

Absence of Sugars in Electrophoretically Purified Cytochrome b_5 Demonstrated by Combined Gas Chromatography–Mass Spectrometry

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ABSTRACT The problem of determining small but significant amounts of carbohydrates, in purified proteins, has been studied using the membrane protein, cytochrome b_5 . A newly developed method that involves direct gas chromatography–mass spectrometry of sugars obtained by hydrolysis of proteins purified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) allows the identification and determination of small amounts of carbohydrates (e.g., 20 μg of glycoprotein containing a minimum of 0.1% monosaccharide), even in the presence of relatively high amounts of impurities. Application of this method to cytochrome b_5 fragments obtained by tryptic digestion from rat liver microsomes and purified by combined gel filtration and ion exchange chromatography, followed by SDS PAGE, has consistently yielded values below 0.07 mol of the individual sugars and aminosugars per mole cytochrome b_5 . It is concluded that cytochrome b_5 , at least its trypsin-released major amino-terminal fragment, is not constitutively glycosylated.

In studies of the synthesis and modification of proteins, especially membrane proteins, the question of the glycosylation of specific proteins is of fundamental importance. Although efficient methods exist for the determination of sugar components present in proteins relatively rich in carbohydrates, the detection of carbohydrates in proteins that are low in relative sugar content and can be obtained in purified form only in limited amounts is still a methodological problem. To clarify the possible existence of sugars in certain proteins, a method has been developed that allows the unequivocal identification and determination of very small amounts of sugars in polypeptides separated and purified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). The value of this method is demonstrated using the example of the widespread membrane protein, cytochrome b_5 , which has been repeatedly reported to be glycosylated. Cytochrome b_5 is a relatively well-characterized membrane constituent of endoplasmic reticulum (ER, e.g., references 8 and 38). Relatively smaller concentrations of this protein have also been found in isolated nuclear envelope (10, 21), outer mitochondrial membrane (9, 12), Golgi apparatus (18, 19; see, however, reference 33), and in several plasma membrane fractions (3, 4, 19; see, however, reference 34). Preparation and purification (29), intramembrane orienta-

tion (31, 38), re-integration into membranes *in vitro* (7), immunological localization (9), and at least some functions (17) of this protein have been described. The complete amino acid sequence of the major tryptic fragment (31) as well as of whole cytochrome b_5 (8) has been established.

In spite of the numerous and intensive studies, it is still not clear whether cytochrome b_5 is a glycoprotein. For human apocytochrome b_5 prepared by the detergent method, Ozols (30) has reported a content of 10–15 glucose equivalents as determined by the phenol-sulfuric acid procedure. The glycoproteinaceous character of rat liver cytochrome b_5 and tryptic peptide fragments derived therefrom has recently been reported by Winqvist et al. using lectin-binding assays (40) and *in vivo* labeling of the sugar moieties and subsequent immunoprecipitation of the protein (5). If proven, this would provide the first case of an identified and purified protein that is glycosylated on the outer (cytoplasmic) side of endomembranes or in the “free” cytosol. Clarification of this question is of special importance also in relation to the discrepant reports on the biosynthesis of this protein (5, 14, 28, 32). Therefore, it was necessary to rigorously purify the protein, with SDS PAGE as the final step, and to examine the sugar content by a method not biased by carbohydrate-containing contaminants. Since

direct application of established gas chromatography (GC) methods for the identification of carbohydrate components is excluded by the presence of considerable amounts of acrylamide contaminants interfering with quantitative evaluation by flame ionization detection, combined GC-mass spectrometry (MS) has to be used.

MATERIALS AND METHODS

Proteins and Chemicals

Rat liver microsomes were prepared as described (19). Tryptic fragments of cytochrome b_5 were purified essentially according to Omura and Takesue (29). Fractions of 10 ml eluted from the Sephadex G-100 column were monitored at 413 nm, and each fraction was examined by spectral analysis in the whole range from 400 to 500 nm. The 15 fractions of the peak containing cytochrome b_5 were further purified on DEAE cellulose (29), and the cytochrome b_5 peak fraction, eluted between 0.2 and 0.37 M KCl (monitored again at 413 nm), was dialyzed, lyophilized, and the protein characterized by difference spectra (Fig. 1). The spectral peaks recorded were at 424, 527, and 556 nm, in agreement with values reported for cytochrome b_5 from mammalian liver (22; for other references see 12 and 19). From the absorption data a heme content of 67 nmol/mg protein (range 65–74) was estimated; using a mean molecular weight of 13,500 (cf. references 4, 12, and 31), this was estimated to represent a relative heme content of 90.5%. From 1,650 mg of washed microsomes, 4.9 mg of "purified cytochrome b_5 " was recovered. Preparations of cytochrome b_5 tryptic fragments either were applied directly to SDS PAGE or were purified by an additional immunoprecipitation step with rabbit IgG against rat liver cytochrome b_5 as used in our laboratory (19). Immunoprecipitates were applied to SDS PAGE. Fetuin was obtained from Calbiochem (San Diego, Calif.), and human transferrin from Miles Inc. (Elkhart, Ind.). Vimentin was prepared from murine 3T3 cells as described (11), and rabbit skeletal muscle actin was a kind gift of Dr. P. Dancker (Max-Planck-Institut für medizinische Forschung, Heidelberg, W. Germany). Reference sugar compounds were of biochemical grade (Merck, Darmstadt, W. Germany), and cytochrome c and neuraminidase were purchased from Boehringer (Mannheim, W. Germany).

