Analytical Study of Microsomes and Isolated Subcellular Membranes from Rat Liver

VII. Distribution of Protein-bound Sialic Acid

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ABSTRACT

Detailed investigations by quantitative centrifugal fractionation were conducted to determine the subcellular distribution of protein-bound sialic acid in rat liver. Homogenates obtained from perfused livers were fractionated by differential centrifugation into nuclear fraction, large granules, microsomes, and final supernate fraction, or were used to isolate membrane preparations enriched in either plasma membranes or Golgi complex elements. Large granule fractions, microsome fractions, and plasma membrane preparations were subfractionated by density equilibration in linear gradients of sucrose. In some experiments, microsomes or plasma membrane preparations were treated with digitonin before isopycnic centrifugation to better distinguish subcellular elements related to the plasma membrane or the Golgi complex from the other cell components; in other experiments, large granule fractions were obtained from Triton WR-1339-loaded livers, which effectively resolve lysosomes from mitochondria and peroxisomes in density gradient analysis. Protein-bound sialic acid and marker enzymes were assayed in the various subcellular fractions. The distributions obtained show that sialoglycoprotein is restricted to some particular domains of the cell, which include the plasma membrane, phagolysosomes, and possibly the Golgi complex. Although sialoglycoprotein is largely recovered in the microsome fraction, it has not been detected in the endoplasmic reticulum-derived elements of this subcellular fraction. In addition, it has not been detected either in mitochondria or in peroxisomes. Because the sialyltransferase activities are associated with the Golgi complex, the cytoplasm appears compartmentalized into components which biogenetically involve the Golgi apparatus and components which do not.

Sialic acid is found within rat liver cells mainly as the terminal sugar of the saccharide chains of glycoproteins and glycolipids. These constituents are abundant at the cell surface, with their sugar residues externally disposed (45).

Several publications have reported that protein-bound sialic acid (sialoglycoprotein) is not confined to the pericellular membrane but also occurs within the cell, in the nuclear membrane (21, 22, 26), mitochondria (13, 14, 35), lysosomal membrane (20), Golgi complex (5, 23), endoplasmic reticulum (5, 7, 19, 24, 28), and cytosol (7) (see also reference 41 for a review). However, in most of these publications it has not been unequivocally established that sialoglycoprotein belongs to the main cell component of the preparation analyzed rather than to a sialoglycoprotein-rich contaminant, for instance the plasma membrane. In fact, the few quantitative investigations of protein-bound sialic acid in liver have yielded contradictory results on the partition of sialoglycoprotein between the nuclear, mitochondrial, microsomal, and final supernate fractions (26, 30).

Whether sialoglycoproteins are confined to the cell surface or also occur in cell organelles raises key questions concerning both their function and their route of biosynthesis. It is generally accepted that after passage through the Golgi complex, which is the sole subcellular organelle unambiguously established as being endowed with sialyltransferase activities (33, 43), glycoproteins are discharged by exocytosis or inserted in the plasma membrane (discussed in reference 32). The present investigation has thus been undertaken to assess, on a quantitative basis, the subcellular distribution of sialoglycoprotein and to determine which intracellular membranes or organelles...
possibly contain constituents of this category. I have used the analytical approach of tissue fractionation (see references 8 and 15 for recent reviews), and the direct chemical assay of sialic acid (39) in subcellular fractions after elimination of the major interfering substances. I present evidence that sialoglycoprotein is associated mainly with the plasma membranes and cannot be detected in mitochondria, peroxisomes, or microsomal elements that derive from the endoplasmic reticulum. Portions of this report have been presented in abstract form (1, 2).

