# THE ISOLATION AND CHARACTERIZATION OF GLYCOPEPTIDES AND MUCOPOLYSACCHARIDES FROM PLASMA MEMBRANES OF NORMAL AND REGENERATING LIVERS OF RATS

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## 1. Introduction

Isolation of the glycopeptides of the O-glycosidic and N-glycosidic types and of heparan sulfate from plasma membranes of ascites hepatoma cells, AH 66 and AH 130, [1,2], prompted us to investigate the complex carbohydrates occuring in plasma membranes of normal and regenerating livers of rats, as normal control cells. In a preliminary study, we reported the isolation of glycopeptides of the N-glycosidic type from the pronase digest of the plasma membranes of normal rat livers which were acidic (sialic acid-containing) and of relatively low mol. wt [3]. At that time, however, the carbohydrate-containing fractions of relatively high mol. wt, which were excluded from a Sephadex G-50 column, were not investigated. Since the O-glycosidic glycopeptides and the mucopolysaccharides from the hepatoma plasma membranes were obtained in these excluded fractions, we decided to reinvestigate the corresponding fractions from normal livers. Regenerating (dividing) liver cells were also used since they are regarded as more adequate than normal (resting) liver cells for use as a normal control to hepatoma cells which are rapidly dividing.

Results reported here show that normal and regenerating livers gave similar glycopeptide patterns, all of which were apparently N-glycosidic and acidic in contrast to the production of both N- and O-glycosidic glycopeptides from the hepatoma membranes. The occurrence of heparan sulfate was, however, common to all the plasma membranes from the livers and hepatomas.

## 2. Materials and methods

Plasma membranes were isolated from male Wistar rats weighing 140–180 g using a combination of the methods of Neville [4] and Ray [5] with some modifications. Livers were homogenized in 10 vols of an isolation medium composed of 1 mM $NaHCO_3$, 0.5 mM $CaCl_2$ and 10 mM sucrose, pH 7.5, in a Dounce type homogenizer with a loosely fitting pestle. The homogenate was diluted to a 1% homogenate with the isolation medium, filtered through 4 layers of gauze, then centrifuged at 1500 *g* for 10 min. The pellet was suspended in the isolation medium of half the previous volume, then centrifuged again at 1500 *g* for 10 min. The pellet was resuspended in the medium of half the previous vol, then centrifuged again at 1500 *g* for 10 min. The final pellet was suspended in a small vol of the medium (about 7 ml per 5–6 g of liver), and the suspension was mixed with 69% sucrose to give a sucrose concentration of 44%. The suspension was poured into a tube fitted to a Hitachi RPS-25 rotor, and onto this about 10 ml of 41 % sucrose and a few ml of the isolation medium were layered stepwise. Centrifugation at 107 000 *g* for 2 h separated plasma membranes at the interphase between the isolation medium and the 41% sucrose. Membranes were collected with a Pasteur pipette and the suspension was layered on a linear sucrose gradient from 5 to 37% which had been layered onto 50% sucrose working as a cushion, then the tubes were centrifuged at 1300 *g* for 40 min. The membranes obtained at the interphase between the cushion and the gradient were

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collected with a Pasteur pipette. The membranes were washed with 1 mM $NaHCO_3$ three times, each time by suspension and centrifugation, to remove sucrose, after which they were lyophilized.

The isolated plasma membranes appeared to be free from cytoplasmic particulates when examined by phase microscopy, the yield being 0.50 mg protein per g liver. They contained various marker enzymes with the following activities, as determined and expressed as described by Ray [5] : 2.28 units/mg protein of alkaline phosphatase [EC 3.1.3.1], an 8-fold increase over the homogenate; 45.6 units/mg protein of 5'-nucleotidase [EC 3.1.3.5], a 14-fold increase; and 3.24 units/mg protein of glucose 6-phosphatase [EC 3.1.3.9], one-third the specific activity of the homogenate. These values indicate that our plasma membrane preparation had a high degree of purity.

