

# Occurrence of $\alpha$ -D-Galactosyl Residues in the Thyroglobulins from Several Species

## LOCALIZATION IN THE SACCHARIDE CHAINS OF THE COMPLEX CARBOHYDRATE UNITS\*

(Received for publication, March 26, 1984)

Robert G. Spiro† and Vishnu D. Bhoyroo

From the Departments of Biological Chemistry and Medicine, Harvard Medical School and the Elliott P. Joslin Research Laboratory, Boston, Massachusetts 02215

Treatment of thyroglobulins from several mammalian sources (calf, sheep, pig, dog, rat, rabbit, guinea pig, and man) with  $\alpha$ -galactosidase demonstrated a species-dependent occurrence of terminal  $\alpha$ -D-galactosyl residues which ranged from 11 mol/mol of protein (23% of total galactose) in calf to a complete absence in man. The presence of the  $\alpha$ -D-galactosyl groups resulted in a partial binding of the thyroglobulins (>70% in calf and sheep) to *Bandeiraea simplicifolia* I-agarose, and this lectin-thyroglobulin interaction could be quantitated by a solid-phase assay utilizing  $^{125}\text{I}$ -labeled *B. simplicifolia* I.

Sequential glycosidase digestions of calf thyroglobulin glycopeptides containing the complex carbohydrate unit (unit B) and characterization of oligosaccharide obtained by partial acid hydrolysis indicated that the  $\alpha$ -D-galactosyl residues are located on oligosaccharide branches with an  $\alpha$ -D-Gal-(1 $\rightarrow$ 3)- $\beta$ -D-Gal-(1 $\rightarrow$ 4)-D-GlcNAc sequence. While mild acid treatment of calf thyroglobulin glycopeptides yielded a disaccharide,  $\alpha$ -D-Gal-(1 $\rightarrow$ 3)-D-Gal, and a trisaccharide,  $\alpha$ -D-Gal-(1 $\rightarrow$ 3)- $\beta$ -D-Gal-(1 $\rightarrow$ 4)-D-GlcNAc, which could be resolved by *B. simplicifolia* I-agarose or thin-layer chromatography, similar hydrolysis of the human unit B-containing glycopeptides did not produce such components.

A study of various glycopeptides indicated that the  $\alpha$ -D-galactosyl residues are unevenly distributed among the multiple complex carbohydrate units of calf thyroglobulin and are preferentially located in units with a relatively low sialic acid content. During affinity chromatography on *B. simplicifolia* I-agarose, glycopeptides with multiple  $\alpha$ -D-galactosyl groups bound more firmly to the lectin than those which contained only a single residue.

In contrast to the  $\alpha$ -D-galactosyl residues,  $\beta$ -linked galactose of calf thyroglobulin was primarily bound in penultimate locations being susceptible to enzymatic release only after prior removal of capping sialyl and  $\alpha$ -D-galactosyl groups. The isolation of *N*-acetylglucosamine and a  $\beta$ -D-Gal $\rightarrow$  $\beta$ -D-GlcNAc $\rightarrow$ D-Man trisaccharide from partial acid hydrolysates helped to position the  $\beta$ -D-galactosyl residues in the oligosaccharide branches of the complex carbohydrate units.

Although thyroglobulin was the first glycoprotein in which the presence of two distinct types of asparagine-linked carbohydrate units, namely the polymannose (unit A) and complex (unit B), was recognized (1, 2), our understanding of the structure of the multiple saccharide chains of this protein is still incomplete. The present investigation, which focuses on the linkage and location of the galactose residues, was undertaken to explain the partial  $\beta$ -galactosidase resistance of desialylated glycopeptides from calf thyroglobulin as well as earlier findings (1, 2) which indicated that the complex carbohydrate units of this protein contain a greater amount of galactose than would be expected if only a single galactose residue occurred in each of the oligosaccharide chains attached to the Man<sub>3</sub>GlcNAc<sub>2</sub> core.

The findings reported in this paper indicate that about 20% of the galactose residues in calf thyroglobulin occur in  $\alpha$ -linked terminal positions and are constituents of an  $\alpha$ -D-Gal-(1 $\rightarrow$ 3)- $\beta$ -D-Gal-(1 $\rightarrow$ 4)-D-GlcNAc<sup>1</sup> sequence. We furthermore observe that the  $\alpha$ -D-galactosyl groups are responsible for interaction of the thyroglobulin with *Bandeiraea simplicifolia* lectin.<sup>2</sup> The content of terminal  $\alpha$ -D-galactosyl groups and the extent of binding to *B. simplicifolia* I differed among the thyroglobulins from several mammalian species, being greatest in the protein from calf and completely absent in that from man.

### EXPERIMENTAL PROCEDURES

**Preparation of Thyroglobulins**—Thyroids from calves, sheep, and pigs were obtained from a local slaughterhouse, while those from guinea pigs, dogs, and rabbits were shipped in the frozen state from Pel-Freez Biologicals. Rat thyroids were dissected in the laboratory from freshly killed animals of the CD strain (Charles River Breeding Laboratories, Inc.), while human thyroids from individuals without known thyroid disease were obtained at autopsy from the Brigham and Women's Hospital. All glands were stored frozen at  $-20^{\circ}\text{C}$  until use. Rat and guinea pig thyroglobulins were prepared by Bio-Gel A-5m filtration (3), while the thyroglobulins from the other species were fractionated with potassium phosphate buffers as previously described (4).

**Preparation of Unit B Glycopeptides**—Glycopeptides from calf and human thyroglobulin containing the complex carbohydrate units (unit B) were obtained by procedures similar to those previously employed (2), including Pronase digestion, filtration on Sephadex G-25, and chromatography on Dowex 50-X2 at pH 3.0. In the case of the calf thyroglobulin digest, the material unadsorbed to the Dowex 50 was fractionated by DE52 cellulose chromatography modified to permit the use of a volatile buffer to yield several unit B glycopeptides. This separation was carried out on a column (2.2  $\times$  78 cm) equili-

\* This work was supported by Grant AM 17325 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed at, the Elliott P. Joslin Research Laboratory, One Joslin Place, Boston, MA 02215.