MATERIALS AND METHODS

Products were obtained from the following sources: UDP-[U-14C]glucose (sp act, 80 Ci/mmol) and [side-chain-2-14C]tyramine bisuconitate (sp act, 60 Ci/mmol), New England Nuclear (Boston, Mass.); [side-chain-2-14C]tyramine hydrochloride (sp act, 50 Ci/mmol), UDP-[U-14C]-acetylgalactosamine (sp act, 300 Ci/mmol), The Radiochemical Center Ltd. (Amersham, England); N-14C]Galactosaminuronic acid (sp act, 20 Ci/mmol), ICN, Isotope and Nuclear Division, (Irvine, Calif.); Dowex AG1-X8, 200-400 mesh, acetate form, and Dowex AG50W-X8, 200-400 mesh, hydrogen form, Bio-Rad Laboratories (Richmond, Calif.); N-acetylgalactosaminuronic acid type IV and deoxyriboseuronic 1 from bovine pancreas (3,800 Kunitz U/mg protein), Sigma Chemical Co. (Saint Louis, Mo.); p-nitrophenyl 5-thymidylate, acid type IV and deoxyribonuclease I from bovine pancreas (3,800 Kunitz U/mg protein), Sigma Chemical Co. (Saint Louis, Mo.); p-nitrophenyl 5-thymidylate, Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, Calif.); Na-

Quantitative Fractionation of Liver by Differential Centrifugation

Blanched livers were homogenized in 0.25 M sucrose solution which contained 3 mM imidazole-HCl buffer at pH 7.4 (henceforth designated 0.25 M sucrose), and fractionated into nuclear fraction, large granules, microsomes, and final supernate as described previously (3).

Subfractionation of Large Granules and Microsomes

Large granules were centrifuged at 40,000 rpm for 30 min in the E-40 rotor (8) loaded with the sample (10 ml), a 32-m sucrose gradient extending linearly from 33.5 to 55.7% (wt/wt), and a 6-ml cushion of density 1.34 (67.3%, wt/wt). All solutions were buffered at pH 7.4 with 3 mM imidazole-HCl and contained 0.1% ethanolic when catalase was assayed. The subfractions were processed for density measurement and biochemical assays as previously described (10). In some experiments, the large granules were obtained from rats injected intraperitoneally with 0.4 ml of 60 mg/ml of heparin. The homogenate was centrifuged at 1,000 U of heparinase solution, successively. The livers were perfused through the portal vein with a solution containing 154 mM NaCl and 25 mM NaHCO3 at 4°C equilibrated with 95% O2 and 5% CO2.

Preparation of Plasma Membranes

The perfused livers from eight rats were minced with steel blades and added with 2.5 ml/g liver of 0.5 M sucrose, 1% dextrose, 5 mM MgCl2, 37.5 mM Tris-maleate, pH 6.5 (29). Each liver was homogenized separately by 25 strokes of the loose-fitting pestle of a Dounce homogenizer. The homogenate was centrifuged at 1,800 rpm for 10 min. The pellet was resuspended and centrifuged at 1,600 rpm for 10 min. This step was repeated once. The combined supernates were centrifuged at 25,000 rpm for 10 min in the Beckman rotor 30. The resulting pellet was washed once, and added with a 38% (wt/wt) sucrose solution to obtain a suspension of density 1.165 at 0°C (sample). The following solutions were layered into the tubes of the Beckman SW25-2 rotor: 10 ml 50.4% (wt/wt), 25 ml sample, 12 ml 31.5% (wt/wt) (density 1.14) and 9 ml 45.8% (wt/wt) sucrose until the appearance of liquid at the center line connection of the rotor. The rotor was then run for 2 h at 45,000 rpm. Fractions of ~10 ml were collected and their densities measured as described in reference 8. The fractions of densities comprised between 1.145 and 1.180 were pooled, diluted twice with 1 mM NaHCO3, and centrifuged at 30,000 rpm for 2 h. The resulting pellet, resuspended in 0.25 M sucrose, constituted the plasma membrane preparation. The other gradient fractions were also pooled for biochemical determinations. All sucrose solutions used contained 1 mM NaHCO3.

Plasma membranes were subfractionated by density gradient centrifugation in a linear gradient of sucrose, as described for microsomes (see above and reference 10). When the plasma membrane preparation was treated with digitonin the conditions described for treatment of microsomes were used.