Gel Electrophoresis and Protein Preparation

Gel electrophoresis was performed using the systems of Laemmli (23; 7.5 and 10% acrylamide slab gels) and Thomas and Kornberg (39; 20% slab gels) after fluorescent labeling of the proteins as described by Eng and Parkes (6). Examples of two different preparations of tryptic fragments of cytochrome b_5 are shown in Fig. 2. The identity of both major polypeptides observed as fragments of cytochrome b_5 was also established by gel electrophoresis, in adjacent slots, of immunoprecipitates with anti-cytochrome b_5 IgG as presented previously (reference 19). For SDS PAGE preparation of polypeptides, including the major tryptic fragments of apocytochrome b_5 , bands were excised from various numbers (10–30) of 9-mm broad slots of slab gels (1.5 mm thick, 13 cm in width). These samples were minced to small pieces of $\sim 1 \text{ mm}^2$ with a razor blade, and the proteins were extracted from the gel with 50 mM barbital buffer, 0.1% SDS, 1% β -mercaptoethanol (pH 8.6) by stirring overnight at room temperature (2). The

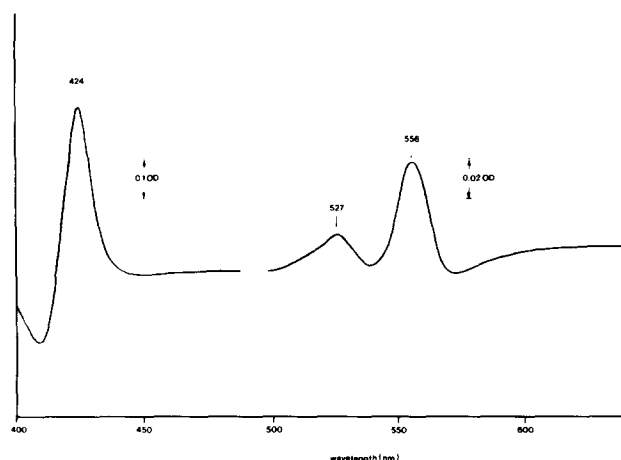


FIGURE 1 Difference spectrum of purified trypsin fragment of cytochrome b_5 . Dithionite-reduced vs. oxidized protein was recorded at room temperature. Optical path was 1 cm and band width 3 mm. For further details, see reference 19.

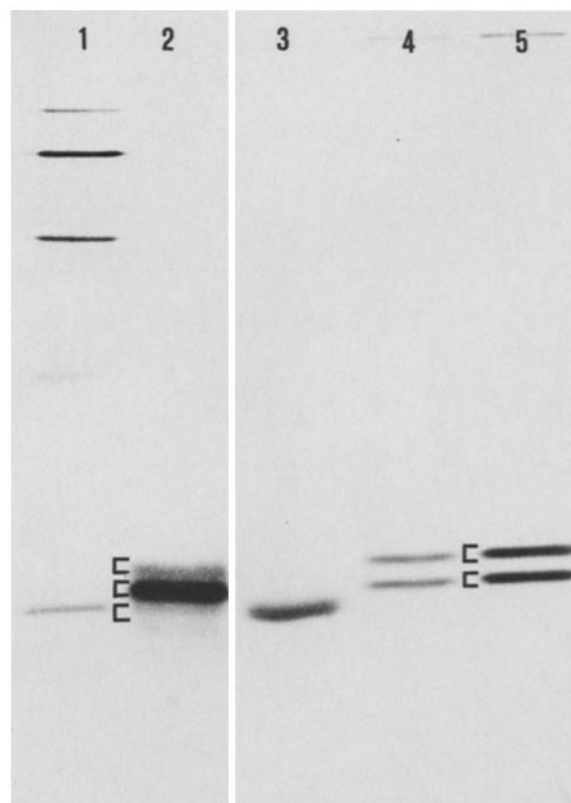


FIGURE 2 SDS polyacrylamide (18%) gel electrophoresis (39) of two different cytochrome b_5 preparations from trypsin-digested rat liver microsomes (slots 2, 4, and 5). Reference proteins are, from top to bottom, phosphorylase A and cytochrome c (from horse heart) in slot 1 and cytochrome c in slot 3. The two different tryptic fragments of cytochrome b_5 resolved in the gel shown in the right (slots 4 and 5, shown at different protein concentrations) of M_r 12,700 and 13,500 were separately excised (brackets) and analyzed in comparison with slices (same size) of adjacent regions (between, below, and above the bands) and apparently protein-free "blanks" from more distant regions of the gel.

protein solution was transferred by a Pasteur pipette into a centrifuge tube, and the gel "fines" were pelleted (10 min at 3,500 g). The supernate was filtered through an "Acrodisc" disposable sterile filter assembly (0.2- μm pore size; Gelman Instrument Co., Ann Arbor, Mich.) into a Teflon-lined screw cap tube (15-mm diameter, Sovirel, Levallois, France) and was precipitated overnight at -20°C by addition of 9 vol of acetone.