Plasma membranes were prepared from regenerating rat livers as follows : male Wistar rats weighing 130–150 g, were 70% hepatectomized and after 33 h the regenerating livers were excised. Some of the hepatectomized rats were injected intraperitoneally 3 h prior to death with 80 $\mu$Ci of [$^3$H]glucosamine (D-[1-$^3$H]-glucosamine-HCl, 3.0 Ci/mmol) to label hexosamines and sialic acid, and with 200 $\mu$Ci of [$^{35}$S]-$Na_2SO_4$ (101 mCi/mmol) to label O- and N-sulfates of mucopolysaccharides. The labelling was effective for monitoring glycopeptides and mucopolysaccharides in the chromatographic fractions, obtained from small amounts of the sample from regenerating livers. Plasma membranes were prepared according to the procedure used for normal livers, with a yield of 0.37 mg protein per g liver. The isolated membranes appeared microscopically similar to those from normal livers and had the following enzyme activities : 17.34 units/mg protein of alkaline phosphatase, a 17-fold increase over the homogenate; and 2.34 units/mg protein of glucose 6-phosphatase, one-third the specific activity of the homogenate. A rise in the specific activity of alkaline phosphatase after partial hepatectomy has been reported [6].

The lyopilized plasma membranes were made lipid-free by extracting the lipids with 50 vols of chloroform : methanol (2 : 1, v/v) three times. The residues were washed with 50 vols of ethanol, then dried in a vacuum desiccator over $CaCl_2$. The lipid-free plasma membranes were digested with pronase in 0.1 M borate buffer, pH 8.0, containing 0.01 M calcium acetate at 37°C by the procedure described previously [7].

To identify the mucopolysaccharides, cellulose acetate electrophoresis using Sepraphore III (Gelman, USA) was carried out (a) in 1 M acetic acid–pyridine buffer, pH 3.5, at a current of 0.5 mA per cm for 30 min according to Seno and Meyer [8], or (b) in 0.3 M calcium acetate at a current of 1 mA per cm for 3 h according to Seno et al. [9]. Strips were stained with 0.5% toluidine blue in 3% acetic acid. Of the reference mucopolysaccharides, the heparan sulfates from the rat kidney (HS-I) and rat lung (HS-II) were gifts from Professor K. Anno of Ochanomizu University, Tokyo. Other mucopolysaccharides and chondroitinase ABC [EC 4.2.2.4] were purchased from Seikagaku Kogyo Co., Tokyo.

To monitor the chromatographic fractions for neutral sugars, the orcinol - $H_2SO_4$ reaction according to Hewitt [10] was used. Sialic acid was determined by the resorcinol method of Jourdian et al. [11]. Hexosamine and amino acids were determined with a Hitachi amino analyzer, KLA-3B, after hydrolysis in 6 N HCl at 100°C for 16 h. N-Sulfate was determined by the nitrous acid oxidation followed by the indole reaction according to Dische and Borenfreund [12]. Protein was determined by the method of Lowry et al. [13] with ovalbumin as the standard. The radioactivity was counted with a Beckman, model LS-100, scintillation counter.

## 3. Results

After pronase digestion of the lipid-free plasma membranes, practically all the glycoproteins and mucoproteins were solubilized, producing glycopeptides and mucopolysaccharides, respectively, as judged by the hexosamine determination for normal livers and the radioactivity determination for regenerating livers. The digest was fractionated on a Sephadex G-50 column, as shown in figs.1 and 2.

Normal and regenerating livers gave similar patterns, characterized by the presence of a significant peak (designated F-I) at the void volume of the column which was positive for neutral sugars, but negative for sialic acid, and peaks (designated F-II) in the area included in the gel which were positive for