<sup>1</sup> All glycosides are presumed to be in the pyranose form. For reduced galactose and *N*-acetylglucosamine, the abbreviations GalH<sub>2</sub> and GlcNAcH<sub>2</sub> are used, respectively.

<sup>2</sup> This lectin is also known as *Griffonia simplicifolia* I.

brated with 2 mM pyridine acetate at pH 5.5; after a wash with this buffer, the bound glycopeptides were eluted with 2200  $\mu$ l of a linear concentration gradient ranging from 20 to 200 mM pyridine acetate at this pH. During this chromatographic procedure, a unit A oligosaccharide emerged with the low-ionic buffer wash (2), while the glycopeptides containing the unit B were eluted with the gradient as seven additional peaks at the following buffer concentrations: peak 2, 34 mM; peak 3, 47 mM; peak 4, 71 mM; peak 5, 96 mM; peak 6, 117 mM; peak 7, 128 mM; and peak 8, 140 mM.

**Radiolabeling of Thyroglobulins and Glycopeptides**—A  $^{14}$ C radiolabel was introduced into the glycoproteins and glycopeptides by reductive alkylation (5). To the material to be labeled (200 nmol of free amino groups) dissolved in 100  $\mu$ l of 0.2 M sodium borate buffer, pH 8.0, was added a 3-fold molar excess of [ $^{14}$ C]formaldehyde (55 mCi/mmol; New England Nuclear) followed by sodium cyanoborohydride (Alfa Inorganics) in a 1.8 molar ratio to the formaldehyde. After 3 h at room temperature, the thyroglobulins were dialyzed against sodium phosphate buffer, pH 7.0, at 2  $^{\circ}$ C, while the glycopeptides were passed through columns of Bio-Gel P-2 equilibrated with 0.1 M pyridine acetate, pH 5.0. The average specific activities of the alkylated thyroglobulins and glycopeptides were  $1.3 \times 10^7$  dpm/mg and  $1.1 \times 10^8$  dpm/ $\mu$ mol, respectively.

**Preparation of Galactose-containing Oligosaccharides by Partial Acid Hydrolysis**—The unit B glycopeptide fractions from calf and human thyroglobulin (3  $\mu$ mol of hexose/ml) were hydrolyzed in 0.1 N sulfuric acid for 1 h at 100  $^{\circ}$ C in sealed tubes. The hydrolysates were passed through coupled columns of Dowex 50-X2 (200–400 mesh, H $^{+}$  form), and Dowex 1-X2 (200–400 mesh, formate form), and the combined effluent and water wash was then placed on charcoal-Celite (Darco G-60-Celite 535, 1:1 by weight) columns (10  $\mu$ mol of hexose/ml-column volume). After application of the sample, the column was washed with water (10 ml/ml-column volume) and then eluted with 15% ethanol (15 ml/ml-column volume) to yield an oligosaccharide fraction. Individual components from this mixture were subsequently chromatographically resolved on a preparative scale either directly on paper or after [ $^3$ H]NaBH $_4$  reduction on cellulose-coated thin-layer plates. Standard  $\alpha$ -D-Gal-(1 $\rightarrow$ 3)-D-Gal and  $\alpha$ -D-Gal-(1 $\rightarrow$ 4)-D-Gal were gifts of Dr. W. W. Watkins, Medical Research Council Clinical Research Center, Harrow, England (6), while standard N-acetylglucosamine was isolated from fetuin (7).

**Reduction of Oligosaccharides with [ $^3$ H]NaBH $_4$** —Reduction was accomplished by reacting 10  $\mu$ mol of [ $^3$ H]NaBH $_4$  (nominally 341  $\mu$ Ci/ $\mu$ mol; New England Nuclear) with oligosaccharide (50–300 nmol) in a volume of 200  $\mu$ l of 0.2 M sodium phosphate, pH 7.6, for 3 h at room temperature. The reaction was terminated by adjustment of the pH to 5.0 with acetic acid; the samples were then passed through coupled columns of Dowex 50 (H $^{+}$  form) and Dowex 1 (acetate form), and the boric acid was volatilized as methyl borate in an Evapomix (Buchler Instruments). Lactose was reduced under the same conditions to permit quantitation of the reduced oligosaccharides after their chromatographic purification.

**Periodate Oxidation**—Oxidation of oligosaccharides (10–25 nmol) was carried out in 100  $\mu$ l of 0.01 M sodium metaperiodate in 0.04 M sodium acetate buffer, pH 4.5, at 4  $^{\circ}$ C for 16 h. The reaction was terminated by the addition of a 2-fold molar excess of ethylene glycol, and after 1 h at room temperature, the samples were brought to pH 7.6 by the addition of sodium phosphate buffer and reduced with [ $^3$ H]NaBH $_4$  as already described. The desalted reduced periodate-oxidized products were then hydrolyzed with 1 N HCl at 100  $^{\circ}$ C for 5 h under nitrogen and passed through a Dowex 1-X8 (acetate form) column to obtain an effluent plus water wash which was examined by thin-layer chromatography. Periodate oxidation of [ $^3$ H]NaBH $_4$ -reduced oligosaccharides was carried out in a similar manner except that unlabeled NaBH $_4$  was employed after the oxidation step.

**Digestion with Glycosidases**—Glycosidase digestion of thyroglobulins (2 mg), glycopeptides (150 nmol), as well as [ $^3$ H]NaBH $_4$ -labeled oligosaccharides (2–20 nmol) was carried out in 200  $\mu$ l of buffer at 37  $^{\circ}$ C in the presence of toluene for 24 h unless otherwise specified. Treatment of glycopeptides and oligosaccharides with coffee bean  $\alpha$ -galactosidase was performed with 0.08 unit in 0.15 M sodium acetate buffer, pH 5.0, while thyroglobulins were treated with 0.2 unit of this enzyme in 0.1 M sodium phosphate buffer, pH 6.5, containing 0.3 mM phenylmethylsulfonyl fluoride. Incubations of oligosaccharides and glycopeptides with 0.12 unit of jack bean  $\beta$ -galactosidase (Sigma, type VII), 0.1 unit of *Aspergillus niger*  $\beta$ -galactosidase (Sigma, type V), 0.7 unit of jack bean  $\beta$ -N-acetylglucosaminidase (Sigma), and 0.08 unit of *Clostridium perfringens* neuraminidase (Sigma, type VIII) were all

carried out in the 0.15 M sodium acetate buffer, pH 5.0. Thyroglobulin digestions were terminated by the addition of ice-cold trichloroacetic acid (final concentration 8% (w/v)), while those of glycopeptides and oligosaccharides were heated at 100  $^{\circ}$ C for 3 min. After desalting by passage through coupled columns of Dowex 50-X2 (200–400 mesh, H $^{+}$  form) and Dowex 1-X2 (200–400 mesh, formate form), the samples were analyzed for released sugars by borate complex anion-exchange chromatography or examined by thin-layer chromatography. When required, enzyme-treated glycopeptides were recovered for further study by filtration on columns of Bio-Gel P-2 equilibrated with 0.1 M pyridine acetate buffer, pH 5.0.