The precipitated protein was centrifuged at 3,500 g for 10 min and the supernate was discarded. The pellet was dried under a stream of nitrogen at room temperature and solubilized by sonication in 1 ml of distilled water. After hydrolysis of *N*-acetyl-neuraminic acid (NANA) in 0.1 N H_2SO_4 (by addition of 50 μl of 2 N H_2SO_4 , 80°C for 1 h), the protein was again precipitated with acetone at -20°C . NANA was determined in the supernate by the periodate-resorcinol method described by Jourdan et al. (20) after evaporation of the organic solvent with nitrogen. Subsequently, the residue was hydrolyzed for sugars and amino sugars (0.6 N HCl, 100°C , 14 h; for release of individual sugars, see reference 25 and, particularly for fetuin, reference 36). Protein was determined as follows: After hydrolysis the sample was centrifuged (3,500 g for 10 min). An aliquot (0.05 or 0.1 vol) of the supernate as well as the total pellet were dried, and each sample was redissolved in 50 μl of 0.1 N NaOH; 500 μl of Lowry reagent C (24) and 50 μl of the Folin reagent, respectively, were added, resulting in an absorbance of ~ 0.035 OD at 500 nm for 2 μg of bovine serum albumin. Recovery of proteins is demonstrated in Fig. 3. After elution of protein from the gels (20–400 μg applied per gel) in the buffer described, centrifugation, filtration, and precipitation in acetone, recoveries in the range from 26 to 38% were routinely found for different types and sizes of proteins, including cytochrome c (M_r : 12,300), fetuin (M_r : 44,300), and transferrin (M_r : 76,000).

Derivatization

The method reported earlier (37) was modified as follows: All evaporations were done only at 40°C by a stream of nitrogen. Before reduction, the internal

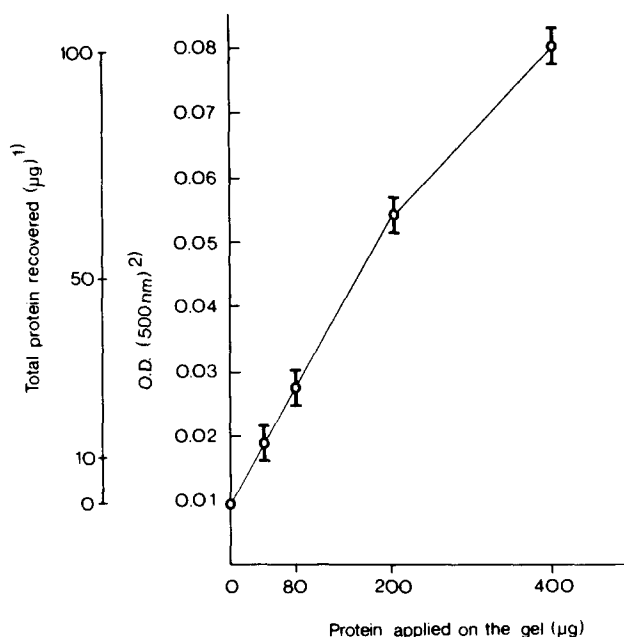


FIGURE 3 Recovery of proteins eluted from the gel. Ordinates present both (1) total amount of protein (μg) recovered from the excised gel slices after elution from the gel, filtration, and acetone precipitation, and (2) absorbance determined in an 0.05 vol aliquot of the supernate obtained after centrifugation. The abscissa presents the amount of protein applied on 10 slots of one gel (13 cm total width, 1.5 mm thick, 2- to 3-mm bands were excised). Total protein (1) was determined by the method of Lowry et al. (24) and calculated as the sum of protein present in the supernate and the pellet obtained after centrifugation of the hydrolysate. For comparison, 5 μg of BSA resulted in 0.084 OD when determined in a final volume of 0.6 ml. Lowry reagent blank (0.008 OD) was always subtracted. Remaining blank absorption was predominantly caused by residual contamination with β -mercaptoethanol.

standard (2-deoxy-D-glucose) was added to the dry hydrolysate. The dry mixture was re-N-acetylated. After this reaction the pyridine/acetic anhydride mixture was immediately evaporated. The thin-layer chromatography step was omitted. From the filtration of the gel eluate, all subsequent operations were performed in the same Sovirel tube (see above). Losses of sugars because of hydrolysis were corrected using different concentrations of equimolar mixtures of sugars (fucose, mannose, glucose, galactose, glucosamine, and galactosamine) that were hydrolysed, mixed with internal standard, and derivatized in the presence of a 10-fold excess of bovine serum albumin (BSA, cf. Fig. 7). Deuterium labeling of glycoproteins containing terminal galactose residues was performed as described for tritium labeling (cf. references 13 and 27). Each sample (200 μg) was incubated for 1 h in 1 ml of phosphate-buffered saline (PBS, pH 7.4) containing 5 U of neuraminidase, 5 U of galactose oxidase, and 5 μl of toluene, at 37°C. Subsequent reduction was performed in 0.1 M NaBD₄ (1 h at room temperature), followed by dialysis against distilled water. After separation on PAGE, the eluted proteins were processed as described above.

