In vitro radiolabeling of galactosyl and N-acetylgalactosaminyl moieties of glycoproteins with carbon-14

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Summary. The primary alcohol group on the carbon 6 of terminal galactosyl and N-acetylgalactosaminyl moieties of glycoproteins can be oxidized to an aldehyde by treatment with galactose oxidase. By reacting these aldehyde groups with ^{14}C-labeled sodium cyanide, ^{14}C-labeled cyanohydrin derivatives were obtained. Similarly, reduction of these aldehyde groups with tritiated sodium borohydride following standard procedures, yields ^{3}H-labeled glycoproteins. ^{14}C- and ^{3}H-labeled derivatives of asialofetuin and asialo ovine submaxillary mucin with high specific radioactivities were prepared using these procedures. Mixtures containing microgram amounts of ^{14}C- and ^{3}H-labeled glycoproteins were subjected to column chromatography and gradient ultracentrifugation and the position of the individual glycoproteins was determined by simultaneous counting for ^{14}C and ^{3}H. These experiments demonstrate the usefulness of this approach for comparative analytical studies using biological specimens available in minute quantities.
Key words. Fetuin; galactose; galactose oxidase; in vitro; N-acetylgalactosamine; ovine submaxillary mucin; radiolabeling.

The detection of small differences in molecular weight, electrical charge and buoyant density of homologous glycoconjugates, e.g. those isolated from control and pathological specimens, is greatly facilitated by the availability of radiolabeled materials. Mixtures of glycoconjugates, one labeled with ^{3}H and the other with ^{14}C can be subjected to analytical procedures, e.g. column chromatography, and the individual elution profiles can be established by simultaneous counting for these isotopes in the eluted fractions.

In vitro radiolabeling is very advantageous because it requires small quantities of radioactive reagents, yields products with high specific activities and circumvents technical and ethical problems associated with in vivo labeling using radioactive metabolic precursors.

Tritium labeling of terminal sialyl and galactosyl residues of glycoconjugates can be readily achieved by reduction with tritiated borohydride of the exopyranosyl aldehyde groups generated by chemical (periodate) or enzymatic (galactose oxidase) oxidation of sialyl[1] and galactosyl[2] residues, respectively.

By reacting the aldehyde groups generated by mild periodate oxidation of sialoglycoproteins with ^{14}C-labeled sodium cyanide (Kiliani reaction) we have prepared ^{14}C-labeled cyanohydrin derivatives of the sialyl residues[3].

In the present paper we describe the in vitro ^{14}C-labeling of galactosyl and N-acetylgalactosaminyl moieties of glycoproteins using a similar approach, i.e., formation of a labeled cyanohydrin on the aldehyde generated at the carbon 6 position following treatment with galactose oxidase (fig. 1). A brief account of this work has been published[4].

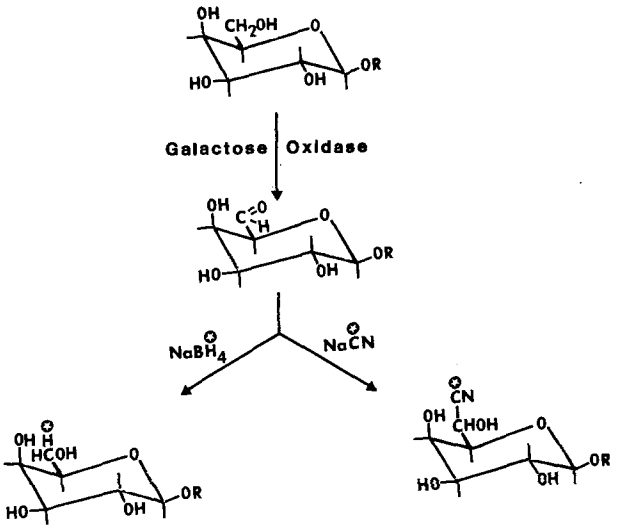

Figure 1. Diagram of the procedure utilized for the in vitro radiolabeling of terminal ß-galactosyl residues such as those present in asialofetuin. The same outline applies to the radiolabeling of the α-N-acetylgalactosaminyl residues of asialo-OSM.

