SPATIAL ORIENTATION OF GLYCOPROTEINS IN MEMBRANES OF RAT LIVER ROUGH MICROSOMES

II. Transmembrane Disposition and Characterization of Glycoproteins

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ABSTRACT

Rat liver microsomal glycoproteins were purified by affinity chromatography on concanavalin A Sepharose columns from membrane and content fractions, separated from rough microsomes (RM) treated with low concentrations of deoxycholate (DOC). All periodic acid-Schiff (PAS)-positive glycoproteins of RM showed affinity for concanavalin A Sepharose; even after sodium dodecyl sulfate (SDS) acrylamide gel electrophoresis, most of the microsomal glycoproteins bound [^{125}I]concanavalin A added to the gels, as detected by autoradiography. Two distinct sets of glycoproteins are present in the membrane and content fractions derived from RM. SDS acrylamide gel electrophoresis showed that RM membranes contain 15–20 glycoproteins (15–22% of the total microsomal protein) which range in apparent mol wt from 23,000 to 240,000 daltons. A smaller set of glycoproteins (five to seven polypeptides), with apparent mol wt between 60,000 and 200,000 daltons, was present in the microsomal content fraction.

The disposition of the membrane glycoproteins with respect to the membrane plane was determined by selective iodination with the lactoperoxidase (LPO) technique. Intact RM were labeled on their outer face with ^{131}I and, after opening of the vesicles with 0.05% DOC, on both faces with ^{125}I. An analysis of iodination ratios for individual proteins separated electrophoretically showed that in most membrane glycoproteins, tyrosine residues are predominantly exposed on the luminal face of the vesicles, which is the same face on which the carbohydrate moieties are exposed. Several membrane glycoproteins are also exposed on the cytoplasmic surface and therefore have a transmembrane disposition. In this study, ribophorins I and II, two integral membrane proteins (mol wt 65,000 and 63,000) characteristic of RM, were found to be transmembrane glycoproteins. It is suggested that the transmembrane disposition of the ribophorins may be related to their possible role in ribosome binding and in the vectorial transfer of nascent polypeptides into the microsomal lumen.

KEY WORDS rough microsomes · transmembrane glycoproteins · concanavalin A lactoperoxidase iodination · ribosome-binding sites

The asymmetric distribution of molecular components within membranes enables these structures to carry out their function as selective permeability barriers between the cell and its environment and

in between subcellular compartments. Studies carried out with erythrocyte membranes indicate that all membrane components, i.e., proteins, lipids, and carbohydrates, are asymmetrically distributed in relation to the phospholipid bilayer (9, 50). Using several experimental approaches, it has been shown that carbohydrates in plasma membranes of erythrocytes are confined to the outer face of the membrane (13, 51, 15). The external localization of carbohydrates in plasma membranes of several mammalian cell types has also been demonstrated, electron microscopically, using ferritin conjugated to lectins (36). In fact, glycoproteins seem to be the only class of proteins exposed on the outer face of the red cell membrane (51, 50, 15). Furthermore, it has been shown that four major glycoproteins of the red cell membrane span the width of the lipid bilayer and are accessible to chemical and enzymatic modifications performed on the cytoplasmic side of the membrane (7, 8, 32, 46, 6, 33, 34, 54, 15).

Although considerable information is available concerning the structural organization of glycoproteins in surface membranes, only a limited number of studies have been carried out on the molecular organization of glycoproteins in intracellular membranes. It has been shown that lectin-binding sites in endoplasmic reticulum (ER) membranes are distributed asymmetrically (17, 39, 41, 55) and that this distribution is topologically equivalent to that found in surface membranes. From the patterns of lectin binding to rough microsomes (RM) (41), and from determinations of the sugar composition of ER membranes (18), it was concluded that carbohydrate chains in microsomal glycoproteins contain mannose-rich cores but lack the terminal sugar chains characteristic of plasma membrane (PM) carbohydrates.

In microsomal vesicles glycoproteins are present within the luminal content and as components of the limiting membranes (24). In this study, we have attempted to characterize electrophoretically these two sets of glycoproteins, after their purification by affinity chromatography on concanavalin A (Con A) Sepharose. Experiments were also performed to establish the exposure of membrane glycoproteins on each membrane face of RM and to determine which glycoproteins, if any, have a transmembrane disposition. It was found that this is the case for several glycoproteins, including two of apparent mol wt 65,000 and 63,000, which appear to play a role in ribosome binding and have received the names of ribophorins I and II (25–27).

MATERIALS AND METHODS

Materials

Acrylamide, methyl bisacrylamide, *N,N,N'N'*-tetramethylethylenediamine (TEMED), and butylated hydroxytoluene (BHT) were purchased from Eastman Kodak Co. (Rochester, N. Y.); deoxycholic acid (DOC) and Coomassie Brilliant Blue from Schwartz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.); anhydrous sodium borohydride, anhydrous L-ascorbic acid, Tris, D-glucose-6-phosphate (G6P) (disodium salt), NADPH (tetrasodium salt), AMP type III (sodium salt), cytochrome *c* type III, glycine, sodium dodecylsulfate (SDS), α-methylmannoside (α-MM) and glucose oxidase (GO) type I (1,000 EU[1]/ml), were acquired from Sigma Chemical Co. (St. Louis, Mo.); lactoperoxidase (LPO) (100 IU[2]/mg) from Calbiochem (La Jolla, Calif.); ^{131}I and ^{125}I from New England Nuclear (Boston, Mass.); Con A and Con A bound to Sepharose beads were obtained from Pharmacia Inc. (Piscataway, N. J.); sodium dimethylarsenate (sodium cacodylate) was purchased from Fisher Scientific Co. (Pittsburgh, Pa.).

Solutions

Low salt buffer (LSB) is 50 mM Tris HCl, pH 7.5, 50 mM KCl, and 5 mM $MgCl_2$; high salt buffer (HSB) is 50 mM Tris HCl, pH 7.5, 500 mM KCl, and 10 mM $MgCl_2$; SLSB is 20% sucrose with the salt composition of LSB.

Methods

A subfraction of rat liver RM, RM_2, (see Table II), which will indistinctly be referred to as RM in this paper, was prepared as described previously (41), except that 1 μg/ml BHT was added to all solutions to prevent lipid peroxidation and to enhance the LPO-catalyzed incorporation of iodine into RM (56). The activity of enzyme markers was measured in microsomal fractions to estimate the degree of contamination with other cell membranes. LPO-catalyzed iodination was always carried out on fresh RM preparations since it was found that storage in glycerol impaired the reaction. RM used for experiments not involving iodination were stored at −70°C in glycerol-LSB (2:1). Before use, RM were washed by centrifugation in HSB for 20 min at 15,000 rpm in a Ti60 rotor (abbreviated as 20 min, 15K, Ti60). RM vesicles were made permeable to macromolecules by the addition of 0.05% DOC, a condition which leaves the vesicular membrane largely intact (22, 41). Protein was determined according to Lowry et al. (29).

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[1] One European unit (EU) catalyzes the iodination of 1 μmol of glucose to gluconic acid and H_2O_2 per minute at pH 5.1 at 35°C.

[2] One international unit (IU) catalyzes the conversion of 1 μmol tyrosine to monoiodotyrosine per minute at 30°C.

Enzymatic Assays

The activity of cytochrome oxidase was assayed according to Smith (49). Horse heart cytochrome *c* (1.2–1.5 mg/ml in 0.1 M phosphate buffer at pH 7.0) was reduced at 5°C under N_2 with $NaBH_4$ until the OD_{550}/OD_{565} ratio was between 9 and 10, followed by extensive dialysis against the same buffer at 5°C under N_2. The reaction mixture contained in 1 ml: 0.8 ml of phosphate buffer, 0.167 ml of the solution of reduced cytochrome *c*, and 0.033 ml of the sample. Incubation was carried out at 25°C and the decrease in absorbancy at 550 nm was followed continuously with a recording spectrophotometer (Aminco 2D spectrophotometer, American Instrument Co., Silver Spring, Md.). 1 U is defined as the amount of enzyme causing a change in log ($A_{550\,nm}$ − $A_{550\,nm}$ for the fully oxidized sample) of 100 per minute.