Instrumental Conditions for GC-MS and Ion Selection

A Varian model 3700 gas chromatograph (Varian Associates, Instrument Div., Palo Alto, Calif.) equipped with a solventless injection device (35; the all-glass modification purchased from LKB, Bromma, Sweden) and a pressure regulator from Siemens (Karlsruhe, W. Germany) were used. A glass capillary (25 m long and 0.35 mm wide, coated with OV 1, made and proposed by H. & G. Jaeggi, Trogen, Switzerland) fitted with graphite ferrules was interfaced via an open-split connection (16) with a Varian MAT 311 A mass spectrometer equipped with turbomolecular pumps and a multi ion selection unit (MIS). At a constant pressure of 0.55 bar (2 ml/min flow of He), the analysis was started (injector temperature 200°C) at 170°C initial temperature (isothermal for 3 min) and increased by 4°C/min up to 240°C final temperature. MS conditions were: electron input mode; source temperature 150°C; electron emission 0.8 mA;

electron energy 80 eV; MIS interval 0.1 s, 1 Hz; resolution 1,500; 10% valley. Because mass spectrometric resolution of 800 is satisfactory, the method described is not restricted to magnetic sector instruments. On the basis of the mass spectra obtained by direct inlet of alditol acetates of reference sugars (Fig. 4 a-c, neutral deoxy and amino sugars), polyacrylamide fines, and hydrolysate of carbohydrate-free proteins (Fig. 4 d and e), masses were selected that are characteristic for the specific sugar derivatives and different from "background." The typical fragment for the deoxy sugars chosen was $m/e = 231$, whereas $m/e = 259$ was found to select for both neutral and amino sugars. MIS recoveries were determined for each experiment using an equimolar mixture of fucose, 2-deoxy-D-glucose, mannose, glucose, galactose, glucosamine, and galactosamine. Especially important was the confirmation of the m/e intensity ratio of 231/259. By exclusion of "background" (i.e., material other than sugar derivatives) at the masses 231 and 259, additional mass recording could be omitted, resulting in a relatively high measuring time interval per channel. The mass fragmentogram of a mixture of alditol acetates of reference sugars, after 20 min of separation, using the nonpolar glass capillary, is shown in Fig. 5. Fig. 6 presents the application of the GC-MS method purified by PAGE (fetuin), and Table I presents a comparison of these data with values reported in the literature using different methods. With this method, nanogram quantities of alditol acetate derivatives can be analyzed. The linear range of the signal recorded in relation to the amount of sugar derivative injected starts with 5 ng of the individual derivative. Recoveries of the individual sugars at different concentrations are demonstrated in Fig. 7. All values given in Results have been corrected using this correction curve after subtraction of background observed in blank preparations from gel pieces without biological material added that have been isolated in parallel.

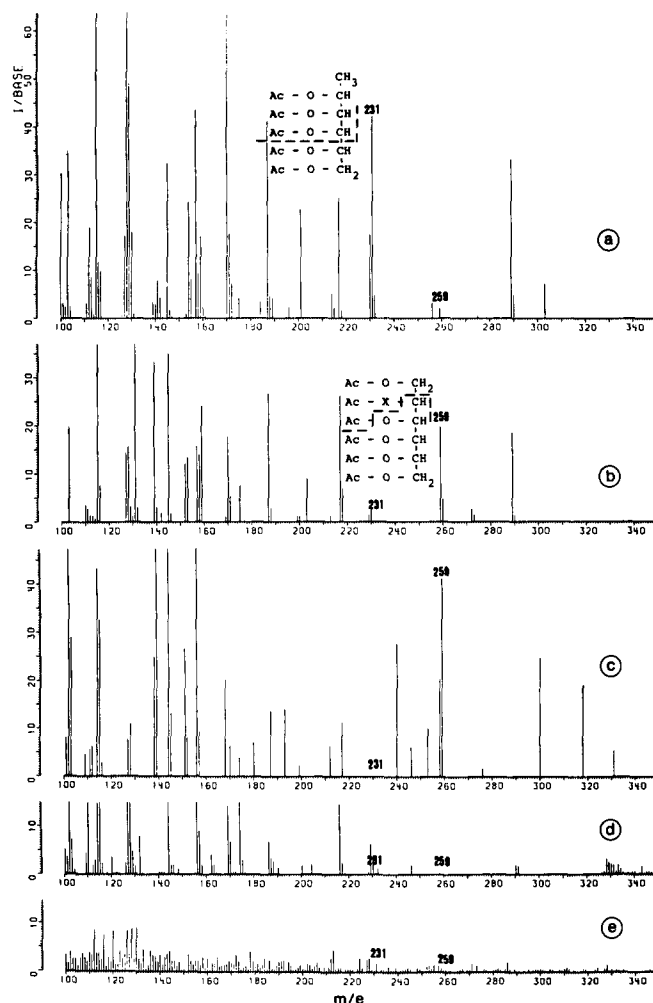


FIGURE 4 Mass spectra of the alditol acetate derivatives characteristic for (a) fucose and 2-deoxy-D-glucose, (b) mannose, glucose, and galactose, (c) glucosamine and galactosamine, and hydrolysates of "background" of blank material from (d) polyacrylamide fines, and (e) a nonglycoprotein (BSA), all obtained by direct insertion probe. X denotes O (for neutral sugars, b) and NH (for amino sugars, spectrum shown in c), respectively.