**Lectin Affinity Chromatography**—A column (0.7  $\times$  13 cm) of *B. simplicifolia* I-agarose (Vector Laboratories) equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl, 0.1 mM CaCl $_2$ , and 0.02% sodium azide was employed for chromatography at 4  $^{\circ}$ C of thyroglobulins as well as glycopeptides and oligosaccharides. The samples were loaded onto the column in a 300- $\mu$ l volume and permitted to interact with the immobilized lectin for 45 min prior to the addition of a buffer wash. A flow rate of 5 ml/h was maintained, and after an extensive wash with the buffer, the adsorbed components were eluted with 10 mM methyl- $\alpha$ -D-galactoside; fractions of 0.6 ml (glycopeptides and oligosaccharides) or 2.1 ml (thyroglobulins) were collected and analyzed for radioactivity. Oligosaccharides which emerged from the column with the buffer wash were desalted for further study by passage through coupled columns of Dowex 50 (H $^{+}$  form) and Dowex 1 (acetate form).

**Solid-phase Lectin Binding Assay**—The interaction of various thyroglobulins with *B. simplicifolia* I was also measured by a procedure in which the lectin (obtained from E. Y. Laboratories), after labeling with Na $^{125}$ I (New England Nuclear) in the presence of 1 mM methyl- $\alpha$ -D-galactoside by the chloramine-T procedure (8), was added to microtiter plates which were coated with the glycoprotein to be assayed. In this assay, 100  $\mu$ l of the glycoprotein (5–300 ng) in 0.05 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl were added to flat-bottom microtiter wells (Immulon II Removawells, Dynatech) and kept at 4  $^{\circ}$ C overnight. The wells were then washed four times with 150  $\mu$ l of the phosphate-buffered saline containing 0.05% Tween 20 and 0.1% bovine serum albumin, and this was followed by the addition of approximately 30 ng of  $^{125}$ I-labeled *B. simplicifolia* I ( $1.4 \times 10^4$  cpm/ng) in 100  $\mu$ l of phosphate-buffered saline containing 0.05% Tween 20, 0.1% serum albumin, and 0.1 mM CaCl $_2$ . After an overnight incubation at 4  $^{\circ}$ C, the wells were again washed with six changes of the phosphate-buffered Tween/albumin solution and assayed for radioactivity in a  $\gamma$ -counter. The procedure was standardized with mouse laminin (Bethesda Research Laboratories) which is known to have terminal  $\alpha$ -D-galactosyl residues (9).

**Chromatographic Procedures**—Chromatography of saccharides was carried out on paper (washed; Whatman No. 1) or on plastic sheets precoated with cellulose (0.1-mm thickness; Merck) in pyridine/ethyl acetate/water/acetic acid (5:5:3:1). Preparative separation of unreduced oligosaccharides was achieved by descending paper chromatography (22 h); the components were eluted with water after they had been located on guide strips with the silver nitrate staining procedure (10). The resolution of [ $^3$ H]NaBH $_4$ -reduced monosaccharides and oligosaccharides was accomplished on thin-layer plates which had a wick of Whatman No. 3 paper clamped to their top; components were located by fluorography. For preparative separation of reduced oligosaccharides, the thin-layer chromatography was performed for 14 h and the radioactive spots were eluted with water. To remove scintillants, the eluted components were extracted with peroxide-free ether and passed through small coupled columns of Dowex 50 (H $^{+}$  form) and Dowex 1 (acetate form).

**Chemical Analyses**—Analyses of neutral sugars released by glycosidase or acid hydrolyses were carried out by borate complex anion-exchange chromatography (11). The various procedures employed for sugar determinations have previously been described (2, 12).

**Radioactive Measurements**—Liquid scintillation counting was performed in Ultrafluor (National Diagnostics) or Bray's reagent (13) with a Beckman LS 7500 instrument, while a Model 1290 Gamma Trac was used for  $\gamma$ -counting.

Radioactive components on thin-layer plates were located by fluorography at  $-70$   $^{\circ}$ C after spraying with ENHANCE (New England Nuclear) using X-Omat AR films (Eastman Kodak).

## RESULTS

**Action of  $\alpha$ -Galactosidase on Thyroglobulins from Several Species**—During a 24-h period of digestion, coffee bean  $\alpha$ -

galactosidase released 23% of the total galactose from calf thyroglobulin (Table I). The galactose liberated by this enzyme which represents 11 mol/mol of thyroglobulin ( $M_r = 670,000$ ) could not be increased by longer digestions with a second addition of enzyme. The content of terminal  $\alpha$ -D-galactosyl residues in thyroglobulin was species dependent (Table I) with the highest values being observed in the calf, sheep, dog, and pig proteins; the human thyroglobulin was notable by the complete absence of  $\alpha$ -galactose-releasable saccharide residues.

**Interaction of Thyroglobulins with *B. simplicifolia* I Lectin—**The various thyroglobulins also differed in the extent to which they were bound to *B. simplicifolia* I-agarose during affinity chromatography (Fig. 1). A large portion (>70%) of calf and sheep thyroglobulin was retained by the immobilized lectin, while the human protein was completely recovered in the void volume of the *B. simplicifolia* I-agarose column. In all cases, the bound thyroglobulin could be eluted at a concentration of 10 mM methyl- $\alpha$ -D-galactoside, although it was not displaced

by a similar concentration of the methyl- $\alpha$ -D-glucoside. While in some of the thyroglobulins (i.e. calf, sheep, man, rabbit, and guinea pig) there was a correlation between the content of terminal  $\alpha$ -D-galactosyl groups and the extent of interaction with *B. simplicifolia* I-agarose, this was not a uniform finding as the dog, pig, and rat proteins bound to the lectin to a lesser degree than might have been anticipated (Table I). Upon rechromatography of the unbound thyroglobulins, they again failed to be retained on *B. simplicifolia* I-agarose, thereby indicating that the capacity of the columns was not limiting.