NADPH cytochrome *c* reductase was assayed by the procedure described by Omura and Takesue (37) using horse cytochrome *c* as the electron acceptor, with a millimolar extinction difference between the reduced and the oxidized forms of 21.0 mM cm^{-1} at 550 nm. The reaction mixture contained 2×10^{-4} M NaCN, 1×10^{-4} M horse cytochrome *c*, 8×10^{-6} M NADPH and the sample, in 1 ml of 0.1 M sodium phosphate buffer (pH 7.5). It has been found that high sucrose concentrations decrease the activity of the enzyme (Dr. T. Morimoto, personal communication); therefore, care was taken to keep the sucrose concentration in the reaction mixture below 2%. Absorbance at 550 nm was followed continuously at 25°C with a recording spectrophotometer, and enzyme activities were calculated from the initial slopes of the curves. 1 U of NADPH cytochrome *c* reductase activity reduces 1 μmol of cytochrome *c* per minute at 25°C.

The activity of 5′-nucleotidase was determined by the procedure described by Widnell and Unkeless (57), measuring P_i directly according to Chen et al. (10) and Ames and Dubin (3). A blank containing the sample but no AMP was run for each assay. 1 U of enzyme was defined as the activity which releases 1 μmol of phosphate as P_i per hour at 37°C.

G6Pase activity was determined using the procedure described by De Duve et al. (11) and Swanson (52), with the slight modification introduced by Leskes et al. (28). Samples were incubated for 20 min at 37°C and the reaction was stopped by the addition of 15% TCA; two blanks for each sample were always included: one to which TCA was added at zero time, and one from which G6P was omitted. Sedimentable membranes were removed by centrifugation, and the supernatant fractions were analyzed for P_i according to Ames and Dubin (3). 1 U of enzyme released 1 μmol of phosphate as P_i per hour at 37°C.

Enzymatic Double Labeling of RM with ^{131}I and ^{125}I

The LPO-catalyzed iodination of RM was carried out at 0°C in LSB containing 10 mM glucose, 1 IU/ml LPO, 0.14 EU/ml GO, 100 μCi/ml ^{131}I or ^{125}I, and 1 μg/ml BHT. After 30 min, the reaction was stopped by centrifugation through SLSB or by the addition of $Na_2S_2O_3$ to a final concentration of 10^{-5} M. Double labeling with ^{131}I and ^{125}I was carried out according to experimental details given in Results.

Radioactivity Determinations

To determine protein bound radioiodine in the incubation mixture, 25-μl aliquots were spotted on glass filters (GF/C, Whatman), which were processed as described for RNA precipitation (31), using ice-cold TCA which contained 10^{-3} M NaI.

Radioactivity was determined with a two-channel Nuclear Chicago Gamma Counter Nuclear-Chicago Corp. (Des Plaines, Ill.) calibrated to measure ^{131}I and ^{125}I simultaneously. Corrections were always made for the amount of ^{131}I radioactivity registered in the ^{125}I channel, which corresponded to 22.4% of the radioactivity measured in the ^{131}I channel. The contribution of ^{125}I to the radioactivity measured in the ^{131}I channel was very small (0.005% of that in the ^{125}I channel) and was not computed. Since there were always intervals of 4 days or longer between the time of labeling and the counting of the gels, ^{131}I radioactivity measurements were corrected to values at the day of labeling.

Glycoprotein Purification

Glycoproteins with affinity for Con A were purified according to Allan et al. (1). RM (5 mg protein/ml) in LSB were solubilized with 2% DOC, the insoluble proteins were removed by centrifugation (60 min, 40K, Ti50), and the supernate was passed through a Con A Sepharose-4B column pre-equilibrated with 1% DOC at pH 8.0. The column was washed thoroughly with 1% DOC. Glycoproteins bound to the column were eluted with 10 mg/ml α-methylmannoside (α-MM) in 1% DOC. The glycoproteins were concentrated and prepared for gel electrophoresis according to Allan et al. (1).

Gel Electrophoresis

Discontinuous SDS acrylamide gel electrophoretic analysis was carried out basically as described by Maizel (30). For the analysis of radioactivity, gels were fractionated into 1-mm slices with a gel slicer (Medical Research Apparatus Corp., Boston, Mass.) and counted without further treatment. Apparent molecular weights of microsomal proteins were estimated by comparing their electrophoretic mobilities in SDS acrylamide gels with those of marker proteins of known molecular weights. The molecular weights assigned in this manner represent only a gross approximation since it has been shown that glycoproteins migrate anomalously in SDS acrylamide gels (45).

Identification of Microsomal Glycoproteins in SDS Acrylamide Gels

Microsomal glycoproteins containing Con A-binding

sites were detected directly in the gels after separation by SDS acrylamide gel electrophoresis by a procedure similar to that described by Tanner and Anstee (53). 1-mm thick slab gels which had been previously stained with Coomassie Blue and destained with acetic acid-methanol solutions were washed with LSB until neutral pH was reached. Gels were then incubated for 90 min in LSB containing 0.1–0.2 mg/ml [^{125}I]Con A (3×10^7 cpm/mg) labeled as previously described (41). To remove most of the free [^{125}I]Con A, the gels were washed four times (for 30 min each) in LSB. After drying, the gels were exposed to Cronex 2 DC X-ray plates (DuPont Instruments, Wilmington, Del.) for 5–10 days.

RESULTS

Distribution of Enzymes in Subcellular Fractions

It has been previously demonstrated that in RM fractions the presence of binding sites for lectins which are specific for sugars distal to the mannose-rich core of glycoproteins is due to minor contamination by membranes derived from organelles other than the rough ER (41). The purity of the heavy fraction of rough microsomes (RM_2) used in the experiments presented in this paper was therefore assessed through determinations of the activity of enzyme markers characteristic of different subcellular membranes. Table I shows these results, as well as the distribution and specific activity of the marker enzymes in the other subfractions obtained during preparation of the RM. It is apparent that the RM_2 fraction, in which only 1.4% of the total protein was recovered, is highly enriched with respect to the homogenate in the microsomal markers G6Pase (6.1 ×) and NADPH cytochrome *c* reductase (2.8 ×). On the other hand, specific activity values for cytochrome oxidase and 5′-nucleotidase in RM_2 are only a fraction (37 and 38%, respectively) of those of the homogenate, indicating low contamination with mitochondria and PM fragments. Assuming that mitochondria contain 16% of the liver homogenate protein (12), the mitochondrial contamination can be estimated to represent only 6% of the protein in RM_2. Since PMs contribute <2% of the liver homogenate protein (35), the contamination of RM_2 with PM proteins is lower than 1%. PM contamination could even be smaller since 5′-nucleotidase may be a constituent of microsomal membranes (57), although its true specific activity in microsomes is thought to be much lower than in PM.