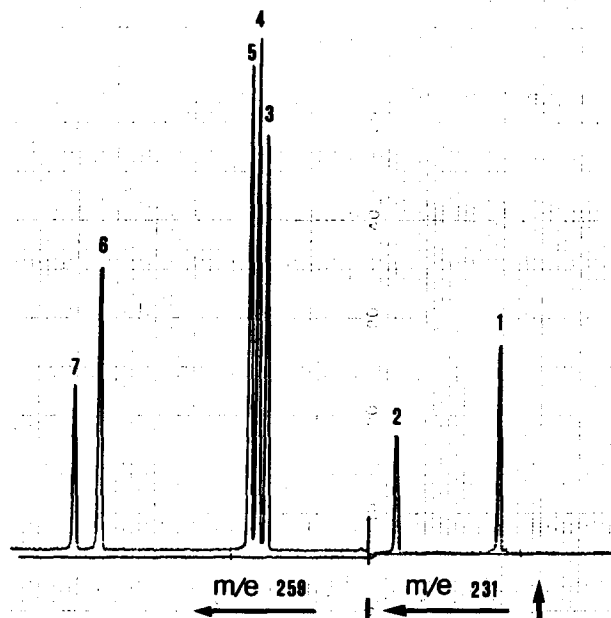


FIGURE 5 Mass fragmentogram of the alditol acetates of an equimolar mixture of (1) fucose, (2) 2-deoxy-D-glucose = internal standard, recorded at m/e 231, and (3) mannose, (4) glucose, (5) galactose, (6) glucosamine, (7) galactosamine, recorded at m/e 259. Separation of the sugar derivatives was performed on an OV 1 capillary column within 20 min after the injection. One peak represents $\sim 0.2 \mu\text{g}$ of the individual sugar. The vertical arrow indicates the recording time of 8 min after injection. Total retention time of galNH_2 (7) is 17 min.

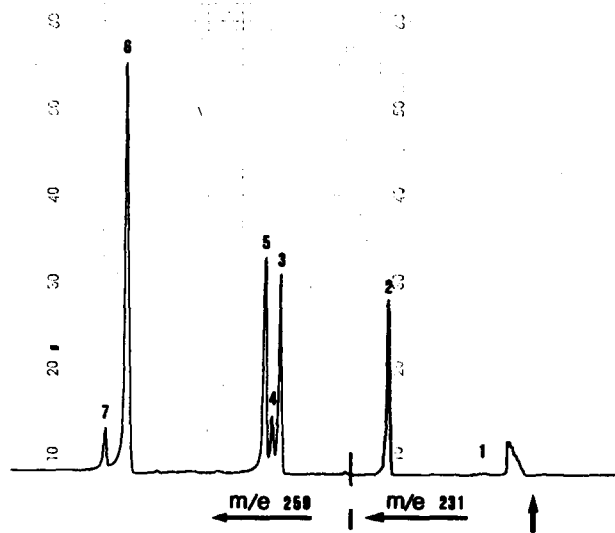


FIGURE 6 Mass fragmentogram of the sugar derivatives of the hydrolysate of fetuin prepared by PAGE (40 μg of protein recovered, 2 μg of internal standard added; for further explanation, see Fig. 3). The asymmetric artifact peak near the region where fucose is normally detected is consistently observed and is derived from acrylamide acetate from the gel; however, it does not interfere with determination of fucose (7).

RESULTS AND DISCUSSION

Cytochrome b_5 fragments (Fig. 2), obtained by tryptic digestion and purified by gel filtration, ion exchange chromatography and PAGE, were characterized by quantitative spectroscopic measurements (cf. Fig. 1) and immunoprecipitation (cf. reference 19) and examined for sugar contents. Some sugars (0.5–2 mol/mol protein) were detected in cytochrome b_5 preparations

obtained by gel filtration and ion exchange chromatography only, with mannose, glucose, galactose, and glucosamine as the major components identified. These small amounts of sugars might include sugars introduced during the preparative procedures. In cytochrome b_5 fragments that were additionally purified by SDS PAGE, no significant amounts of sugars could be detected. Substoichiometric trace amounts of mannose, galactose, glucosamine, and galactosamine never exceeded 0.2 mol of sugar/mol cytochrome b_5 (Fig. 8), and this represented only 0.07 mol of sugars, if corrected for background levels found in apparently protein-free "blank" regions of the same gel. Identical values of low carbohydrate contents (0.07 mol/mol protein) were obtained when proteins from the different bands of cytochrome b_5 fragments were analyzed (see Fig. 2, cf. reference 19). Glucose, which was present in such preparations in 0.2- to 0.8- μg quantities, was not proportionally correlated with increasing protein concentrations and was probably caused by contamination from various polyglucan materials (e.g., dialysis bag, Sepharose, paper, dust particles) introduced during the preparation of the protein. Similar trace amounts of "background" sugars were encountered in nonglycosylated proteins such as actin, cytochrome c , and vimentin purified by SDS PAGE analyzed in parallel.