Materials and methods

$NaB^{3}H_4$ (specific activity 341 mCi/mmol) and $Na^{14}CN$ (specific activity 7.9 mCi/mmol) were purchased from New England Nuclear. Galactose oxidase Type V from Dactylium dendroides (182 units per mg protein) was purchased from Sigma. Fetuin (prepared by the method of Spiro) was purchased from Gibco and further purified by gel filtration chromatography[3].

Ovine submaxillary mucin (OSM) was isolated from ovine submaxillary glands[5] and purified by gel filtration chromatography[6] as previously described[3]. To remove terminal sialic acid residues the glycoproteins were subjected to mild acid hydrolysis with 0.05 M sulfuric acid at 80 °C for 1 h. The hydrolysates were neutralized with a saturated solution of barium hydroxide and centrifuged to sediment the barium sulfate. The clear supernatants were dialyzed against distilled water to remove free sialic

acid and the asialoglycoproteins were recovered by lyophilization.

Total sialic acid was measured by the resorcinol assay[7] and free sialic acid by the thiobarbituric acid method[8]; crystalline N-acetylneuraminic acid was used as the standard. Proteins were determined by the method of Lowry et al.[9] using crystalline bovine serum albumin as the standard.

Preparative CsBr gradient ultracentrifugation was carried out as described by Bhaskar and Reid[10]. Radioactivity measurements were done in Handifluor (Mallinckrodt) using a Tracor Analytical Mark III scintillation counter.

Results and discussion

Tritium radiolabeling was carried out by a modification of the method described by Morell and Ashwell[11]. In a typical experiment, 15 mg of asialoglycoprotein dissolved in 30 ml of 0.1 M phosphate buffer pH 7.0 was incubated with galactose oxidase (50 units for asialofetuin; 150 units for asialo-OSM) at 25 °C for 24 h. The incubation mixture was saturated with toluene to inhibit bacterial growth. Following dialysis against phosphate-buffered saline (0.15 M NaCl in 0.05 M phosphate buffer pH 7.4) one half of the dialyzed solution (15 ml) containing 7.5 mg of oxidized asialoglycoprotein, was treated with 100 μmoles of NaB^3H_4 dissolved in 1.6 ml of 0.01 M NaOH. After 30 min at room temperature in a ventilated fume hood, 20 mg of non-radioactive $NaBH_4$ in 10 ml of 0.01 M NaOH was added and the reduction was allowed to proceed for another 30 min at room temperature. The reaction was terminated by dropwise addition of excess acetone (1 ml) to consume the unreacted borohydride and prevent evolution of $^3H_2$ during dialysis. The reaction mixture was dialyzed exhaustively first against phosphate-buffered saline, then against distilled water and lyophilized. The specific radioactivity of the tritiated glycoproteins, expressed as cpm/mg dry, was 7.68×10^6 for asialofetuin and 2.23×10^7 for asialo-OSM.

The second half of the oxidized asialoglycoprotein solution was dialyzed against distilled water to remove the phosphate-buffered saline and cyanohydrin formation was performed using a mixture of equimolar amounts of $Na^{14}CN$ and NH_4Cl[12]. A 10% excess, i.e., 16 μmoles each of NH_4Cl and $Na^{14}CN$, in aqueous solution (0.3 ml each) were added to asialofetuin and 48 μmoles each to asialo-OSM. These stoichiometries were estimated from the initial sialic acid content assuming complete oxidation of the galactosyl and N-acetylgalactosaminyl residues exposed after desialylation of fetuin and OSM, respectively. Cyanohydrin formation was allowed to proceed overnight at room temperature in a ground-glass-stoppered flask placed in a ventilated fume hood. The reaction mixture was then dialyzed against distilled water and lyophilized. The specific radioactivity of the ^{14}C-labeled glycoproteins, expressed as cpm/mg dry, was 2.99×10^6 for asialofetuin and 7.27×10^6 for asialo-OSM.