The interaction of thyroglobulin with *B. simplicifolia* I was also evaluated by a solid-phase binding assay in which the  $^{125}$ I-labeled lectin was added to glycoprotein-coated microtiter wells (Fig. 2). Native calf thyroglobulin was found to bind the *B. simplicifolia* I lectin in a concentration-dependent fashion (50–300 ng of thyroglobulin); this interaction was completely abolished when the  $\alpha$ -galactosidase-treated protein was tested (Fig. 2), and furthermore, the bound  $^{125}$ I-labeled *B. simplicifolia* I could be displaced (91% reduction of radioactivity) by a wash with 10 mM methyl- $\alpha$ -D-galactoside. As anticipated on the basis of terminal  $\alpha$ -D-galactosyl content, pig thyroglobulin interacted with *B. simplicifolia* I to a lesser extent than calf, while the human protein manifested no detectable binding (Fig. 2). Laminin bound about three times as much *B. simplicifolia* I as the calf thyroglobulin, consistent with its previously reported (9) high content of terminal  $\alpha$ -D-galactosyl residues (66 nmol/mg).

**Presence of  $\alpha$ -D-Galactosyl Residues in Unit B Glycopeptides of Calf Thyroglobulin—**Since calf thyroglobulin was found to have the highest content of  $\alpha$ -D-galactosyl residues, this protein was chosen for studies designed to localize these saccharide groups. The investigations were facilitated by previous studies (1, 2) which indicated that all of the galactose of the

TABLE I

Terminal  $\alpha$ -D-galactosyl residue content and affinity for *B. simplicifolia* I-agarose of thyroglobulins from several species

| Species    | Total galactose <sup>a</sup> | Galactose released by $\alpha$ -galactosidase <sup>b</sup> |                | Bound to BSI <sup>c</sup> |
|------------|------------------------------|------------------------------------------------------------|----------------|---------------------------|
|            | nmol/mg <sup>d</sup>         | nmol/mg <sup>d</sup>                                       | % <sup>e</sup> | %                         |
| Calf       | 73                           | 17                                                         | 23             | 71                        |
| Sheep      | 68                           | 11                                                         | 16             | 73                        |
| Pig        | 67                           | 8.5                                                        | 13             | 14                        |
| Man        | 73                           | 0                                                          | 0              | 0                         |
| Dog        | 56                           | 9.4                                                        | 17             | 38                        |
| Rabbit     | 71                           | 0.5                                                        | 1              | 2                         |
| Rat        | 50                           | 4.8                                                        | 10             | 5                         |
| Guinea pig | 79                           | 3.3                                                        | 4              | 9                         |

<sup>a</sup> Determined after acid hydrolysis as described under "Experimental Procedures."

<sup>b</sup> Conditions for  $\alpha$ -galactosidase digestion are described under "Experimental Procedures."

<sup>c</sup> Values represent the percentage of thyroglobulin which bound to *B. simplicifolia* I (BSI)-agarose and was eluted with methyl- $\alpha$ -D-galactoside under the conditions described under "Experimental Procedures."

<sup>d</sup> Values are expressed per mg of thyroglobulin.

<sup>e</sup> Percentage of total galactose release by the enzyme.

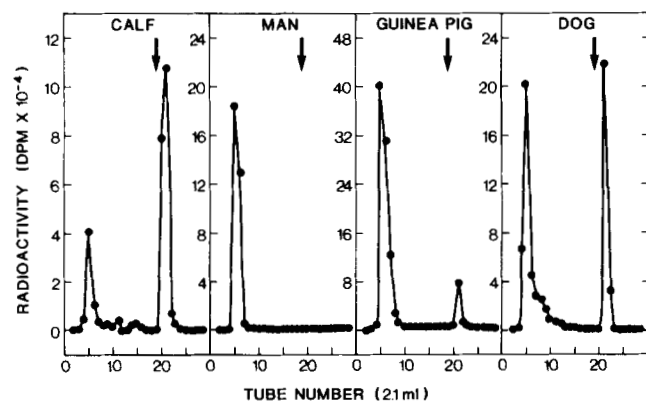


FIG. 1. Affinity chromatography on *B. simplicifolia* I-agarose of  $^{14}$ C-labeled thyroglobulins from several species. The proteins (25–75  $\mu$ g) were placed on the column ( $0.7 \times 13$  cm) and chromatographed in 0.1 M sodium phosphate, pH 7.0, containing 0.15 M NaCl and 0.1 mM  $\text{CaCl}_2$  as described under "Experimental Procedures." After washing with this buffer at a flow rate of 5 ml/h, the column was eluted with 10 mM methyl- $\alpha$ -D-galactoside in buffer as indicated by the arrows. Fractions of 2.1 ml were collected, and aliquots were taken for the measurement of radioactivity.

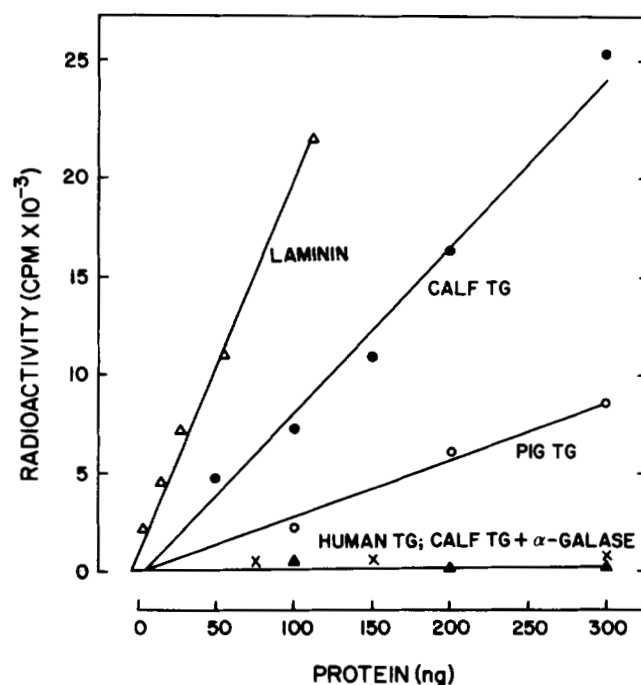


FIG. 2. Determination of *B. simplicifolia* I-glycoprotein interaction by solid-phase lectin binding radioassay. The binding of  $^{125}$ I-labeled *B. simplicifolia* I to microwells coated with increasing amounts of the designated protein was determined as described under "Experimental Procedures." The proteins assayed were thyroglobulins (TG) from calf (●), pig (○), and human (▲) thyroids as well as mouse laminin (Δ) and  $\alpha$ -galactosidase-treated calf thyroglobulin (×).

calf thyroglobulin molecule is present in the carbohydrate unit B.