TABLE I
Comparison of Values Obtained by GC-MS Analysis of PAGE-Purified Glycoproteins with Data Obtained by Conventional Methods Reported in the Literature

Glycoproteins, 50–100 μg	Transferrin*	Fetuin‡
	$\mu\text{g}/\text{mg protein}$	
Fucose	0 (0.7, tr)	0 (0, 0)
Mannose	8 (8, 12)	29 (30, 24)
Galactose	11 (16, 12)	34 (46, 41)
Glucosamine	26 (20, 20)	65 (56, 73)
Galactosamine	0	6 (7, 11)
Total hexose	19 (24, 23.5)	63 (76, 65)
Total amino sugars	26 (20, 20)	71 (63, 84)

Values are corrected according to the calibration curve shown in Fig. 5. *tr*, Traces.

* Values in parentheses are from references 15 and 26.

‡ Values in parentheses are from references 36 and 1.

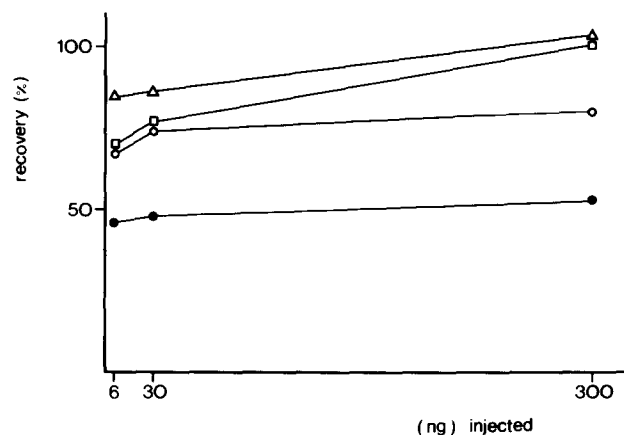


FIGURE 7 Recoveries of the individual sugars determined by comparison of the peak areas observed with the expected ones, relative to 1 μg of 2-deoxy-D-glucose (internal standard) injected. Δ , Neutral hexoses; \square , fucose; \circ , gluNH_2 ; and \bullet , galNH_2 . These data have been obtained after hydrolysis and derivatization of the 20-fold amounts of sugars given in the curve and in the presence of 100 μg of BSA in each experiment.

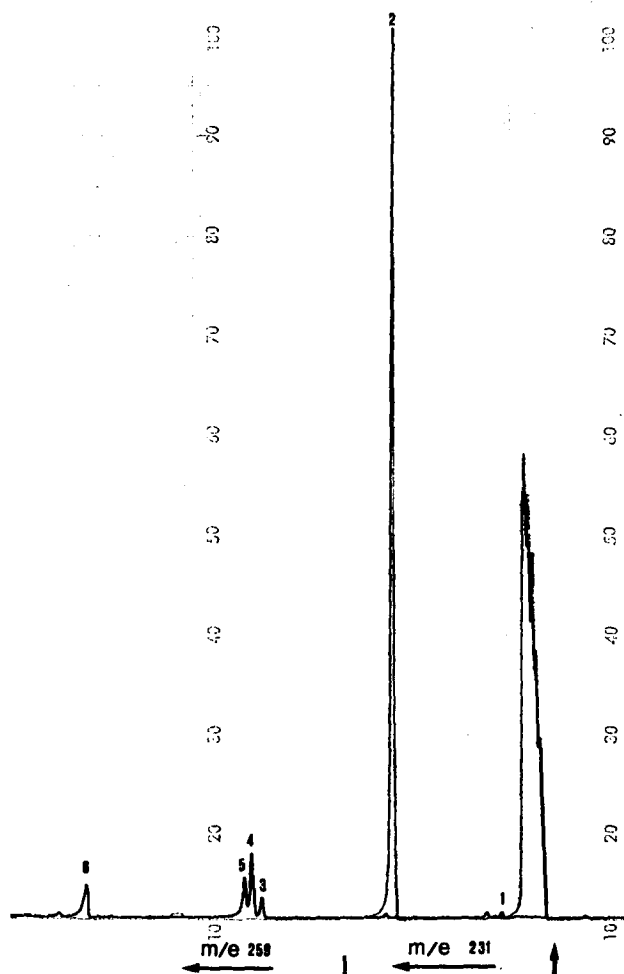


FIGURE 8 Typical mass fragmentogram from a high concentration of cytochrome b_5 (100 μg), purified by SDS PAGE (10 μg of internal standard [2] added; identical amplification for m/e 231 and 259 as in Fig. 5). The sugars recovered represent 0.07 μg (mannose, 3), 0.2 μg (glucose, 4), 0.15 μg (galactose, 5), and 0.2 μg (glucosamine, 6); all values uncorrected. After correction for "background" (see Results), these sugars represent 0.02 μg (mannose), 0.02 μg (glucose), 0.05 μg (galactose), and 0.07 μg (glucosamine), corresponding to <0.07 mol of each of the individual sugar/mol cytochrome b_5 . In this experiment the high amount of cytochrome b_5 has been applied to demonstrate traces of substoichiometric amounts of sugars, probably small contaminants in the preparation.