Preparation of Golgi Complex

This preparation was made according to the procedure of Wibo et al. (43), slightly modified as follows. The perfused livers from six rats were minced with steel blades and added with 2.5 ml/g liver of 0.5 M sucrose, 1% dextrose, 5 mM MgCl2, 37.5 mM Tris-maleate, pH 6.5 (29). Each liver was homogenized separately by 25 strokes of the loose-fitting pestle of a Dounce homogenizer. The homogenate was centrifuged at 1,800 rpm for 10 min. The pellet was resuspended and centrifuged at 1,600 rpm for 10 min. This step was repeated once. The combined supernates were centrifuged at 25,000 rpm for 10 min in the Beckman rotor 30. The resulting pellet was washed once, and added with a 38% (wt/wt) sucrose solution to obtain a suspension of density 1.165 at 0°C (sample). The following solutions were layered into the tubes of the Beckman SW25-2 rotor: 10 ml 50.4% (wt/wt), 25 ml sample, 12 ml 31.5% (wt/wt) (density 1.14) and 9 ml 45.8% (wt/wt) sucrose until the appearance of liquid at the center line connection of the rotor. The rotor was then run for 2 h at 45,000 rpm. Fractions of ~10 ml were collected and their densities measured as described in reference 8. The fractions of densities comprised between 1.145 and 1.180 were pooled, diluted twice with 1 mM NaHCO3, and centrifuged at 30,000 rpm for 2 h. The resulting pellet, resuspended in 0.25 M sucrose, constituted the plasma membrane preparation. The other gradient fractions were also pooled for biochemical determinations. All sucrose solutions used contained 1 mM NaHCO3.

Biochemical Determinations

SIALIC ACID: A volume of subcellular fraction containing 50-100 mg of liver protein and 0-100 μg of sialic acid was added to an equal volume of ice-cold 5% TCA and centrifuged. The pellet was washed twice by resuspension in distilled water followed by centrifugation. The final pellet was suspended in 1 ml of water and extracted with 12.5 ml of chloroform/methanol (2/1, vol/vol). The chloroform and aqueous phases were discarded. The sialic acid was released from the insoluble material by hydrolysis in 0.05 M H2SO4 for 3 h at 80°C. The hydrolysate was neutralized with a saturated solution of NaOH and added with 100 μl (±8,000 cpm) of [N-14C]N-acetylgalactosaminuronic acid. The precipitate was removed by centrifugation and washed twice with distilled water. The pooled supernates were passed over a column poured with 2 ml of Dowex AG1-X8. The resin was rinsed with 100 ml of water. Sialic acid was eluted with 10 ml of 1 M acetic acid buffer, pH 4.6. The 10-ml eluate was passed over a second column containing 7.6 mEq of Dowex AG50W-X8. The eluate was lyophilized and the dried residue was dissolved in 0.5 ml of water.

The radioactivity was determined on a 10-μl aliquot diluted with 10 ml of Insta-Gel (Packard Instrument Co., Inc., Downers Grove, Ill.). Sialic acid was determined on 10- to 200-μl aliquots (0-60 μg of sialic acid) by the thiosulfuric acid procedure (39). The absorption spectra of pigment formed were scanned from 450 to 600 nm in a Cary model 15 recording spectrophotometer (Cary Instruments, Monrovia, Calif.). In some subcellular fractions, particularly the nuclear fraction, the absorbancy spectrum showed a marked interference by deoxy sugars. To suppress this interference, the fraction from 1.2 g of liver was incubated in a medium containing, in a final volume of 10 ml, 100 mM acetic acid buffer, pH 5, 5 mM MgCl2, and 25 U of deoxyriboseuronic 1 unit/L protein. After a 3-h incubation at 37°C, the suspension was centrifuged for 1 h at 39,000 rpm. The supernate was discarded and the sialoglycoprotein was assayed on the pellet as described above.

For the calculations, Waren's formula (40) was used, and it was assumed that in all cases sialic acid was present as N-acetylgalactosaminuronic acid. The values were corrected for recovery of [N-14C]N-acetylgalactosaminuronic acid in the samples analyzed (77 ± 10%, mean ± standard deviation of 189 assays).

N-acetylgalactosaminuronictransferase was assayed at 37°C as described by Wibo et al. (44). The 60-μl incubation medium contained 100 mM cacodylate-HCl buffer, pH 6.4, 0.11 mM UDP-[U-14C]N-acetylgalactosamine (40,000 cpm), 4% (wt/vol) ovomucin, 5 mM ADP, 20 mM MgCl2 and 0.4% (wt/vol) Triton X-100.