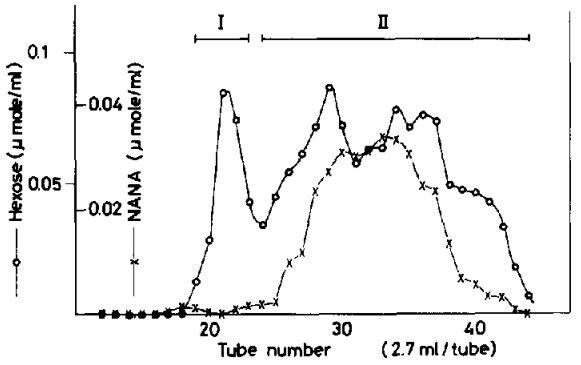


Fig.1. Gel filtration of the pronase digest of plasma membranes of normal rat livers. A digest corresponding to 30 mg of lipid-free membrane was dissolved in 3 ml of 0.05 M pyridine - acetic acid buffer, pH 5.0, and applied to a Sephadex G-50 column (1.4 X 102 cm) equilibrated with the same buffer. Elution was carried out with the same buffer at a flow rate of 10 ml per h. 'Hexose' stands for an equimolar mixture of galactose and mannose, and 'NANA' for *N*-acetylneuraminic acid. I and II denote F-I and F-II, respectively.

both neutral sugars and sialic acid. F-I from regenerating livers contained both $^{3}H$ and $^{35}S$ radioactivities, indicative of the presence of mucopolysaccharides. These patterns for normal and regenerating livers contrasted with the corresponding patterns for the plasma membranes of ascites hepatoma cells, AH 66 and AH 130, in which a significant amount of sialic acid constituting the O-glycosidic glycopeptides was found in the peak at the void volume [1,2].

Analysis of F-I from normal livers showed that glucosamine was the only hexosamine in this fraction, and about half (49.5%) of the glucosamine was estimated to be N-sulfated. These analyses, though they could not be carried out on the F-I from regenerating livers due to the small amounts of material, suggested that the F-I would contain heparin or heparan sulfate, but no O-glycosidic glycopeptides. Actually, F-I, from both normal and regenerating livers, gave a single spot on electrophoresis, using two different systems, at a position between two reference heparan sulfates (fig. 3). They resisted the action of chondroitinase ABC. From the amount of glucosamine in F-I, the heparan sulfate was estimated to correspond to about 10% of the total glucosamine in the plasma membranes of normal or regenerating livers.

All the glycopeptides in F-II appeared to be acidic, as seen from the approximate coincidence of the peaks for neutral sugars and sialic acid (fig.1) or more clearly from the coincidence of the peaks for sialic acid and $^{3}H$ radioactivity (fig.2). No O-glycosidic glycopeptides seemed to be present in F-II since no galactosamine was detected.

The orcinol positive substance found in F-II was not identified. Note that, in the corresponding frac-

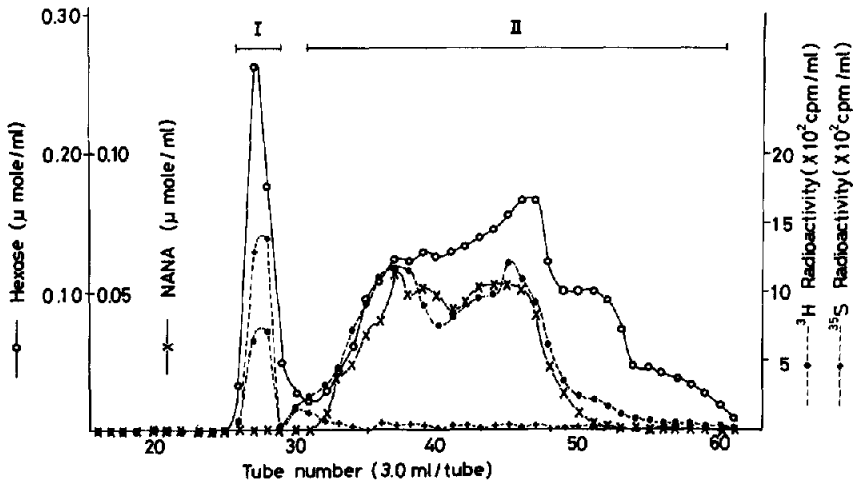


Fig.2. Gel filtration of the pronase digest of plasma membranes of regenerating rat livers. A digest corresponding to 60 mg of lipid-free membrane was dissolved in 4 ml of 0.05 M pyridine–acetic acid buffer, pH 5.0, and applied to a Sephadex G-50 column (1.6 X 95 cm). Other conditions are the same as in fig.1.

tion from the plasma membranes of the ascites hepatoma cells, dextran was detected which contaminates the sucrose used for preparing the membranes.