The demonstrated absence of stoichiometric amounts of sugars in purified cytochrome b_5 appears to be in contrast to observations reported by other authors (for references, see Introduction). While the hexose contents determined by Ozols (30) using the phenol sulphuric acid procedure could be caused by the presence of some sugar contaminants introduced during the column chromatography involved, the sugar contents reported by Elhammer et al. (5) and Winqvist et al. (40) are more difficult to explain. The nonquantitative data of these authors critically rely on the specificity of the immunoprecipitation step included in their preparation. However, the purity of the immunoprecipitates obtained cannot be adequately assessed from the published data in which whole rabbit antisera were used (5). Furthermore, the specificity of the antibody preparations used was examined only by double immunodiffusion (Ouchterlony) tests (5, cf. reference 17), a method that cannot unequivocally demonstrate the specific and exclusive reaction with only one protein. Therefore, it is not excluded that the

galactose and glucosamine contents reported by Elhammer et al. (5) could be derived from co-precipitated glycoprotein(s). Moreover, the reported introduction of radioactivity into cytochrome b_5 by treatment with galactose oxidase and tritiated sodium borohydride cannot be considered as a demonstration of carbohydrate content per se as long as it is not excluded that the label is in amino acids (cf. reference 13).

The present studies show that highly purified cytochrome b_5 fragments do not contain stoichiometric amounts of carbohydrates. This seems to be in accord with the general concept that mechanisms of co- and post-translational protein glycosylation are membrane-located in such a way that proteins present in the cytosol or oriented toward the cytoplasmic phase are not covalently linked to carbohydrate chains.

Our determinations, however, do not exclude the presence of carbohydrate residues that may be embedded in the membrane along with the carboxy-terminal portion of cytochrome b_5 which is not included in the trypsin-released major fragment of the molecule (cf. references 8, 30, 31, and 38). The procedure developed to examine possible low carbohydrate content in polypeptides purified by SDS PAGE allows a sensitive and unequivocal determination of sugars and amino sugars in gel-purified components, and this should be of general value in analyses of defined protein components in cell biology.

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REFERENCES

- Bahl, O. P., and K. M. L. Agrawal. 1969. Glycosidases of *Aspergillus niger*. I. Purification and characterization of α - and β -galactosidase and β -N-acetylglucosaminidase. *J. Biol. Chem.* 244:2970-2978.
- Blomberg, F., R. S. Cohen, and P. Siekevitz. 1977. The structure of postsynaptic densities isolated from dog cerebral cortex. II. Characterization and arrangement of some of the major proteins within the structure. *J. Cell Biol.* 74:204-225.
- Bruder, G., A. Fink, and E.-D. Jarasch. 1978. The b-type cytochrome in endoplasmic reticulum of mammary gland epithelium and milk fat globule membranes consists of two components, cytochrome b, and cytochrome P-420. *Exp. Cell Res.* 117:207-217.
- Bruder, G., A. Bretscher, W. W. Franke, and E.-D. Jarasch. 1980. Plasma membranes from intestinal microvilli and erythrocytes contain cytochromes b, and P-420. *Biochim. Biophys. Acta.* 600:739-755.
- Elhammer, A., D. Dallner, and T. Omura. 1978. Glycosylation of rat liver cytochrome b, on the ribosomal level. *Biochem. Biophys. Res. Commun.* 84:572-580.
- Eng, P. R., and C. O. Parkes. 1974. SDS electrophoresis of fluorescamine-labeled proteins. *Anal. Biochem.* 59:323-325.
- Enoch, H. G., P. J. Fleming, and P. Strittmatter. 1979. The binding of cytochrome b, to phospholipid vesicles and biological membranes. Effect of orientation on intermembrane transfer and digestion by carboxypeptidase Y. *J. Biol. Chem.* 254:6483-6488.
- Fleming, P. J., H. A. Daily, D. Corcoran, and P. Strittmatter. 1978. The primary structure of the nonpolar segment of bovine cytochrome b,. *J. Biol. Chem.* 253:5369-5372.
- Fowler, S., J. Remacle, A. Trouet, H. Beaufay, J. Berthet, M. Wibo, and P. Hauser. 1976. Analytical study of microsomes and isolated subcellular membranes from rat liver. V. Immunological localization of cytochrome b, by electron microscopy: methodology and application to various subcellular fractions. *J. Cell Biol.* 71:535-550.
- Franke, W. W., B. Deumling, B. Ermen, E.-D. Jarasch, and H. Kleinig. 1970. Nuclear membranes from the mammalian liver. I. Isolation procedure and general characterization. *J. Cell Biol.* 46:379-395.
- Franke, W. W., E. Schmid, M. Osborn, and K. Weber. 1978. Different intermediate-sized filaments distinguished by immunofluorescence microscopy. *Proc. Natl. Acad. Sci. U. S. A.* 75:5034-5038.
- Fukushima, K., and R. Sato. 1973. Purification of cytochrome b,-linked hemoprotein associated with outer mitochondrial membrane of rat liver. *J. Biochem.* 74:161-173.
- Gahmberg, C. G. 1978. Tritium labeling of cell-surface glycoproteins and glycolipids using galactose oxidase. *Methods Enzymol.* 50:204-206.
- Harano, T., and T. Omura. 1978. Biogenesis of endoplasmic reticulum membrane in rat liver cells. III. Biosynthesis of NADPH-cytochrome c reductase and cytochrome b, by loosely-bound ribosomes. *J. Biochem.* 84:213-223.
- Heimburger, N., K. Heide, H. Haupt, and H. E. Schultz. 1964. Bausteinanalysen von Humanserumproteinen. *Clin. Chim. Acta.* 10:293.