Treatment of the entire unit B-containing glycopeptide fraction with coffee bean  $\alpha$ -galactosidase resulted in the release of 24% of the galactose, a value in close accord with that observed with the native glycoprotein (Table I). When the DEAE-cellulose-fractionated unit B glycopeptides were examined for their  $\alpha$ -D-galactosyl content, a pronounced microheterogeneity in regard to these terminally positioned sugar residues became evident (Table II). The  $\alpha$ -D-galactosyl groups were more abundant in the glycopeptides which emerged early from the column (glycopeptides 2-4) and which contained relatively few sialic acid residues. In general, the sum of  $\alpha$ -D-galactosyl and sialyl groups in each glycopeptide fraction was similar to its  $\alpha$ -galactosidase-resistant galactose residues; the latter which ranged from about 2 to 4 in number are believed to be in  $\beta$ -linkage (see below) and provide an index of the number of oligosaccharide branches/carbohydrate unit.

Chromatography of the unit B glycopeptide fraction on *B. simplicifolia* I-agarose revealed a pattern (Fig. 3) quite distinct from that obtained with the intact calf thyroglobulin. While 47% of the glycopeptides were retained on the column, most of these were not tightly bound and emerged with the buffer wash; only a relatively small proportion of the glycopeptides required the methyl- $\alpha$ -D-galactoside for displacement from *B. simplicifolia* I-agarose (Fig. 3). When individual glycopeptide fractions were chromatographed on the lectin column, not unexpectedly glycopeptide 2, which contains 41% of its galactosyl residues in  $\alpha$ -linked terminal positions, demonstrated the most extensive binding (Fig. 4). Over 90% of the radioactivity in this fraction was retained by the column, and of that, about 30% was bound tightly enough to require the methyl- $\alpha$ -D-galactoside for elution.

**Localization of Galactose Residues in the Carbohydrate Units**—Two of the major unit B glycopeptide fractions (2 and 4) were taken for glycosidase digestions in order to determine the position of both the  $\alpha$ - and  $\beta$ -linked galactose residues (Table III). These studies indicated that in both glycopeptides all of the  $\alpha$ -D-galactosyl residues were in nonreducing terminal positions; neither prior neuraminidase nor  $\beta$ -galactosidase treatment increased the amount of galactose release by  $\alpha$ -

TABLE II

Distribution of  $\alpha$ -D-galactosyl and sialyl residues among glycopeptides obtained from Pronase digest of calf thyroglobulin

| Glycopeptide <sup>a</sup>         | Total galactose <sup>b</sup> | Galactose released by $\alpha$ -galactosidase <sup>c</sup> | Sialic acid <sup>d</sup> |
|-----------------------------------|------------------------------|------------------------------------------------------------|--------------------------|
| mol/mol glycopeptide <sup>e</sup> |                              |                                                            |                          |
| 2                                 | 5.9 (10) <sup>f</sup>        | 2.4                                                        | 1.2                      |
| 3                                 | 3.3 (11)                     | 1.1                                                        | 1.3                      |
| 4                                 | 4.2 (38)                     | 1.1                                                        | 1.9                      |
| 5                                 | 3.4 (24)                     | 0.5                                                        | 2.9                      |
| 6                                 | 3.3 (7)                      | 0.4                                                        | 3.0                      |
| 7                                 | 3.2 (6)                      | 0.4                                                        | 2.6                      |
| 8                                 | 3.5 (4)                      | 0.4                                                        | 2.5                      |

<sup>a</sup> Glycopeptide fractions containing unit B carbohydrate units were prepared by DE52 cellulose chromatography of Pronase-digested calf thyroglobulin as described under "Experimental Procedures"; fraction 1 which contains unit A carbohydrate unit is not listed.

<sup>b</sup> Determined after acid hydrolysis of glycopeptide.

<sup>c</sup> Conditions for coffee bean  $\alpha$ -galactosidase treatment of glycopeptides are described under "Experimental Procedures."

<sup>d</sup> Fucose was evenly distributed among glycopeptides ranging from 0.90 to 1.06 mol/mol of glycopeptide.

<sup>e</sup> Expressed on the basis of 3 mannose residues/carbohydrate unit.

<sup>f</sup> Values in parentheses represent the percentage of the total galactose of calf thyroglobulin present in each glycopeptide fraction.

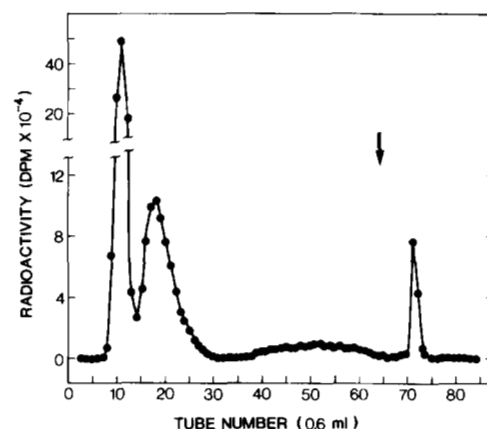


FIG. 3. Affinity chromatography on *B. simplicifolia* I-agarose of the total  $^{14}\text{C}$ -labeled unit B glycopeptides from calf thyroglobulin. The glycopeptides (25 nmol), prepared as described under "Experimental Procedures," were chromatographed under similar conditions as described in the legend to Fig. 1 except that fractions of 0.6 ml were collected. The arrow designates the beginning of elution with 10 mM methyl- $\alpha$ -D-galactoside. The recovery of radioactivity from the column was 92%.