Because of the high specific radioactivity of the labeled asialoglycoprotein, elution profiles from gel filtration chromatography could be obtained with very small specimens, i.e. 3–4 μg. The elution profiles for ^{14}C- and ^3H-labeled asialofetuin were the same when a mixture of the two glycoproteins was chromatographed on a column of Sephadex G-200 (data not shown). Similar results were obtained with mixtures of labeled asialo-OSM. A mixture of ^{14}C-asialo-OSM and ^3H-asialofetuin was then chromatographed on the same column. Simultaneous counting for ^{14}C and ^3H in the column effluent allowed us to follow the individual elution profiles (fig. 2). Each protein peak in the mixture eluted in the same position observed when they were chromatographed individually.

Since OSM contains about 20% of sialic acid we decided to assess the effect of desialylation on the elution profile. To this effect, ^{14}C-labeled asialo-OSM was mixed with OSM which had been labeled with ^3H on the sialyl moiety as previously described[3]. As shown in figure 3 the difference in molecular weight is reflected in the elution profiles which show OSM eluting before asialo-OSM.

The results of CsBr gradient ultracentrifugation of a mixture of ^3H-labeled asialo-OSM and ^{14}C-labeled asialofetuin are shown in figure 4A. The initial glycoprotein solution contained a 0.42 weight fraction of CsBr[10]. Following ultracentrifugation asialofetuin was concentrated in the upper half of the gradient with its peak at density 1.324. Asialo-OSM, on the other hand, displayed a wide peak with its maximum at density 1.396. The higher density of asialo-OSM is consistent with its higher carbohydrate content, whereas the shape of the peak is consistent with the microheterogeneity of OSM[13]. Since solubilization of some complex biological mixtures of glycoconjugates often requires the addition of guanidine hydrochloride, we investigated the effect of this compound on the

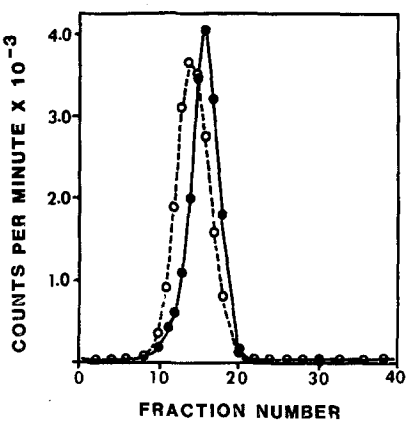

Figure 2. Chromatography on a column of Sephadex G-200 (0.7 × 16 cm) of a mixture containing ca 3 μg (i.e. 20,000 cpm) each of ^{14}C-labeled asialo-OSM (--o--o--) and ^3H-labeled asialofetuin (–●–●–). Elution with 0.1 M phosphate buffer pH 7.0; fraction volume, 0.37 ml. A 0.1-ml aliquot was utilized for radioactivity measurements.

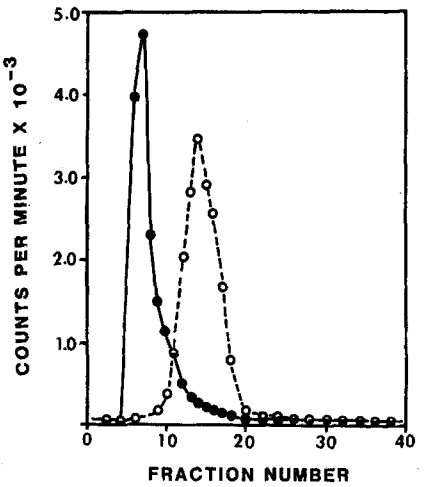

Figure 3. Chromatography of a mixture of 3.6 µg (26,000 cpm) of ^{14}C-labeled asialo-OSM (--o--o--) and 2.8 µg (28,500 cpm) of OSM labeled with ^{3}H on the sialyl moiety (–●–●–). Same experimental conditions as in fig. 2.