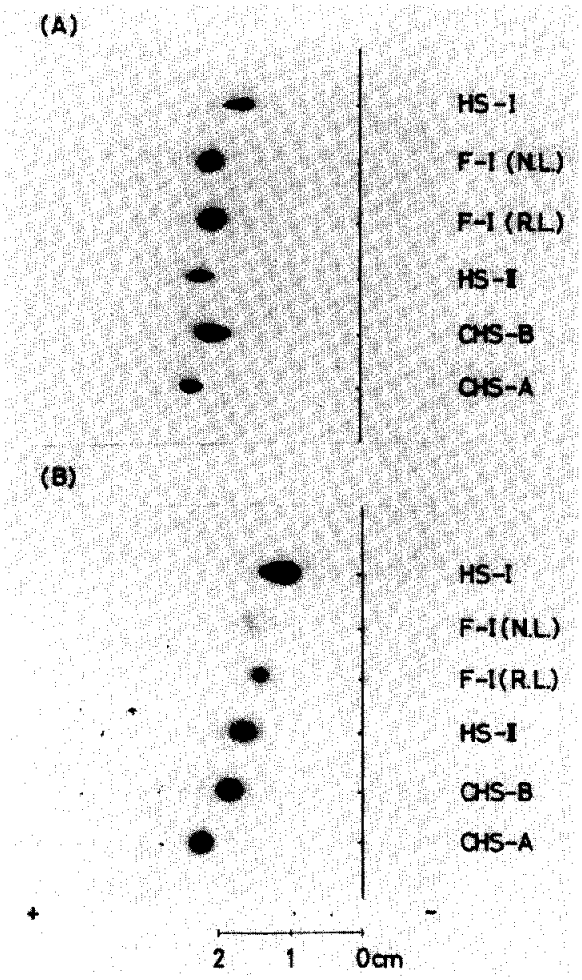


Fig.3. Electrophoresis of isolated mucopolysaccharides on a cellulose acetate strip. Electrophoresis was carried out as described in the text. F-I (N.L.) and F-I (R.L.) are the fractions (I) (see figs.1 and 2) from normal and regenerating livers, respectively. Reference mucopolysaccharides are heparan sulfates (HS-I and HS-II, as described in the text); chondroitin sulfate B (CHS-B); and chondroitin sulfate A (CHS-A). (A) : 1 M acetic acid–pyridine buffer, pH 3.5; and (B) 0.3 M calcium acetate. In (B), only a limited amount of F-1 (N.L.) could be applied to the strip since some unidentified substances precipitable with calcium acetate interfered with the electrophoresis.

## 4. Discussion

Our results showing that heparan sulfate was the only mucopolysaccharide detected in the pronase digest of the plasma membranes of normal and regenerating rat livers differ somewhat from the findings of Kojima et al. [14] who claimed that chondroitin sulfate B occured in the plasma membranes of normal rat livers in addition to heparan sulfate, at an approximate ratio of 1 : 30, and that its amount increased in regenerating livers reaching nearly the level of heparan sulfate 28 h after hepatectomy. The reason for this inconsistency is not yet known.

No glycopeptides with O-glycosidic linkages were detected in the pronase digest of the plasma membranes of either normal or regenerating livers. This is in contrast to their abundance in the digest of the plasma membranes of ascites hepatomas, AH 66 and AH 130. Sanford et al. [15] showed some evidence that O-glycosidic glycoproteins, the parent molecules which produce O-glycosidic glycopeptides on proteolytic digestion and occur in the plasma membranes of TA3-Ha cells, a subline of the TA3 mammary adenocarcinoma, may be involved in masking histocompatibility (H-2) antigens at the cell surface. This explains the low strain-specificity of TA3-Ha cells with O-glycosidic glycoproteins in contrast to the high strain-specificity of another cell line, TA3-St with no O-glycosidic glycoprotein. The O-glycosidic glycopeptides of high molecular weight, which we isolated from the ascites hepatoma cells, are very similar to the O-glycosidic glycopeptides from the TA3-Ha cells in size and structure [1]. Their presence in hepatomas which have the capacity to grow in randomly bred rats and their absence in hepatocytes which are tissue-specific cells appear to be consistent with the above conclusion.

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