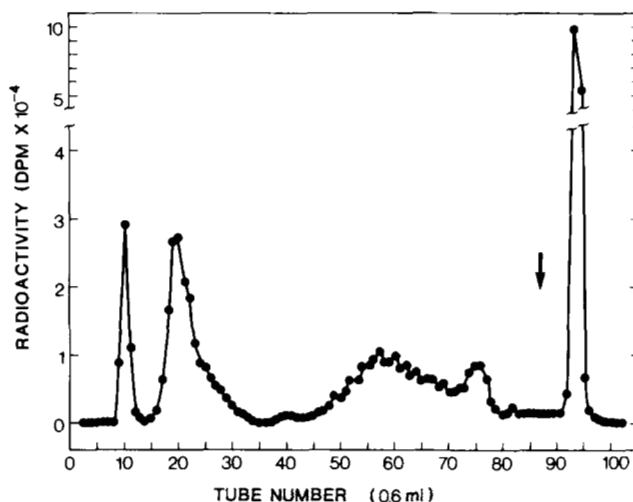


FIG. 4. Affinity chromatography on *B. simplicifolia* I-agarose of  $^{14}\text{C}$ -labeled  $\alpha$ -galactosyl-rich unit B glycopeptide fraction from calf thyroglobulin. Glycopeptide 2 (13 nmol) obtained by DEAE-cellulose chromatography of Pronase-digested thyroglobulin (Table I) was chromatographed in the same manner as described in the legend to Fig. 3. The recovery of radioactivity from the column was 90%.

galactosidase digestion. In contrast, the  $\beta$ -D-galactosyl groups were primarily situated in internal positions of the oligosaccharide chains, being substituted with either sialyl or  $\alpha$ -D-galactosyl residues (Table III). On the basis of total number of terminal sugar residues, it would appear that the carbohydrate units in glycopeptide fraction 2 are more extensively branched than those in glycopeptide 4 (Table III).

**Isolation of Galactose-containing Oligosaccharides**—Further information about the location of galactose in calf thyroglobulin was obtained by submitting unit B glycopeptides to partial acid hydrolysis. Thin-layer chromatography of the charcoal-Celite-fractionated,  $[^3\text{H}]\text{NaBH}_4$ -reduced released saccharides revealed, in addition to *N*-acetylglucosaminitol, four oligosaccharide components which were designated A-D (Fig. 5). After a similar hydrolytic treatment of glycopeptides from human thyroglobulin, only oligosaccharides A and D were observed upon chromatography (Fig. 5), and this differ-

TABLE III

Location of galactose residues in glycopeptides from calf thyroglobulin

| Glyco-peptide <sup>a</sup> | Location of galactose               |                                    |                                                       |                                               | Total chain termini <sup>e</sup> |
|----------------------------|-------------------------------------|------------------------------------|-------------------------------------------------------|-----------------------------------------------|----------------------------------|
|                            | Terminal $\alpha$ -Gal <sup>b</sup> | Terminal $\beta$ -Gal <sup>b</sup> | $\alpha$ -Gal $\rightarrow$ $\beta$ -Gal <sup>c</sup> | NeuAc $\rightarrow$ $\beta$ -Gal <sup>d</sup> |                                  |
| 2                          | 2.4                                 | 0.4                                | 2.5                                                   | 1.0                                           | 4.0                              |
| 4                          | 1.1                                 | 0.2                                | 1.0                                                   | 1.9                                           | 3.2                              |

<sup>a</sup> Glycopeptide fractions containing unit B carbohydrate units were prepared by DE52 cellulose chromatography of Pronase-digested calf thyroglobulin as described under "Experimental Procedures."

<sup>b</sup> Galactose released by coffee bean  $\alpha$ -galactosidase or jack bean  $\beta$ -galactosidase digestions of unmodified glycopeptides.

<sup>c</sup> Galactose released by  $\beta$ -galactosidase digestion of  $\alpha$ -galactosidase-treated glycopeptide minus terminal  $\beta$ -linked galactose of unmodified glycopeptide. Sequential digestions indicated that no  $\beta$ -Gal $\rightarrow$  $\alpha$ -Gal occurred in either glycopeptide.

<sup>d</sup> Galactose release by  $\beta$ -galactosidase digestion of neuraminidase-treated glycopeptide minus terminal  $\beta$ -linked galactose of unmodified glycopeptide. Sequential digestions indicated that no NeuAc $\rightarrow$  $\alpha$ -Gal occurred in either glycopeptide.

<sup>e</sup> Sum of terminal  $\alpha$ -Gal, terminal  $\beta$ -Gal, and sialic acid residues. It may be noted that the values for chemically analyzed sialic acid (Table II) were similar to the enzymatically determined NeuAc $\rightarrow$  $\beta$ -Gal.

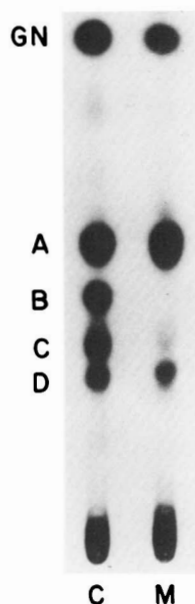


FIG. 5. Thin-layer chromatography of oligosaccharides released by partial acid hydrolysis from calf and human thyroglobulin unit B glycopeptides. The saccharides released by 0.1 N  $\text{H}_2\text{SO}_4$  (1 h, 100 °C) were fractionated on charcoal-Celite and reduced with  $[^3\text{H}]\text{NaBH}_4$  as described under "Experimental Procedures" prior to being applied to a cellulose-coated plate. Chromatography was carried out for 10 h on material originating from 0.2 mg of calf (lane C) and human (lane M) thyroglobulin under the conditions specified under "Experimental Procedures," and the components were visualized by fluorography. A, B, C, and D designate oligosaccharides which are discussed in the text. GN refers to *N*-acetylglucosaminitol; this sugar appeared in the oligosaccharide fraction because of its retention by charcoal-Celite.

ence was attributed to the absence of  $\alpha$ -D-galactosyl groups in that protein (Table I).