16. Henneberg, D., U. Henrichs, H. Husmann, and G. Schomburg. 1978. High-performance gas chromatograph-mass spectrometer interfacing: investigation and optimization of flow and temperature. *J. Chromatogr.* 167:139-147.
17. Hirokata, Y., A. Shigematsu, and T. Omura. 1978. Immunochemical study on the pathway of electron flow in reduced nicotinamide adenine dinucleotide-dependent microsomal lipid peroxidation. *J. Biochem.* 83:431-440.
18. Ichikawa, Y., and T. Yamano. 1970. Cytochrome b_5 and CO-binding cytochromes in the Golgi membranes of mammalian livers. *Biochem. Biophys. Res. Commun.* 40:297-305.
19. Jarasch, E.-D., J. Kartenbeck, G. Bruder, A. Fink, D. J. Morre, and W. W. Franke. 1979. B-type cytochromes in plasma membranes isolated from rat liver, in comparison with those of endomembranes. *J. Cell Biol.* 80:37-52.
20. Jourdan, G. W., L. Dean, and S. Roseman. 1971. The sialic acids. XI. A periodate-resorcinol method for the quantitative estimation of free sialic acids and their glycosides. *J. Biol. Chem.* 246:430-435.
21. Kasper, C. B. 1971. Biochemical distinctions between the nuclear and microsomal membranes from rat hepatocytes. *J. Biol. Chem.* 246:577-581.
22. Klingenberg, M. 1958. Pigments of rat liver microsomes. *Arch. Biochem. Biophys.* 75:376-386.
23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
24. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
25. Marshall, R. D., and A. Neuberger. 1972. In *Glycoproteins. Qualitative and quantitative analysis of the component sugars*. B. B. A. Library, Vol. 5A. A. Gottschalk, editor. Elsevier, Amsterdam. 224-299.
26. Montreuil, J., G. Spik, M. Monsigny, J. Descamps, G. Biserte, and M. Deutrevauy. 1965. Etude comparée de la composition en oses et en amino-acides de la transferrine et de la lactotransferrine humaines. *Experientia (Basel)*. 21:254-256.
27. Morell, A. G., and G. Ashwell. 1972. Tritium-labeling of glycoproteins that contain terminal galactose residues. *Methods Enzymol.* 50:204-206.
28. Okada, Y., D. D. Sabatini, and G. Kreibich. 1979. Sites of synthesis of rat liver cytochrome b_5 and NADPH-cytochrome P-450 reductase. *J. Cell Biol.* 83(2, Pt. 2):437a (Abstr.).
29. Omura, T., and S. Takesue. 1970. A new method for simultaneous purification of cytochrome b_5 and NADPH-cytochrome c reductase from rat liver microsomes. *J. Biochem. (Tokyo)*. 67:249-257.
30. Ozols, J. 1972. Cytochrome b_5 from a normal human liver. Isolation and partial amino acid sequence. *J. Biol. Chem.* 247:2242-2245.
31. Ozols, J., and C. Gerard. 1977. Primary structure of the membranous segment of cytochrome b_5 . *Proc. Natl. Acad. Sci. U. S. A.* 74:3725-3729.
32. Rachubinski, R. A., D. P. S. Verma, and J. J. M. Bergeron. 1980. Synthesis of rat liver microsomal cytochrome b_5 by free ribosomes. *J. Cell Biol.* 84:705-716.
33. Remacle, J. 1978. Binding of cytochrome b_5 to membranes of isolated subcellular organelles from rat liver. *J. Cell Biol.* 79:291-313.
34. Remacle, J. 1980. The binding of cytochrome b_5 to plasma membranes of rat liver. Its implication for membrane specificity and biogenesis. *Biochim. Biophys. Acta.* 597:564-576.
35. Ros, A. 1965. Method for dry-sampling in gas chromatography. *J. Gas Chromatogr.* 3:252.
36. Spiro, R. G. 1962. Studies on the monosaccharide sequence of the serum glycoprotein fetuin. *J. Biol. Chem.* 237:646-652.
37. Stadler, J. 1976. Quantitative analysis of total membrane-bound sugars and amino sugars as alditol acetates by combined thin-layer chromatography, gas-liquid chromatography, and radiogas chromatography. *Anal. Biochem.* 74:62-72.
38. Strittmatter, P., M. J. Rogers, and L. Spatz. 1972. The binding of cytochrome b_5 to liver microsomes. *J. Biol. Chem.* 247:7188-7194.
39. Thomas, J. O., and R. D. Kornberg. 1975. An octamer of histones in chromatin and free in solution. *Proc. Natl. Acad. Sci. U. S. A.* 72:2626-2630.
40. Winqvist, L., L. C. Eriksson, and G. Dallner. 1979. Interaction of lectins with proteins of the endoplasmic reticulum and Golgi system or rat liver. *J. Cell Sci.* 39:101-116.