Cytosol oxidative was assayed at 25°C in a medium containing 30 mM K phosphate buffer, pH 7.4, 41 μM cytochrome c reduced up to 80-90% by Na
dithionite, 1 mM EDTA, 0.03% (wt/vol) Triton X-100, 0.1% (wt/vol) Tween 80, and 0.01% (wt/vol) digitonin. Except for the presence of detergents in the incubation medium, this assay was carried out as described by Beaufay et al. (9).

Monoamine oxidase was assayed by the procedure of Wurtman and Axelrod (46) with either [3H]tyramine or [3H]tryptamine as substrate. No difference in enzyme activity because of the nature of the substrate was observed.

Other enzymes and chemical constituents were assayed as previously described (9).

Presentation of Results

Results of differential or density gradient centrifugation experiments are presented as described in reference 8.

RESULTS

Biochemical Characteristics of Perfused Liver

In all experiments, the livers were perfused to eliminate blood sialoglycoproteins which could become adsorbed onto subcellular components. Perfusion did not alter significantly the biochemical characteristics of the organ. The content in protein, phospholipid, cholesterol, RNA, and the various marker enzymes (listed in the tables and figures) was comparable to that reported previously for nonperfused rat liver (3). The content of liver in sialic acid amounted to 240 ± 56 µg/g tissue, and 1.03 ± 0.02 µg/mg protein (mean ± standard deviation of 18 experiments). Mean values reported in the literature for rat liver are: 1.17 ± 0.27 (18), 1.39 ± 0.45 (13), and 2.01 ± 0.33 (26) µg/mg protein. Differences in animal strains, and a more complete elimination of substances that interfere with quantification of sialic acid in subcellular fractions (see Materials and Methods) may be responsible for the lower value reported in this work.

Distribution of Sialoglycoprotein in Primary Liver Fractions

Fractionation of perfused liver by differential centrifugation was used to establish the distribution of sialoglycoprotein between the nuclear fraction, large granules, microsomes, and final supernate. The results are shown in Fig. 1 under the form of mean distribution profiles for protein-bound sialic acid and five marker enzymes. Acid phosphatase (lysosomes), glucose 6-phosphatase (endoplasmic reticulum), galactosyltransferase (Golgi complex), and alkaline phosphodiesterase I (plasma membrane) show the distributions previously observed for nonperfused liver (3); cytochrome oxidase (mitochondria) is recovered with slight excess in the nuclear fraction.

The major portion of protein-bound sialic acid (55 ± 7.5%, mean ± standard deviation of 10 experiments) sediments with microsomes. In this fraction, the average specific content in sialic acid was 2.9 µg/mg protein. The rest of sedimentable sialoglycoprotein was shared evenly between the nuclear and large granule fractions: 18 and 20%, respectively.

Subfractionation of Microsomes

Microsomes are complex in their composition. They consist mainly of endoplasmic reticulum vesicles, plasma membranes, Golgi complex fragments, and also contain elements of other origin (4, 10). For assessment of the partition of sialoglycoprotein between these subcellular entities, microsomes were centrifuged to equilibrium in sucrose gradients and the distribution of protein-bound sialic acid in the gradient fractions was compared to that of characteristic enzymes of the three main microsomal components.

FIGURE 1 Mean distribution patterns of sialic acid and marker enzymes after fractionation of perfused liver by differential centrifugation. Nuclear fraction, large granules, microsomes, and final supernate are shown by blocks, from left to right, respectively. The ordinate scale gives the relative specific activity of the constituent (percentage of total amount/percentage of total protein). The abscissa scale gives cumulatively the percentage of protein. Figures in parentheses are the numbers of experiments. Recovery of sialoglycoprotein was 97 ± 6% (mean ± standard deviation).