Preparative thin-layer chromatography of the reduced oligosaccharide from the calf hydrolysate yielded four distinct components (Fig. 6) with the following chromatographic migration relative to lactitol: A, 1.28; B, 1.08; C, 0.87; and D, 0.76. Hydrolysis (1 N HCl, 4 h, 100 °C) of these radiolabeled oligosaccharides permitted identification of their reducing terminal residue, while their total composition was deter-

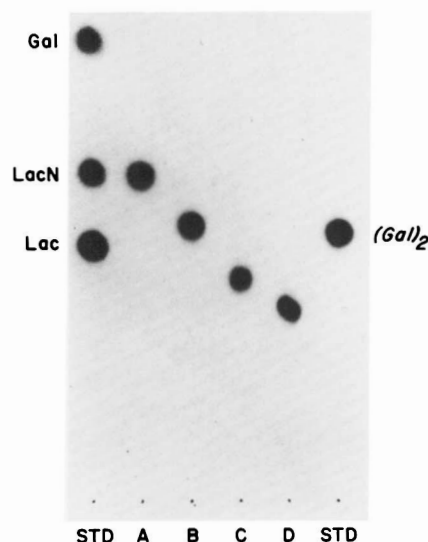


FIG. 6. Thin-layer chromatography of purified  $[^3\text{H}]\text{NaBH}_4$ -reduced oligosaccharides from calf thyroglobulin unit B glycopeptides. The isolated oligosaccharides A-D (10,000 dpm; 102 pmol) were applied to a cellulose-coated plate and chromatographed for 14 h as described under "Experimental Procedures." The components were detected by fluorography, and their migration was compared to  $[^3\text{H}]\text{NaBH}_4$ -reduced standards (STD) of lactose (Lac), *N*-acetylglucosamine (LacN), galactose (Gal), and  $\alpha$ -D-Gal-(1 $\rightarrow$ 3)-D-Gal ( $(\text{Gal})_2$ ).

mined by analysis of the unreduced forms which were isolated by preparative paper chromatography. These studies indicated that components A and B were Gal $\rightarrow$ GlcNAc and Gal $\rightarrow$ Gal disaccharides, respectively, while C and D were trisaccharides with the former containing 2 galactose residues attached to an *N*-acetylglucosamine at the reducing end and the latter consisting of galactose, *N*-acetylglucosamine, and a reducing terminal mannose. On thin-layer plates, component A co-migrated with *N*-acetylglucosaminitol, while B moved to the same position as the reduced form of  $\alpha$ -D-Gal-(1 $\rightarrow$ 3)-D-Gal (Fig. 6). The yields of the oligosaccharides expressed as nanomoles/100 mg of thyroglobulin were: A, 99; B, 62; C, 45; and D, 23. The same components were evident over hydrolysis times ranging from 20 min to 3 h, but the yields were smaller during shorter periods, while more extended treatments decreased the recovery of B and C while increasing that of A and D.

**Structure of Galactose-containing Oligosaccharides**—The sequence and anomeric configurations were explored by glycosidase digestions of the  $[^3\text{H}]\text{NaBH}_4$ -reduced oligosaccharides. Component B was shown to be an  $\alpha$ -linked galactobiitol on the basis of its ready cleavage by coffee bean  $\alpha$ -galactosidase and resistance to hydrolysis by *A. niger*  $\beta$ -galactosidase (Fig. 7). Since oligosaccharide C yielded upon  $\alpha$ -galactosidase treatment a component which migrated to the same position as component A (Fig. 7), it appeared to consist of a galactose in  $\alpha$ -linkage to *N*-acetylglucosaminitol. As anticipated from its chromatographic mobility, *A. niger*  $\beta$ -galactosidase digestion of the  $\alpha$ -galactosidase product from oligosaccharide C resulted in the release of *N*-acetylglucosaminitol, and this sugar was also liberated by such treatment of component A (Fig. 7). In contrast to the *A. niger* enzyme, the jack bean  $\beta$ -galactosidase was found to be ineffective in cleaving the galactosidic bond to a reduced *N*-acetylglucosamine.

Treatment of oligosaccharide D with jack bean  $\beta$ -galactosidase led to the appearance on thin-layer chromatography of a component which had a mobility of 1.08 relative to *N*-acetylglucosaminitol, and this newly formed product was in

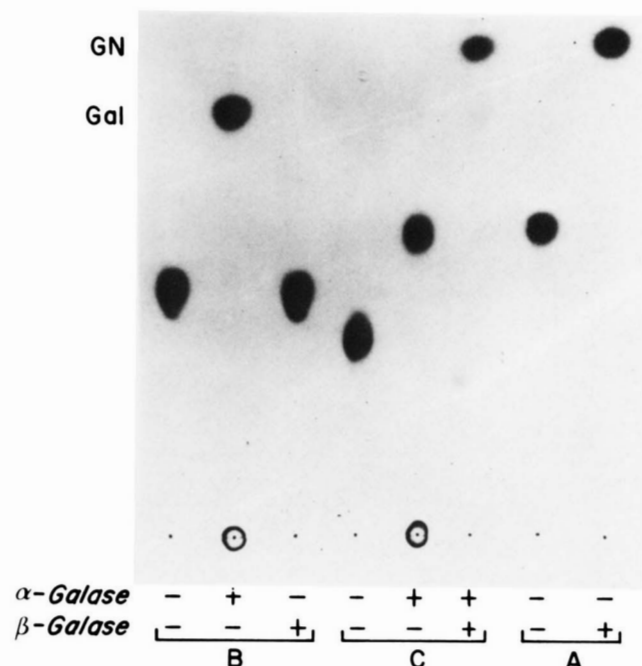


FIG. 7. Effect of  $\alpha$ - and  $\beta$ -galactosidase digestion on the thin-layer chromatographic migration of [ $^3$ H]NaBH $_4$ -reduced oligosaccharides from calf thyroglobulin. After treatment with coffee bean  $\alpha$ -galactosidase ( $\alpha$ -Galase) and/or *A. niger*  $\beta$ -galactosidase ( $\beta$ -Galase) as described under "Experimental Procedures," oligosaccharides (A-C) were chromatographed for 9 h on cellulose-coated plates alongside the undigested components; visualization was achieved by fluorography. The symbols + and - indicate nature of glycosidase treatment, while GN and Gal are the abbreviations for N-acetylglucosaminitol and galactitol, respectively.

turn slowly cleaved by jack bean  $\beta$ -N-acetylglucosaminidase to yield mannitol (Fig. 8). These interconversions suggested that oligosaccharide D had  $\beta$ -D-Gal $\rightarrow$  $\beta$ -D-GlcNAc $\rightarrow$ ManH $_2$  sequence.