Fractionation of Microsomal Glycoproteins by Affinity Chromatography on Con A Sepharose and Electrophoretic Characterization of Membrane and Content Subfractions

Glycoproteins were isolated from RM fractions, following the scheme given in Fig. 1, by affinity chromatography on Con A Sepharose according to Allan et al. (1). Table II shows the relative amounts of protein in the fractions obtained during purification by this procedure. RM (3 mg of

TABLE I

*Distribution of Enzymatic Markers in Subcellular Fractions**

Fraction‡	Protein§	Cytochrome oxidase		G6Pase		NADPH-cytochrome *c* reductase		5′-Nucleotidase	
		U	U/mg (× 10^{-3})	U	U/mg	U	U/mg (× 10^{-3})	U	U/mg
	%	%		%		%		%	
Homogenate	100.0	100.0	4.03	100.0	10.47	100.0	3.33	100.0	5.87
PNS	86.5	86.2	4.03	91.0	11.03	94.3	3.63	88.1	6.00
Nuclei	8.2	3.5	1.71	6.3	8.09	4.1	1.63	7.3	5.25
Mitochondria	32.9	77.0	9.43	42.0	13.39	27.7	2.77	46.9	8.56
PMS	44.7	7.2	0.60	52.8	12.43	69.7	5.23	39.6	5.15
RM_1	1.3	0.7	2.09	5.1	41.38	4.2	10.70	1.2	5.56
RM_2	1.4	0.5	1.49	8.3	64.00	3.9	9.42	0.5	2.22
SM	2.9	1.9	2.71	8.5	31.40	10.7	12.30	13.4	27.70
Soluble	31.3	2.2	0.29	20.7	6.96	30.6	4.36	17.1	3.21

* Results are the average of two experiments.

‡ PNS: postnuclear supernate, PMS: postmitochondrial supernate, RM_1 and RM_2: rough microsomal fractions 1 and 2, SM: smooth microsomes.

§ 100% of protein corresponds to 8,300 mg.

TABLE II

*Protein Recovery during Purification of RM Membrane Glycoproteins**

Fraction‡	Protein	Percent
	mg	
RM	50.9	100.0
Mb	44.8	88.0
Cont.	5.2	10.2
$Mb_{insol.}$	4.0	7.9
$Mb_{sol.}$	38.4	75.4
$Mb_{excl.}$	27.2	53.4
Mb_{bound}	6.8	13.4

* Results are the average of two experiments.

‡ Abbreviations used are those defined in Fig. 1.

protein/ml) were treated with 0.05% DOC in LSB, and membranes bearing ribosomes (Mb) were separated by centrifugation from content proteins (Cont.) which constituted ~10–12% of the total. The content-free RM (Mb) were then treated with 2% DOC, which resulted in 85–91% of the membrane proteins becoming nonsedimentable after centrifugation at 10^5 *g* for 60 min ($Mb_{sol.}$). The fraction insoluble in 2% DOC ($Mb_{insol.}$) contained mainly a protein of 34,000 daltons, which has been identified as urate oxidase and which is present in peroxisomal cores contaminating the RM fraction (26). To separate the glycoproteins, the solubilized membrane protein fraction ($Mb_{sol.}$) was passed slowly (7 ml/min) through a Con A Sepharose column (1 cm × 7 cm). The protein fraction which did not bind to the lectin ($Mb_{excl.}$) was collected and the column was washed with 1% DOC, pH 8, until the absorbance at 280 nm decreased to low levels, similar to those measured before the application of the sample (representing mainly a basal release of Con A from the column). Approximately 29% of the membrane protein soluble in 2% DOC ($Mb_{sol.}$) was retained by the Con A column. Most of this material (>60%) was eluted with α-MM (10 mg/ml; 0.5 bed vol/h) and will be referred to as Mb_{bound}. A similar procedure, but using only 0.05% DOC, was employed to separate glycoproteins ($Cont._{bound}$) from other proteins ($Cont._{excl.}$) in the content fraction.

SDS acrylamide gel electrophoretic analysis of proteins in the membrane and content fractions and in the subfractions obtained during the purification procedure (Fig. 2) demonstrated that the Con A Sepharose chromatography resulted in the purification of two distinct subsets of microsomal glycoproteins. The Coomassie Blue-staining patterns (Fig. 2*a–h*) showed that glycoproteins of different electrophoretic mobilities were present in the membrane and content fractions (compare Fig. 2*e* and *h*). In the purified glycoprotein fractions, periodic acid-Schiff (PAS)-staining patterns agreed well with patterns obtained after staining with Coomassie Blue (Fig. 3*c* and *c'*). On the other hand, no PAS-positive polypeptides were detected in the membrane protein fraction which was excluded from the Con A column (Fig. 3*b* and *b'*). PAS-staining patterns of membrane glycoproteins were also in good agreement with the autoradiographic patterns obtained after binding of [^{125}I]Con A to purified glycoproteins separated by SDS acrylamide gel electrophoresis (compare Fig. 2*e'* and Fig. 3*c'*).

Most of the glycoproteins capable of binding to Con A Sepharose retained their ability to bind to Con A even after separation by SDS acrylamide

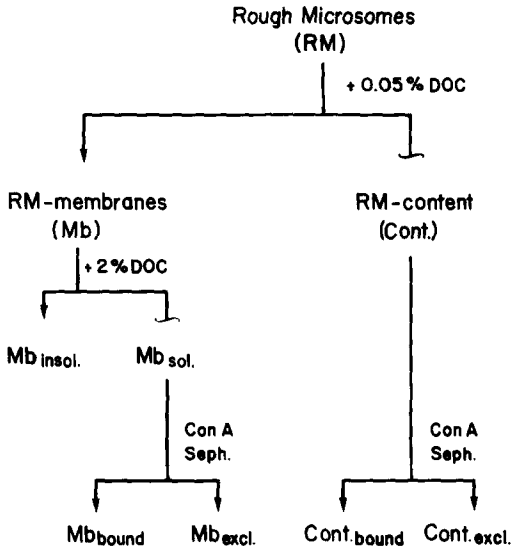

FIGURE 1 Schematic description of the procedure for purifying membrane and content glycoproteins from RM. RM membranes and RM content were obtained from RM by treatment with 0.05% DOC. Abbreviations defined in this figure are used in the text and in all subsequent legends: *RM*, rough microsomes; *Mb*, content-free RM; $Mb_{insol.}$, sedimentable fraction (10^5 *g*, 60 min) obtained after treatment of Mb with 2% DOC; $Mb_{sol.}$, nonsedimentable fraction obtained after treatment of Mb with 2% DOC; $Mb_{excl.}$, protein fraction from $Mb_{sol.}$ which did not bind to the Con A Sepharose column; Mb_{bound}, glycoprotein fraction from $Mb_{sol.}$ which was retained by the Con A Sepharose column, and was eluted with α-MM. $Cont._{bound}$ and $Cont._{excl.}$ are separated by chromatography on Con A Sepharose from RM-content.