FIGURE 2 Density distribution of sialoglycoprotein, compared to that of other constituents, after isopycnic centrifugation of microsomes in a sucrose gradient. The represented portion of histograms extends from 1.07 to 1.30 and corresponds to >95% of each constituent. Percentages refer to the yield of the corresponding constituents in the microsomal fraction subjected to density gradient centrifugation. For protein and sialoglycoprotein these values were 22 and 62% of the liver content, respectively. The distribution of sialoglycoprotein is represented by the shaded area superimposed on each enzyme profile; recovery from the gradient was 97% of sialoglycoprotein in the microsome fraction.
The results of a typical experiment are shown in Fig. 2. In spite of a rather extensive overlapping of the density distributions, sialoglycoprotein is clearly different from glucose 6-phosphatase and galactosyltransferase, regarding both the shape and the average density. Sialoglycoprotein almost coincides with 5'-nucleotidase and alkaline phosphatase. These two enzymes have previously been classified in a particular group, designated a2, on the basis of their behavior in centrifugal fractionation of microsomes (4, 10). This group also includes cholesterol and alkaline phosphodiesterase I. The slight differences noted here within group a2, in particular the higher density of microsomal alkaline phosphodiesterase I, are identical to those found in nonperfused liver (10, 15).

Under appropriate conditions, the various membrane components of the microsome fraction react differently to treatment with digitonin (4, 37). As a result, group a2 constituents undergo a characteristic increase in their equilibrium density in sucrose gradients (<0.03 U), galactosyltransferase is shifted to a lesser extent, and enzymes associated with endoplasmic reticulum elements are practically unaffected. Fig. 2 shows that the density distribution of sialoglycoprotein still closely fits those of 5'-nucleotidase and of the other constituents of group a2 when microsomes are brought to density equilibrium after treatment with digitonin. Concomitantly, sialoglycoprotein dissociates in a more clear-cut fashion from galactosyltransferase than in the case of untreated microsomes. By comparison with Fig. 2 it is also seen that sialoglycoprotein is shifted relative to glucose 6-phosphatase. In addition, at the most 6% of the microsomal content in sialoglycoprotein was released from glucose 6-phosphatase. In addition, at the most 6% of the liver content, respectively. Recovery of sialoglycoprotein in the microsomal subfractions was 94%.

Figure 3 Density distribution of sialoglycoprotein, compared to that of other constituents of the group a2 (Table I), which may reflect the presence of sialic acid in mitochondria, lysosomes, or peroxisomes. For clarification of this matter, large granules were subjected to isopycnic centrifugation in sucrose gradient to compare the behavior of this sialoglycoprotein material to that of various reference enzymes, including, in addition to cytochrome oxidase, catalase (peroxisomes), acid phosphatase, and N-acetyl-beta-glucosaminidase (lysosomes).

The distributions of these enzymes (Fig. 4) are as expected from previous studies in this laboratory (11). Sialoglycoprotein has a broad density distribution stretched from 1.1 to 1.3, contrasting with the narrow profile shown by cytochrome oxidase and with the high equilibrium density of catalase. It mimics acid phosphatase, somewhat less exactly alkaline phosphodiesterase I, N-acetyl-beta-glucosaminidase, and 5'-nucleotidase which all are broadly distributed through the gradient. These data make it unlikely that a significant fraction of sialoglycoprotein be associated with mitochondria and peroxisomes.

To establish this point more conclusively, we conducted another experiment in which rats were injected with Triton WR-1339 before isolation of the large granules. In agreement with earlier reports (25, 42), mitochondria and peroxisomes were not significantly altered in their density (Fig. 5), contrary to lysosomes which were shifted from density 1.21-1.22 to density 1.12-1.13 (median values of acid phosphatase and N-acetyl-beta-glucosaminidase). 5'-Nucleotidase was shifted similarly and almost coincides with the lysosomal enzymes. Sialoglycoprotein also equilibrated at a lower density than in experiments carried out on normal animals, but did not behave like the lysosomal enzymes anymore. Their profile was similar to that of alkaline phosphodiesterase I, the median equilibrium densities being 1.156 for sialoglycoprotein and 1.154 for alkaline phosphodiesterase I, as compared to 1.20 and 1.19, respectively, in large granules from normal rats.

Analysis of Plasma Membrane Preparations

The results reported so far closely link sialoglycoprotein to enzymes of the group a2 in the microsome and large granule fractions which, together, account for 75% of sialoglycoprotein in liver. Because of the relationship of group a2 with the cell surface (4, 10, 15) and of the high content of sialoglycoprotein in plasma membranes (5, 17, 38, reviewed in references 41 and 45), the behavior of this constituent upon subfractionation of plasma membrane preparations by isopycnic centrifugation has been determined.