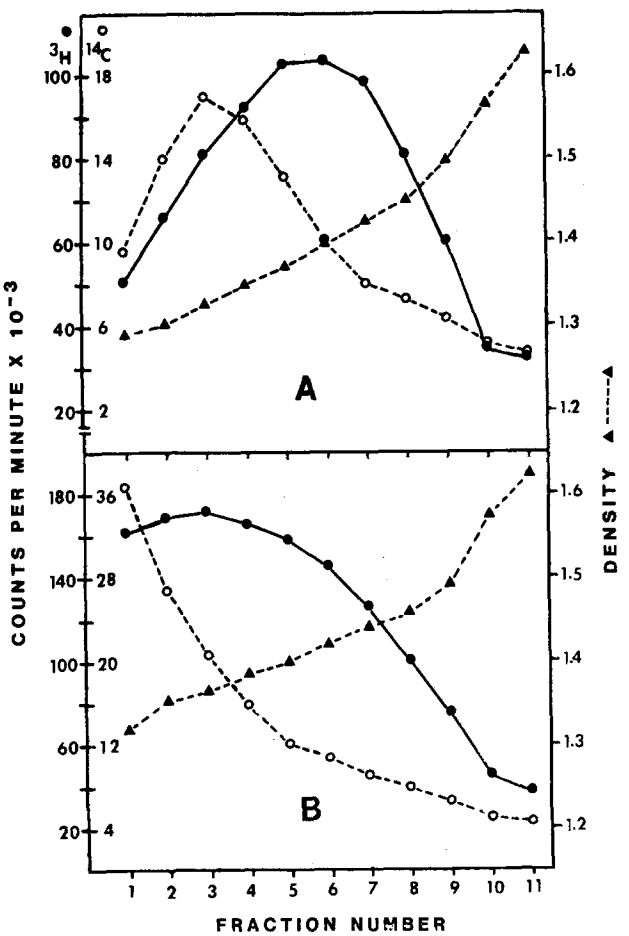

Figure 4. *A* Cesium bromide gradient ultracentrifugation of a mixture of 173 µg (3.85×10^6 cpm) of ^{3}H-labeled asialo-OSM (–●–●–) and 230 µg (6.88×10^5 cpm) of ^{14}C-labeled asialofetuin (--o--o--). The solution was prepared in phosphate-buffered saline pH 6.8, solid CsBr was added to obtain a 0.42 weight fraction and the solution (11 ml) was subjected to ultracentrifugation at 105,000 g for 72 h at 15 °C. One-milliliter fractions were removed from the top of the gradient. Densities were determined using a pycnometer and 0.3-ml aliquots were utilized for radioactivity measurements. *B* As in *A*, except that the initial buffer solution contained 4 M guanidine hydrochloride and CsBr was added to obtain a 0.37 weight fraction.

CsBr gradient ultracentrifugation profiles. As shown in figure 4 B, when the mixture of labeled asialoglycoproteins was dissolved in buffer containing 4 M guanidine hydrochloride and CsBr was added to a 0.37 weight fraction (to achieve the same initial density as in figure 4 A, i.e. ca 1.470), both glycoproteins were displaced toward the top of the gradient. This decrease in the buoyant density is consistent with our previous observations with labeled sialylated fetuin and OSM [3]. Similarly, Carlstedt et al.[14] reported a decrease in the buoyant density of human cervical mucin subjected to CsCl gradient ultracentrifugation in presence of 4 M guanidine hydrochloride.

In conclusion, our procedure for the in vitro radiolabeling of galactosyl and N-acetylgalactosaminyl residues with ^{14}C complements published procedures for ^{3}H labeling of these residues in glycoproteins [2, 11]. Mixtures of glycoproteins radiolabeled with ^{3}H and with ^{14}C can be subjected to a variety of analytical procedures for detailed comparative studies [3]. Because of the high specific radioactivities obtained, this approach is particularly advantageous for studies with biological materials available in small quantities. In addition to studies with macromolecular glycoconjugates described here, a similar approach can be utilized for comparative studies of oligosaccharides and of prosthetic groups isolated from glycoproteins. ^{14}C-cyanohydrin derivatives of galactosyl and N-acetylgalactosaminyl residues present in prosthetic groups obtained by reductive ß-elimination of gastric mucins, have been utilized to detect minor components in autoradiograms during two-dimensional fingerprinting on thin layer plates [15].

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