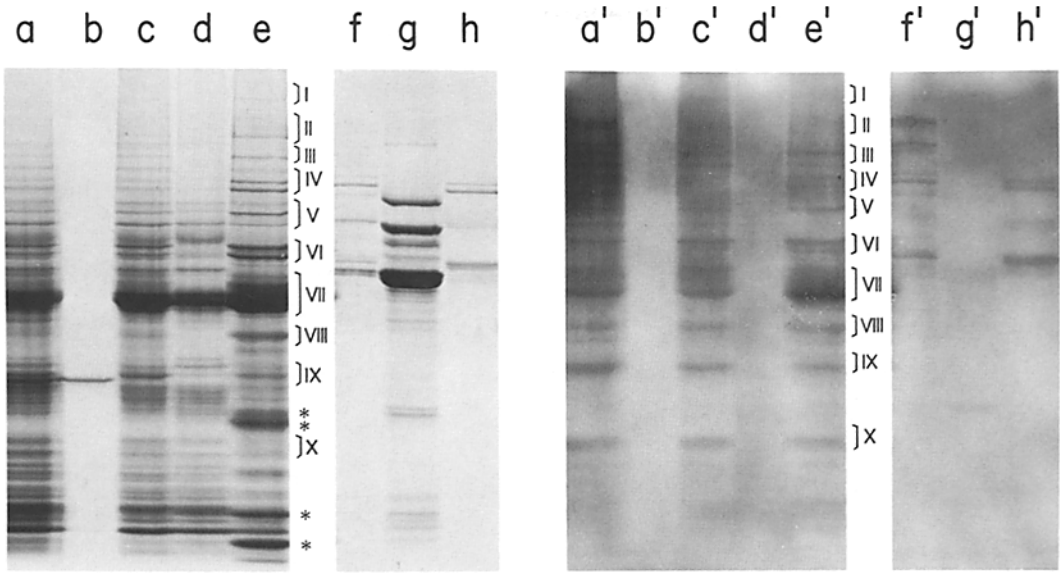

FIGURE 2 Electrophoretic analysis of microsomal subfractions obtained during purification of glycoproteins from RM. Proteins and glycoproteins isolated from RM membrane and content fractions (see Fig. 1) were analyzed by SDS acrylamide electrophoresis (1 mm thick, 8–12% in acrylamide concentration). The abbreviations for protein fractions are those introduced in Fig. 1: (*a*, *a'*) Mb; (*b*, *b'*) $Mb_{insol.}$; (*c*, *c'*) $Mb_{sol.}$; (*d*, *d'*) $Mb_{excl.}$; (*e*, *e'*) Mb_{bound}; (*f*, *f'*) Cont.; (*g*, *g'*) $Cont_{excl.}$; (*h*, *h'*) $Cont_{bound}$. Gels *a-h* show the Coomassie Blue-staining patterns, while *a'-h'* represent autoradiographs of the same gels after incubation of stained gels with [^{125}I]Con A. In gel *e* the asterisks indicate the positions of subunits of Con A released from the column used for the purification of the glycoproteins. The same bands are seen in gel *g*. The amount of protein loaded in gel (*a*, *a'*), was 300 μg. For other samples amounts were selected to maintain the proportion contributed by each fraction to the total RM protein as determined by the Lowry procedure, except in the case of samples (*e*, *e'*) and (*g*, *g'*) where 3× and 4× the corresponding amounts were loaded. Roman numerals (*I–X*) indicate the glycoprotein groups described in Fig. 4.

gel electrophoresis (Fig. 2*a'–h'*). As expected, proteins which were excluded from the column did not show detectable levels of Con A binding after separation by gel electrophoresis (Fig. *d'* and *g'*). Identification through the binding of [^{125}I]Con A to gels also allowed the recognition of the characteristic subsets of membrane and content glycoproteins (Fig. 2, compare *e'* and *h'*), even on gels of unfractionated microsomes or of microsomal fractions before the affinity chromatography step (see Fig. 2*a'*, *c'*, and *f'*).

The fact that gels of the membrane protein fraction ($Mb_{sol.}$) and of the purified glycoproteins (Mb_{bound}) gave similar patterns of PAS staining and [^{125}I]Con A binding demonstrated that no major membrane glycoproteins were selectively retained by the column (compare Fig. 2*c'* and Fig. 3*a'* with Fig. 2*e'* and Fig. 3*c'*, respectively). It should be noticed, however, that two high molecular weight glycoproteins of the content fraction were retained by the Con A Sepharose column and could not be eluted with α-MM (compare Fig. 2*f'* and *h'*). Taken together, these results suggest that a mannose-rich core containing Con A-binding sites is a feature common to all microsomal glycoproteins.

As was estimated by SDS acrylamide gel electrophoresis, apparent molecular weights of RM membrane glycoproteins ranged from 23,000 to 240,000 daltons. Although within this range 10 main groups could be recognized (Fig. 4), the major membrane glycoproteins had mol wt between 65,000 and 35,000 daltons (groups VI, VII, and VIII). Microsomal content glycoproteins had molecular weights within the range of 50,000 to 200,000 (positions which would correspond to content glycoproteins are indicated by arrows in Fig. 4). Ribophorins I and II, two integral membrane proteins (65,000 and 63,000 daltons) that may be involved in the attachment of ribosomes to the membranes (25–27), are found within group VI of membrane glycoproteins. It has been

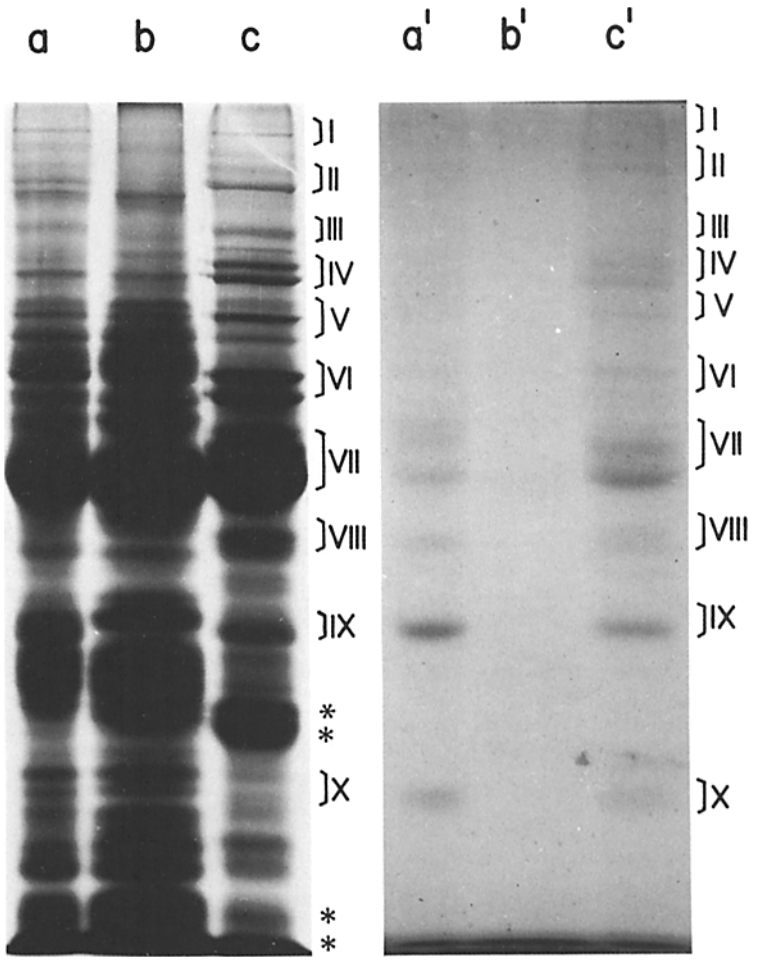

FIGURE 3 Electrophoretic analysis of glycoproteins isolated from membranes of RM. Rough microsomal membrane proteins solubilized in 2% DOC ($Mb_{sol.}$) were fractionated by affinity chromatography on Con A Sepharose and analyzed by electrophoresis on a discontinuous polyacrylamide gel (5 mm thick, 8%). Gels were first stained with the PAS procedure for carbohydrates, photographed (*a'-c'*), and then stained with Coomassie Blue (*a-c*). In gel *c* the asterisks indicate the position of subunits of Con A released from the column used for the purification of the glycoproteins. (*a*, *a'*) $Mb_{sol.}$ (800 μg protein); (*b*, *b'*) $Mb_{excl.}$ (1,200 μg); (*c*, *c'*) Mb_{bound} (1,400 μg). Numbers within parentheses indicate amounts of protein loaded.

shown that the presence of ribophorins represents the major polypeptide difference detectable by SDS acrylamide electrophoresis between membranes of RM from which ribosomes have been removed by treatment with HSB and puromycin and smooth microsomal membranes (26).

Group VII contains at least three membrane glycoproteins with mol wt ranging from 48 to 56,000 daltons. These are likely to represent polypeptides related to cytochrome P-450, since various species of cytochrome P-450 fall within this molecular weight range (2, 42, 16), and it has been recently reported (16) that some cytochrome P-450 species are glycoproteins containing two mannose residues and one N-acetylglucosamine residue linked to asparagine. This carbohydrate composition is compatible with affinity for Con A (47).

