. T. & Singer, S. J. (1971) *J. Cell Biol.* **51**, 265–272.
7. Avrameus, S. (1969) *Immunochemistry* **6**, 43–52.
8. Rambourg, A. (1971) *Int. Rev. Cytol.* **31**, 57–114.
9. Poretz, R. D. & Goldstein, I. J. (1970) *Biochemistry* **9**, 2890–2896.
10. Drysdale, R. G., Herrick, P. R. & Franks, D. (1968) *Vox Sang.* **15**, 194–202.
11. Singer, S. J. & Nicolson, G. L. (1972) *Science* **175**, 720–731.
12. Marchesi, V. T. & Palade, G. E. (1967) *J. Cell Biol.* **35**, 385–404.
13. Kornberg, R. K. & McConnell, H. M. (1971) *Biochemistry* **10**, 1111–1128.
14. Frye, C. D. & Edidin, M. (1970) *J. Cell Sci.* **7**, 319–335.
15. Devaux, P. & McConnell, H. M. (1972) *J. Amer. Chem. Soc.*, in press.
16. Palade, G. (1959) in "Subcellular Particles," ed. Hayashi, T. (Ronald Press, New York), p. 64.
17. Rambourg, A., Hernandez, W. & Leblond, C. P. (1969) *J. Cell Biol.* **40**, 395–414.
18. Bennett, G. & Leblond, C. P. (1970) *J. Cell Biol.* **46**, 409–416.
19. Stockem, W. (1969) *Histochemie* **18**, 217–240.
20. Wise, G. E. & Flickinger, C. J. (1970) *Exp. Cell Res.* **61**, 13–23.
21. Ray, T. K., Lieberman, I. & Lansing, A. I. (1968) *Biochem. Biophys. Res. Commun.* **31**, 54–65.
22. Spiro, R. G. (1970) *Annu. Rev. Biochem.* **39**, 599–638.
23. Kraemer, P. M. (1971) in *Biomembranes*, ed. Manson, L. A. (Plenum Press, New York), Vol. I, pp. 67–190.
24. Molnar, J. & Sy, D. (1967) *Biochemistry* **6**, 1941–1947.
25. Whur, P. & Herscovics, A. (1968) *Anat. Record* **160**, 450.
26. Melchers, F. & Knopf, P. M. (1967) *Cold Spring Harbor Symp. Quant. Biol.* **32**, 255–262.
27. Zagury, D., Uhr, J. W., Jamieson, J. D. & Palade, G. E. (1970) *J. Cell Biol.* **46**, 52–63.
28. Choi, Y. S., Knopf, P. M. & Lennox, E. S. (1971) *Biochemistry* **10**, 668–679.
29. Whur, P., Herscovics, A. & Leblond, C. P. (1969) *J. Cell Biol.* **43**, 289–311.
30. Wise, G. E. & Flickinger, C. J. (1970) *J. Cell Biol.* **46**, 620–626.