The results of such experiments are summarized in Figs. 6 and Table I.

| Constituent | No. of exp | Percent of liver content* |
|-------------|------------|---------------------------|
| Sialoglycoprotein | 21 | 18.6 ± 4.9 |
| 5'-Nucleotidase | 23 | 15.1 ± 3.2 |
| Alkaline phosphatase | 11 | 10.0 ± 2.5 |
| Cholesterol | 11 | 9.1 ± 2.4 |
| Alkaline phosphodiesterase I | 19 | 8.6 ± 2.8 |

* Percent of the sum of contents in nuclear, large granule, microsome, and final supernate fractions. Statistics refer to the means ± standard deviations. Values are different from sialoglycoprotein at P < 0.01 (5'-nucleotidase), or at P < 0.001 (other constituents).

**Subfractionation of Large Granules**

About 20% of the liver content in sialoglycoprotein is recovered in the large granule fraction. This level is higher than that of other constituents of the group a2 (Table I), which may reflect the presence of sialic acid in mitochondria, lysosomes, or peroxisomes. For clarification of this matter, large granules were subjected to isopycnic centrifugation in sucrose gradient to compare the behavior of this sialoglycoprotein material to that of various reference enzymes, including, in addition to cytochrome oxidase, catalase (peroxisomes), acid phosphatase, and N-acetyl-beta-glucosaminidase (lysosomes).
FIGURE 4 Mean density distribution of sialoglycoprotein, compared to that of other constituents, after isopycnic centrifugation of large granule fractions in a sucrose gradient. The represented portion of histograms extends from 1.05 to 1.30 and corresponds to >95% of each constituent. Percentage refers to the yield of the corresponding constituent in the large granule fractions subjected to density gradient centrifugation (mean of three experiments). This value was 19% for sialoglycoprotein which is represented by the shaded area superimposed on each enzyme profile. Mean recovery of sialoglycoprotein in the gradient fractions was 83%.

and 7 which refer to original and to digitonin-treated plasma membranes, respectively, and give both the biochemical characteristics of the preparations used and the density distribution of some constituents. The two preparations were similar with regard to the relative specific activities of enzymes (14-15 for alkaline phosphodiesterase I) and relative specific content in sialoglycoprotein (7-8). The difference in yield between sialoglycoprotein and alkaline phosphodiesterase I is not a consequence of digitonin treatment, since the same difference was found in untreated fractions.

In the control plasma membrane preparation (Fig. 6), sialoglycoprotein, cholesterol, and the a2 enzymes peak at the density 1.18. However, their density profiles are not identical; the sharpest difference is seen when 5'-nucleotidase and alkaline phosphatase are compared. The meaning of this difference, which has been found consistently in similar experiments carried out in collaboration with other investigators in this laboratory, will be examined in a subsequent paper (44). In spite of a large degree of overlapping, monoamine oxidase and galactosyltransferase differ from sialoglycoprotein by the shape of the density distribution and by a lower average density. This difference is more clearly seen after treatment of membranes with digitonin (Fig. 7), because the distribution of sialoglycoprotein is then shifted to higher densities (median density: 1.21), whereas that of monoamine oxidase is not altered and that of galactosyltransferase is shifted to a lesser extent. In contrast, the digitonin treatment shifted the distributions of cholesterol, 5'-nucleotidase, alkaline phosphodiesterase I, and alkaline phosphatase in a manner very similar to that noted for sialoglycoprotein.

Sialoglycoprotein in a Golgi Complex Preparation

The biochemical properties of a Golgi complex preparation, including its content in protein-bound sialic acid, are given in Table II. Galactosyltransferase and N-acetylglucosaminyltransferase are enriched 80- and 89-fold, respectively, in this subcellular preparation, indicating substantial purification. Enzymes of group a2 occur, however, in this preparation, but with much lower yields and relative specific activities than the glycosyltransferases. Sialoglycoprotein is present with a yield that is intermediate between the yield of galactosyltransferase and that of 5'-nucleotidase. Therefore, these data are compatible with 9% of the liver sialoglycoprotein being associated with the glycosyltransferase-bearing component. Consistent with this estimate is the value of 8.8 μg N-acetylneuraminic acid/mg protein reported by others (5) for the Golgi complex.