LPO-Catalyzed Iodination of RM

The RM fraction is composed mainly of closed vesicles with ribosomes attached to the outer face of the membranes. The predominant orientation of the membranes in RM is therefore equivalent

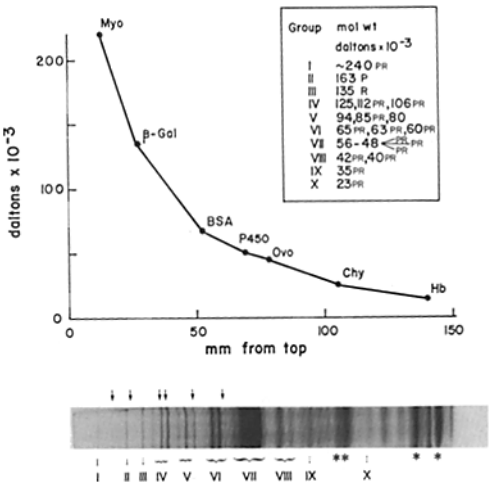

FIGURE 4 Apparent molecular weights of rough microsomal glycoproteins estimated by SDS acrylamide gel electrophoresis. The electrophoretic pattern of RM membrane glycoproteins (Mb_{bound}) (200 μg), analyzed on SDS acrylamide gel (1 mm, 8–11%) and stained with Coomassie Blue is shown at the bottom. Roman numerals (*I-X*) were assigned to groups of glycoproteins which run with close mobilities. Arrows indicate the positions which would correspond to microsomal content glycoproteins ($Cont_{bound}$). The upper part of the figure represents a calibration curve obtained in the same gel system using myoglobin (*Myo*), β-galactosidase (*β-Gal*), bovine serum albumin (*BSA*), cytochrome P-450 (*P450*), ovalbumin (*Ovo*), chymotrypsin (*Chy*), and Hemoglobin subunits (*Hb*) as markers. In the *inset*, the apparent molecular weights for glycoproteins in each group (*I-X*) are listed; the description to the right of each mol wt value indicates whether the band is PAS-positive (*P*) and/or binds [^{125}I]Con A, as demonstrated by autoradiography after incubation of the gel with the iodinated lectin (*R*). The asterisks represent Con A polypeptides released from the column during elution.

to the one in rough ER cisternae. Since microsomal vesicles are impermeable to the passage of macromolecules, the exposure of proteins on the surfaces of the membranes can be coveniently studied by determining their accessibility to iodination with an LPO-catalyzed reaction (56, 23).

To study the disposition of glycoproteins in the microsomal membranes, double-labeling experiments with ^{131}I and ^{125}I were carried out in which RM were first exposed to the LPO iodination system as intact vesicles (Fig. 5), and then as vesicles opened by treatment with a low DOC concentration. During the first iodination, which was carried out with ^{131}I, only membrane proteins exposed on the cytoplasmic face of the vesicles should be labeled, while during the second, carried out with ^{125}I, membrane proteins exposed on both faces, as well as content proteins, should become accessible. The use of two different isotopes greatly increased the reliability of the method since it allowed accurate comparisons to be made between both iodination patterns.

Fig. 6 shows the kinetics of incorporation during each of the two iodinations, in intact RM and in microsomes treated with two DOC concentrations. It is evident that although in intact RM ^{131}I incorporation appeared to reach saturation (Fig. 6*a*), this did not represent labeling of all the accessible sites since at least an equivalent amount of ^{125}I would still be incorporated during a second incubation (Fig. 6*b*). Actual levels of maximum incorporation were somewhat variable from experiment to experiment. When the second iodination was carried out in the presence of 0.05 or 1% DOC (Fig. 6*b*), the total incorporation of ^{125}I increased by ~30%. This is an effect which, as will be seen below, may be attributed to several causes, including the labeling of proteins present in the lumen of the microsomal vesicles, and the labeling of membrane proteins exposed on the inner aspect of RM membrane.

Proteins in the vesicular content, which in intact RM were protected from labeling during both iodination cycles (Fig. 7*a*), incorporated high

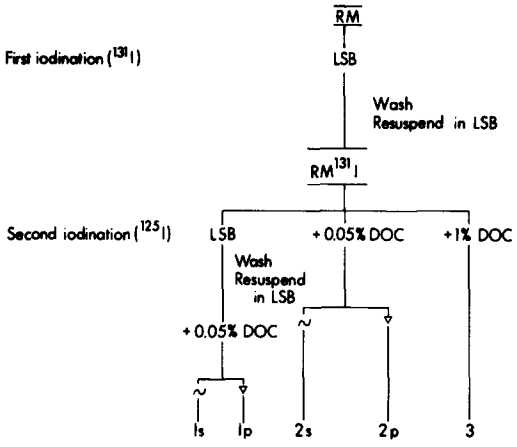

FIGURE 5 Schematic description of the procedure for the LPO-catalyzed double iodination with ^{131}I and ^{125}I. A first iodination with ^{131}I was always carried out on intact RM. The second iodination cycle with ^{125}I was performed on intact microsomes and on microsomes treated with two different concentrations of DOC. This procedure was used for the experiments described in Figs. 6–9.

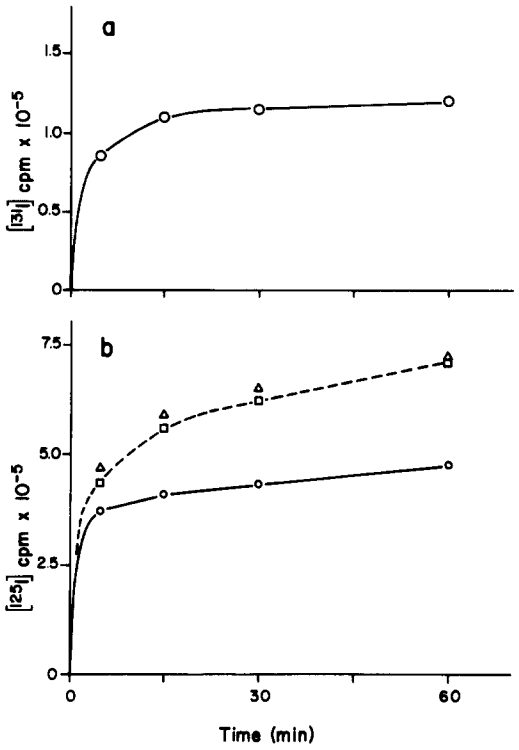

FIGURE 6 Time-course of the enzymatic iodination of RM. RM (3 mg protein/ml) were incubated with the LPO-catalyzed iodination system described in Materials and Methods. At different times, 25-μl samples were taken from the incubation mixture and spotted on glass fiber filters for radioactivity determinations. (*a*) Incorporation of ^{131}I during the first iodination of intact RM. (*b*) Incorporation of ^{125}I during the second iodination, carried out with no DOC (○); 0.05% DOC (□); or 1% DOC (△) in the incubation mixture. It should be noted that the counting efficiency of ^{131}I is approximately half of that for ^{125}I.

levels of ^{125}I when the second iodination was carried out in the presence of 0.05% DOC (Fig. 7*b*). This demonstrates that the microsomal membrane is an effective barrier to the iodinating system, in agreement with previous results obtained with single-label experiments (23).

The accessibility of membrane proteins and glycoproteins to iodination in intact and opened vesicles can be judged from the results presented in Figs. 8 and 9 (two separate experiments). ^{131}I and ^{125}I iodination patterns for RM membrane and ribosomal proteins were almost identical when both iodinations were carried out in the absence of DOC (Figs. 8*a* and 9*a*). However, when the second iodination was carried out in the presence of 0.05% DOC (Figs. 8*b* and 9*b*), an enhancement of the incorporation of ^{125}I into several proteins, particularly into proteins with molecular weights in the neighborhood of 50,000 daltons, was observed. Sometimes (Fig. 8 but not in Fig. 9) DOC produced a small but uniform stimulation of ^{125}I labeling in all microsomal proteins (compare ratios of ^{125}I and ^{131}I radioactivity scales in Fig. 8 with (*b* and *d*) and without (*a* and *c*) DOC).

Since carbohydrate chains of RM glycoproteins are located only on the cisternal side of the membrane (41), analysis of proteins purified by Con A chromatography in combination with double-labeling experiments should allow the identification of RM glycoproteins which have a transmembrane disposition and are exposed on the cytoplasmic side of the membrane. Iodination patterns of glycoproteins purified by Con A Sepharose chromatography after two successive iodinations are shown in Figs. 8*c* and *d* and 9*c* and *d*. It should be noted that most glycoproteins were labeled in the first iodination and therefore are exposed on the cytoplasmic side of the vesicles. When both iodinations were carried out on intact RM, very similar patterns of ^{125}I and ^{131}I were obtained for all the different glycoprotein groups (Figs. 8*c* and 9*c*). On the other hand, when RM iodinated in the intact condition with ^{131}I were exposed to the LPO system and ^{125}I, in the presence of 0.05% DOC (Figs. 8*d* and 9*d*), several microsomal glycoproteins showed dramatically different ^{125}I and ^{131}I incorporation ratios. These glycoproteins (see groups III, IV, VI, VII, VIII, and X in Figs. 8*d* and 9*d*; see also Table III) are therefore also exposed, on the inner (luminal) surface of the RM, where the carbohydrate groups are located. In spite of their high molecular weights, several minor membrane glycoproteins (groups I and II) showed only small changes in incorporation ratios caused by treatment with 0.05% DOC.

DISCUSSION

The results of this report indicate that RM contain a complex set of glycoproteins, which may be purified through their affinity for con A and represent up to 22% of the total protein in the microsomal fraction. By SDS acrylamide gel electrophoresis, this set of glycoproteins was resolved into more than 20 polypeptides with apparent mol wt ranging between 23,000 and 240,000 daltons.

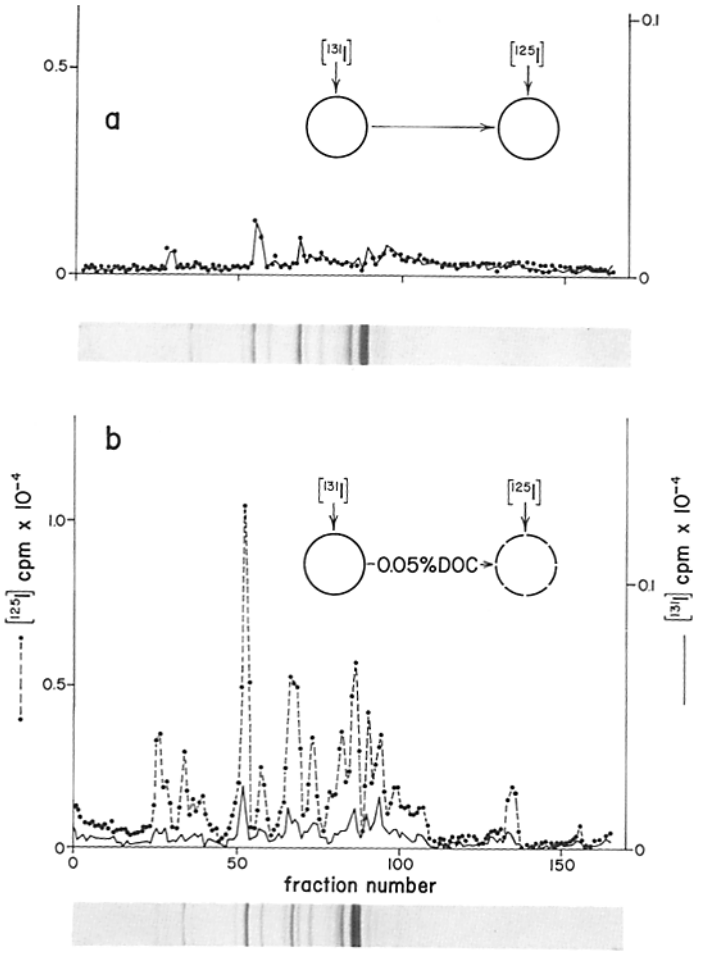

FIGURE 7 Effect of DOC on the enzymatic iodination of microsomal content proteins. RM was first labeled with ^{131}I as intact vesicles and in a second step with ^{125}I, either as intact vesicles (*a*) or in the presence of 0.05% DOC (*b*). After the second iodination, sample *a* was recovered by centrifugation, resuspended in LSB, and treated with 0.05% DOC. Released content proteins from *a* and *b* were separated from rough microsomal membranes by centrifugation (60 min, 40K, Ti50) and analyzed by SDS acrylamide gel electrophoresis in a 7–12% gradient gel. The gel was stained with Coomassie Blue, photographed, sliced, and counted as described in Materials and Methods. The continuous line (—) represents the distribution of ^{131}I throughout the gel. Filled circles and dashed line (●- -●) represent ^{125}I incorporated during the second incubation. The dashed line is omitted when the points (●) lay very near or on the continuous line. Photographs of stained gels and plots of the radioactivity distribution were aligned so that radioactivity peaks can be related with the corresponding stained bands.

Although after electrophoretic separation most of these polypeptides could be demonstrated with the PAS method, the staining intensity of the microsomal glycoprotein bands was much lower than for PM glycoproteins, an observation which indicates a lower content of carbohydrates per polypeptide chain in the microsomal glycoproteins and is also expected from the absence of sialic acid in RM (41). A major fraction of the glycoprotein which was bound to a Con A Sepharose column (~60%) could be released with the specific inhibitor of binding to Con A, α-MM. There were no indications that glycoproteins were present in the protein fraction which did not bind to the Con A Sepharose column, since the electrophoretic gels of excluded fractions did not contain bands which stained with PAS or bound [^{125}I]Con A. These observations demonstrate that most microsomal