DISCUSSION

These experiments demonstrate that, in perfused liver, the overwhelming bulk of the sedimentable, protein-bound sialic acid may be categorized in the group of constituents previously designated a2 (4, 10), which includes 5'-nucleotidase, alkaline...
phosphatase, alkaline phosphodiesterase I, and cholesterol. The most characteristic property of these constituents is an exquisite sensitivity to digitonin which has been established by density gradient analysis of the microsome fraction (Figs. 2 and 3), and of plasma membrane preparations obtained from low-speed sediments (Figs. 6 and 7). In addition, in all the subcellular preparations subjected to density gradient analysis, including the large granule fraction (Figs. 4 and 5), comparison of the density distributions reveals a close correlation between sialoglycoprotein and one or several a2 enzymes.

The meaning of this centrifugation behavior is obviously that sialoglycoprotein largely belongs to plasma membranes. The presence of 5'-nucleotidase, alkaline phosphatase, and alkaline phosphodiesterase I in plasma membranes is widely documented (4, 10, 15, 17, 38, see also reference 16 for a review) and the plasma membrane preparations of the type used in this study consist mainly of large membrane fragments that show recognizable bile canaliculi and junction complexes. Furthermore, plasma membrane-rich preparations have a distinctly higher content in protein-bound sialic acid than liver homogenates. Expressed in microgram sialic acid/milligram protein, values of 3.0 (7), 10.1 (17), 10.4 (20), 10.8 (5), 14.3 and 14.8 (38) have been reported. Compared to those found in liver homogenates (see above), they are generally consistent with the sevenfold purification of sialoglycoprotein in the plasma membrane preparations used in this work (Figs. 6 and 7).

The key question to examine is whether sialoglycoprotein is exclusively located at the cell periphery or also occurs elsewhere in the cytoplasm. It is similar to that raised by the centrifugation behavior of cholesterol (15). Sialoglycoprotein and cholesterol are recovered with higher yield in microsomes than in the nuclear fraction, whereas enzymes of group a2 show a clear-cut nucleo-microsomal distribution in differential centrifugation (3). Similarly, the percent yield of sialoglycoprotein in plasma membrane preparations is close to that of cholesterol (Figs. 6 and 7) but lower than the yield of the a2 enzymes. The average percentage ratio of sialoglycoprotein to alkaline phosphodiesterase I is 0.48 in the nuclear fraction (Fig. 1), 0.47 in the plasma membrane preparations (Figs. 6 and 7), and 1.04 in the microsome fraction (Fig. 1). The possibility that, in addition to plasma membrane, microsomes contain another sialoglycoprotein-rich membrane therefore has to be scrutinized.
It is unlikely that this membrane derives from the rough endoplasmic reticulum, because the amount of sialoglycoprotein present in dense microsome subfractions is extremely small and not greater than the amount of a2 enzymes (Fig. 2). Similarly, the data of Fig. 3 rule out the vesicles derived from the smooth endoplasmic reticulum as a sialoglycoprotein-rich component of the microsome fraction. After mild treatment with digitonin, these vesicles are still recovered at low density, in subfractions that contain much less sialoglycoprotein than the corresponding subfractions from untreated microsomes, and do not show any excess of sialoglycoprotein over the a2 enzymes. Our conclusion is consistent with the demonstration made by using iodinated lectins that, in the liver endoplasmic reticulum, membrane glycoproteins do not have the terminal trisaccharide: N-acetylgalactosamine-galactose-sialic acid (32). It is at variance with the reports of others describing sialoglycoprotein as a genuine constituent of the endoplasmic reticulum (5, 7, 12, 19, 23, 28). However, this latter conclusion is based upon the biochemical properties of microsomal fractions in which the vesicles derived from the endoplasmic reticulum were probably contaminated by sialoglycoprotein-rich components.