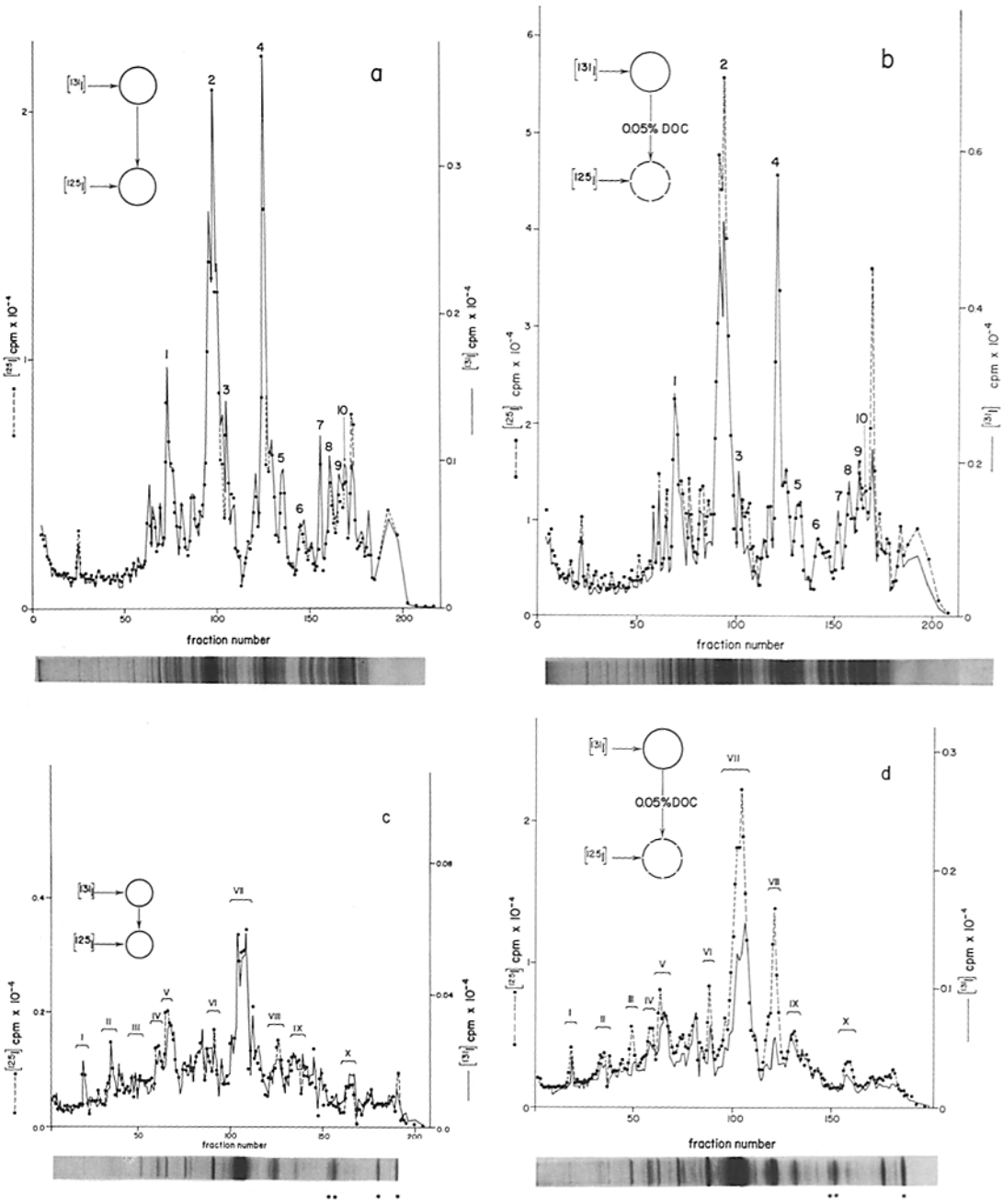

FIGURE 8 Effect of DOC on the enzymatic iodination of rough microsomal membrane proteins and glycoproteins. RM were iodinated first with ^{131}I, and then with ^{125}I in the absence (*a* and *c*) and in the presence (*b* and *d*) of 0.05% DOC. Membrane proteins were separated from content proteins as described in the legend to Fig. 7. Membrane glycoproteins were purified by affinity chromatography on Con A Sepharose. Membrane proteins (*a* and *b*) and glycoproteins (*c* and *d*) were analyzed in SDS acrylamide gels, which were sliced to determine the distribution of ^{131}I (—) and ^{125}I (●- -●) radioactivities. Roman numerals in Fig. 8*c* and *d* indicate the glycoprotein groups (*I-X*) as defined in Fig. 4. Arabic numerals in Fig. 8*a* and *b* were introduced to indicate the major iodinatable proteins (peaks *1-10*). The radioactivity scales in Fig. 8*a* and *b* were chosen to match the height of ^{131}I and ^{125}I radioactivity peaks nos. *4-10*, which represent ribosomal proteins (peaks *5-10*) and microsomal contaminants such as urate oxidase (peak *4*), which are always exposed to the iodinating system, irrespectively of the presence of DOC. The same $^{125}I/^{131}I$ scale ratios were used for Fig. 8*a* and *c* and for Fig. 8*b* and *d*. The difference between the scale ratios used for Fig. 8*a* and *c* (absence of DOC) and Fig. 8*b* and *d* (0.05% DOC) reflects a small but uniform stimulation of ^{125}I incorporation caused by DOC which was observed in this experiment but not in the one shown in Fig. 9. Asterisks represent Con A polypeptides released from the column during elution of glycoproteins.

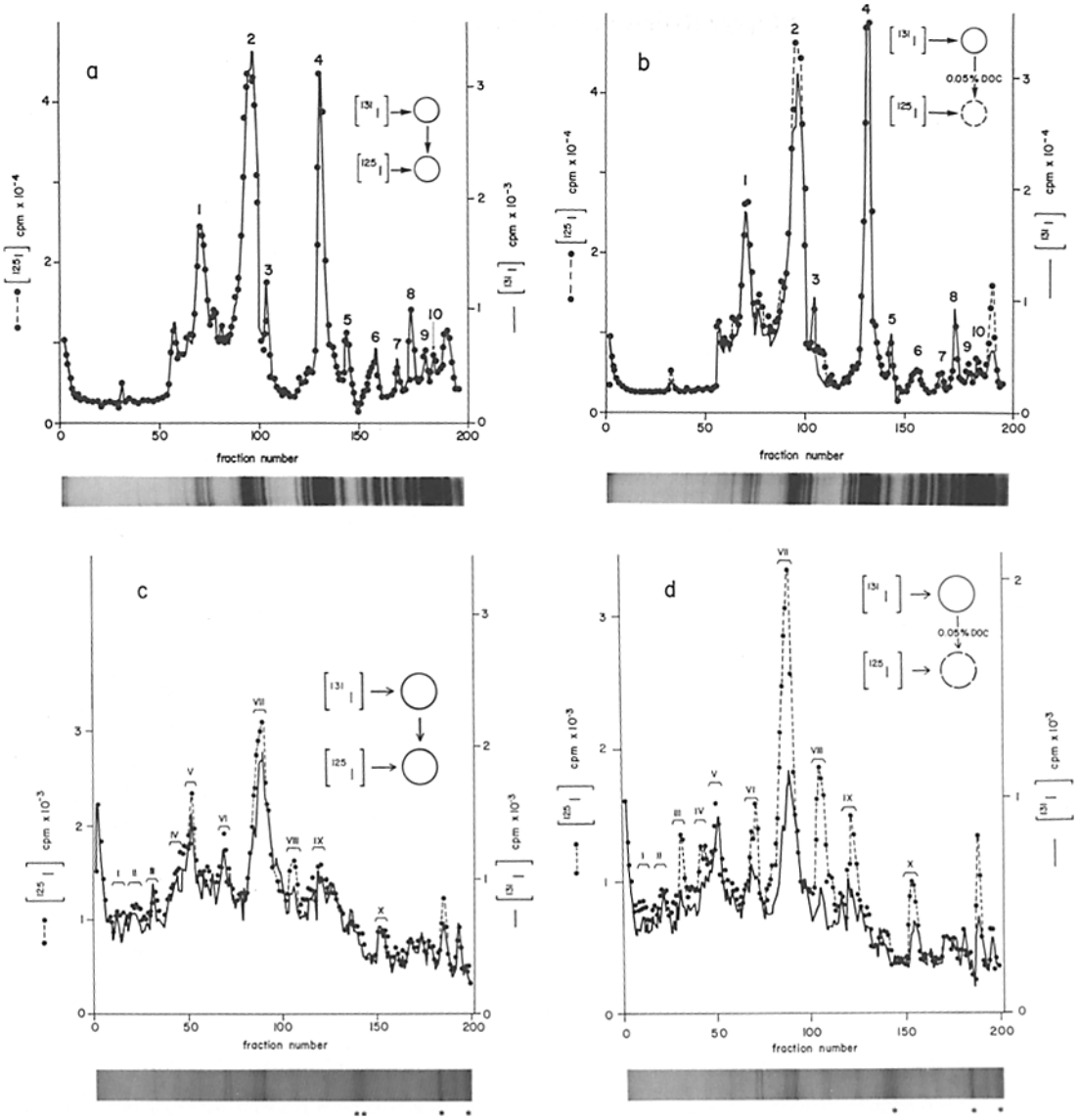

FIGURE 9 Effect of DOC on the enzymatic iodination of rough microsomal membrane proteins and glycoproteins. RM were iodinated with ^{131}I and then with ^{125}I in the absence (*a* and *c*) and in the presence (*b* and *d*) of 0.05% DOC as described in the legend to Fig. 8. A quantitative evaluation of the changes in the $^{125}I/^{131}I$ ratios due to the opening of vesicles by adding the low DOC concentration (0.05%) is shown in Table III. Asterisks represent Con A polypeptides released from the column.

glycoproteins have affinity for Con A, a fact which should be contrasted with the behavior of glycoproteins from the erythrocyte membrane where some, like band III, have affinity for Con A (14), while others, like glycophorin, do not (53). The finding that all microsomal glycoproteins have affinity for Con A is in agreement with electron microscope evidence showing that only lectins with specificity for mannose cores in glycoproteins, such as Con A and Lens culinaris, bind to rough microsomal membranes (17, 41). Several lectins with abilities to bind to different receptors in plasma membranes (40, 41) have no binding sites in RM.