Golgi elements possibly account for a small part of the sialoglycoprotein in microsomes but certainly not for the whole of it. Sialoglycoprotein largely dissociates from galactosyltransferase after treatment with digitonin (Fig. 3). In addition, the biochemical properties of Golgi-rich preparations (Table II) indicate that a maximum of 10% of liver sialoglycoprotein may be attributed to the galactosyltransferase-bearing membranes. This would account for 6–7% of the total amount of sialoglycoprotein in microsomes, not the 25–30% apparent excess found in this fraction.

Having excluded possible alternative explanations, we conclude that the a2 group is noticeably heterogeneous, and that the underlying component in the microsome fraction is relatively rich in protein-bound sialic acid and cholesterol in comparison with alkaline phosphodiesterase 1, 5'-nucleotidase, and alkaline phosphatase. Heterogeneity within the group a2 is reproducibly observed in subcellular fractions derived from perfused (this work) and nonperfused livers (4, 10). It undoubtedly reflects functional differences along the cell surface of hepatocytes, and the presence of nonparenchymal cells. Plasma membrane preparations obtained from low-speed sediments are clearly more representative of the pericanalicular, functional, and lateral portions of hepatocytes than of other plasma membrane domains of the liver (16). This probably holds for the nuclear fraction. In view of the heterogeneity existing within group a2, the true origin of the excess of sialoglycoprotein over several enzymes of this group in the Golgi-rich preparation remains uncertain.

As envisaged earlier (4, 10), there are intracellular components, e.g. secretory vesicles and endocytic vacuoles, that might have biochemical characteristics similar to those of the plasma membrane and perhaps contribute to the biochemical heterogeneity of group a2. The density distribution of sialoglycoprotein present in the large granule fraction (Fig. 4) and the shift noted after treatment of the animals with Triton WR-1339 (Fig. 5) are compatible with part of the protein-bound sialic acid being associated with endocytic vacuoles and lysosomes. The similarity of behavior between 5'-nucleotidase and the authentic lysosomal enzymes argues for the presence of a genuine 5'-nucleotidase activity in lysosomes (6). The presence of sialoglycoprotein and alkaline phosphodiesterase 1 in subcellular components altered in their density when the large granules are obtained from Triton WR-1339-loaded livers may result from the interiorization of membrane material thought to occur concomitantly with the endocytic uptake (34, 36). Mitochondria and peroxisomes do not contain sialoglycoprotein in significant amount. In spite of the minor overlap of sialoglycoprotein and cytochrome oxidase, or catalase, persisting in large granules from Triton WR-1339-loaded livers, the density distributions are shaped quite differently and demonstrate that the presence of sialoglycoprotein in mitochondrial preparations (13, 14, 35) results from contamination by sialoglycoprotein-containing subcellular entities.

In this work, the protein-bound sialic acid has been studied in bulk; therefore minor sialylated glycoproteins may have escaped detection. Nevertheless, it is remarkable that sialoglycoprotein is largely confined to some particular domains of the cell which include plasma membranes, phagolysosomes, the Golgi complex, and perhaps secretory vesicles. In contrast, we have been unable to detect protein-bound sialic acid in endoplasmic reticulum, in mitochondria, and in peroxisomes. The confinement of protein-bound sialic acid to particular domains may have its origin in the biogenetic pathway of glycoproteins. The Golgi apparatus is the only cell component in which the presence of sialyltransferases is firmly established (33, 43); in this respect it is meaningful that, referring to a membrane flow concept, sialoglycoprotein occurs in the cell downstream from the Golgi apparatus. The conclusion we arrive at casts doubt upon the existence of an autonomous biosynthesis of sialoglycoprotein in mitochondria (13) and upon the back flow theory for the synthesis and insertion of sialoglycoproteins into the endoplasmic reticulum membrane (7).

I wish to express my gratitude to Dr. Christian de Duve for his helpful and critical advice. Special appreciation is extended to Dr. Henri Beaufay for many suggestions during the course of this work and during the writing of the manuscript. I gratefully acknowledge the advice of Dr. Maurice Wibo in the isolation of Golgi elements and the expert technical assistance of Marie-Christine Hamaide.

Received for publication 18 August 1980, and in revised form 2 December 1980.

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