It has been shown that RM fractions are usually contaminated with membranes derived from other

TABLE III

Ratios of $^{125}I/^{131}I$ Incorporated into Microsomal Membrane Proteins and Glycoproteins

Total proteins			Glycoproteins		
Peak no.	−DOC	+0.05% DOC	Group no.	−DOC	+0.05% DOC
1	1.39	1.54	I	1.50	2.03
2	1.40	1.64	II	1.53	1.94
3	1.25	1.31	III	1.53	2.69
4	1.43	1.57	IV	1.51	2.23
5	1.28	1.27	V	1.51	1.84
6	1.22	1.14	VI	1.52	2.33
7	1.25	1.34	VII	1.57	2.61
8	1.19	1.20	VIII	1.75	3.50
9	1.57	1.64	IX	1.50	3.62
10	1.35	1.54	X	1.46	2.48
$\bar{X}$ (n)	1.33 (10)	1.42 (10)	$\bar{X}$ (n)	1.54 (10)	2.53 (10)
s	0.12	0.18	s	0.08	0.61
P	>0.2		P	<0.001	

The ratios between ^{125}I and ^{131}I incorporation into microsomal membrane proteins (Fig. 9 *a* and *b*) and into the membrane glycoproteins purified by affinity chromatography (Fig. 9 *c* and *d*) were computed for each peak after separation by SDS acrylamide gel electrophoresis. In the membrane protein fraction, peaks *1*, *2*, and *3* correspond to membrane proteins, peak *4* corresponds to a nonmicrosomal contaminant, urate oxidase; while peaks *5-10* represent $^{125}I/^{131}I$ ratios in ribosomal proteins. Roman numerals correspond to the nomenclature for glycoproteins presented in Fig. 4. $\bar{X}(n)$ and s represent the mean (number of values) and the standard deviation of the mean, respectively. P is the probability for the null hypothesis obtained with the Student's t test.

organelles, which contain lectin-binding sites (41). Therefore, the possibility was considered that a significant fraction of the glycoproteins purified from the microsomal fractions is contributed by those contaminants or represents secretory or extracellular glycoproteins which are adsorbed to the microsomal vesicles during cell fractionation.

Contamination with soluble secretory or extracellular glycoproteins is unlikely, since a wash with high ionic strength buffers of microsomal membranes was always included in the preparation procedure. Measurements of enzymatic markers indicate that contamination with mitochondria and plasma membrane fragments represented <6 and 1%, respectively, of the total protein in RM fraction. Thus, even if it is assumed that glycoproteins constitute 40% of the proteins in these membranes, as is the case in the red cell membrane (50), no more than 5% of the glycoprotein content of the RM fraction should be expected to represent contamination from mitochondria and plasma membranes. Electron microscope evidence and quantitative studies on the binding of labeled lectins to rat liver RM (41) indicate that, although a small proportion of high affinity sites (20% of the total) is present in contaminating smooth membranes, most of the Con A-binding sites in the RM fraction are contributed by carbohydrate chains exposed on the luminal face of the microsomal membranes or by proteins within the microsomal vesicles. The weak PAS staining of most of RM glycoproteins also distinguishes them from glycoproteins in Golgi or plasma membrane fractions, which are highly reactive with this staining procedure (our own unpublished observations).

Most glycoproteins identified in the RM fraction were found to be membrane proteins. SDS acrylamide gel patterns of purified glycoproteins showed that this content subfraction consisted of six to eight glycoproteins with apparent mol wt ranging from 60,000 to 200,000, while 13-15 glycoproteins were isolated from RM membranes ranging in apparent mol wt from 23,000 to 240,000 daltons. Although no major membrane glycoproteins were detected in the content fraction, it is possible that content glycoproteins were recovered in association with the membranes since four to five bands in both subfractions had similar mobilities. The dual distribution of these glycoproteins may be caused by incomplete separation of the content set of proteins by DOC or may have a physiological significance. In relation to the latter possibility, it is relevant to mention the

observation by Redman and Cherian (38) that, during the early stages of biosynthesis, secretory glycoproteins may remain attached to the ER membrane for a certain period before being released into the microsomal lumen. Recently, Blackburn and Kasper (5) have purified and characterized several membrane proteins from rat liver microsomes with apparent mol wt ranging from 17,000 to 170,000 daltons. These workers, however, did not observe differences between the gel electrophoretic patterns of glycoproteins from rough and smooth microsomes (4) and therefore failed to recognize two of the glycoproteins described by us. They are the ribophorins in group VI, of mol wt 65,000 and 63,000 daltons, which have been shown to represent the major polypeptide compositional difference between rough and smooth microsomes (25, 26).

The results of double-labeling experiments with the LPO-catalyzed iodinating system indicated that iodinatable tyrosine groups in most membrane glycoproteins are predominantly exposed on the luminal surface of the microsomal membranes. The luminal exposure of the glycoproteins is in agreement with previous observations on the location of lectin-binding sites, indicating that carbohydrates are present only on the cisternal face of RM vesicles (17, 39–41, 55). Some glycoproteins, such as those in groups V, VI, and VII, also appeared to be exposed on the cytoplasmic face of the microsomal membranes, since they incorporated ^{131}I when RM were labeled as intact vesicles. An independent demonstration of the transmembrane disposition of at least one of the glycoproteins in group VI has been provided by its accessibility to trypsin when the protease was added to intact RM vesicles (26). A transmembrane disposition of at least one of the "ribophorins" should be of considerable interest, since these proteins appear to be related to ribosome binding (26, 27) and, therefore, are likely to participate in the vectorial transfer of nascent polypeptides across ER membranes.

The asymmetric distribution of microsomal glycoproteins and the transmembrane disposition of some of these glycoproteins represent structural arrangements topologically equivalent to those of glycoproteins in plasma membranes. Because hydrophilic groups of proteins cannot easily traverse the phospholipid barrier within membranes (48), the orientation of membrane proteins is thought to be related to the mechanisms by which they are inserted into the membrane (c.f. reference 43). The equivalent orientation of glycoproteins in rough ER and PM is that expected for a mechanism of transport of membrane proteins involving flow in the membrane plane or membrane fusion. The finding that carbohydrate chains in the ER membranes lack sugars distal to the mannose core, which are present in plasma membrane glycoproteins (40, 41), together with the role of membrane-bound ribosomes in the synthesis of integral membrane proteins (c.f. reference 43), are consistent with a model in which all sugars of ER glycoproteins are added within the ER itself. Recent experiments tracing the migration of the vesicular stomatitis virus envelope glycoprotein (19–21) suggest that plasma membrane glycoproteins may also have their sugars added in the ER, from where they would be transferred to the surface by a process which allows further glycosidation. The subcellular distribution of glycosyl transferases (44) also indicates that terminal glycosidation should follow transfer of glycoproteins from the ER into other subcellular components. It may be speculated that the addition of terminal sugars plays an important role in the selection of glycoproteins to be transferred to the cell surface.

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