Periodate oxidation studies provided information pertaining to the position of glycosidic substitution in the oligosaccharides (14). Treatment of unreduced oligosaccharide B with sodium metaperiodate followed by [ $^3$ H]NaBH $_4$  reduction and acid hydrolysis resulted in the formation of radiolabeled components which migrated to the positions of standard lyxitol and glycerol (Fig. 9, lane 3), while hydrolysis of the [ $^3$ H]NaBH $_4$ -reduced oligosaccharide without oxidation yielded only galactitol (Fig. 9, lane 2). This indicated that oligosaccharide B is an  $\alpha$ 1 $\rightarrow$ 3-linked galactobiose, and indeed this was substantiated by finding that the oxidation products of standard  $\alpha$ -D-Gal-(1 $\rightarrow$ 3)-D-Gal (Fig. 9, lane 4) and the thyroglobulin disaccharide were the same. In contrast, oxidation of standard  $\alpha$ -D-Gal-(1 $\rightarrow$ 4)-D-Gal led to the formation of glycerol and threitol (Fig. 9, lane 5). Standard  $\alpha$ 1 $\rightarrow$ 2- and  $\alpha$ 1 $\rightarrow$ 6-linked disaccharides, namely kojibiose and melibiose, which were periodate oxidized under the same conditions yielded, as expected, glycerol as the only radioactive component (not shown).

The results of periodate oxidation together with the aforementioned glycosidase studies indicated that an  $\alpha$ -D-Gal-(1 $\rightarrow$ 3)-D-Gal linkage also occurred in oligosaccharide C as part of an  $\alpha$ -D-Gal-(1 $\rightarrow$ 3)- $\beta$ -D-Gal-(1 $\rightarrow$ 4)-D-GlcNAcH $_2$  sequence. Consistent with an  $\alpha$ 1 $\rightarrow$ 3 linkage between the two galactose residues was the finding that half the galactose in the unreduced form of this trisaccharide was spared destruction during periodate oxidation. Moreover, when the [ $^3$ H]NaBH $_4$ -reduced oligosaccharide was submitted to a Smith periodate degrada-

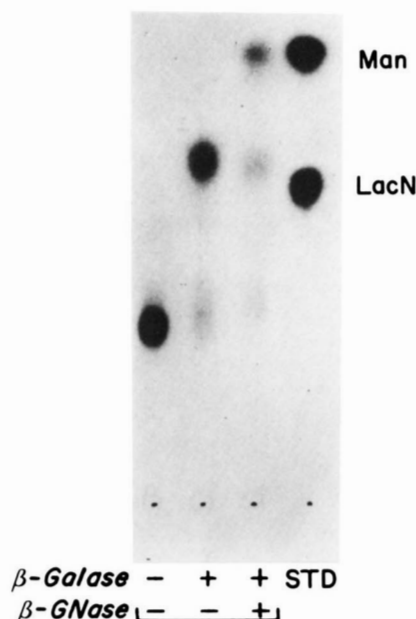


FIG. 8. Effect of glycosidase digestions on [ $^3$ H]NaBH $_4$ -reduced oligosaccharide D from calf thyroglobulin as evaluated by thin-layer chromatography. The oligosaccharide, untreated or after digestion with the jack bean enzymes,  $\beta$ -galactosidase ( $\beta$ -Galase) and  $\beta$ -N-acetylglucosaminidase ( $\beta$ -GNase), was chromatographed for 10 h on a cellulose-coated plate as described under "Experimental Procedures"; the symbols + and - specify the nature of treatment. The components were detected by fluorography, and their migration was compared to [ $^3$ H]NaBH $_4$ -reduced standards (STD) of mannose (Man) and N-acetylglucosamine (LacN).

tion (NaBH $_4$  reduction of oxidation products followed by hydrolysis with 0.1 N H $_2$ SO $_4$  at 80  $^{\circ}$ C for 1 h), a radiolabeled component was observed on thin-layer chromatography with a mobility of 1.21 relative to N-acetylglucosaminitol, which upon complete acid hydrolysis (1 N HCl, 5 h, 100  $^{\circ}$ C) yielded xylosaminitol (not shown). This pentosaminitol would be the expected radiolabeled product if the reduced terminal N-acetylglucosamine was substituted at C-4 (15), and indeed periodate oxidation of [ $^3$ H]NaBH $_4$ -reduced oligosaccharide A and standard N-acetylglucosamine also gave rise to xylosaminitol after hydrolysis with acid (not shown). The identification was made by thin-layer chromatography in the pyridine/ethyl acetate/water/acetic acid (5:5:3:1) solvent system in which the xylosaminitol migrated with a mobility of 1.15 relative to glucosaminitol; standard threosaminitol, prepared from N-acetylchondrosinitol (16), which would be the expected oxidation product from a C-3 substituted terminal hexosaminitol (15), was found to have a migration of 1.34 relative to glucosaminitol in this system.

**Interaction of Oligosaccharides with *B. simplicifolia* I Lectin**—Chromatography on *B. simplicifolia* I-agarose of the [ $^3$ H]NaBH $_4$ -reduced saccharide fraction obtained by partial acid hydrolysis of calf thyroglobulin unit B glycopeptides led to the resolution of two peaks of radioactivity (peaks 2 and 3) which were substantially retarded from the material which emerged in the void volume of the column (Fig. 10). Only a very small amount of saccharide was bound to the immobilized lectin tightly enough to require displacement with the methyl- $\alpha$ -D-galactoside (Fig. 10). Thin-layer chromatographic examination of the materials in the two retarded column fractions (peaks 2 and 3) indicated that the former contained trisaccharide C, while disaccharide B was present in the latter peak (Fig. 11). It is evident that the two  $\alpha$ -D-galactosyl-containing components of the saccharide fraction were retained by the

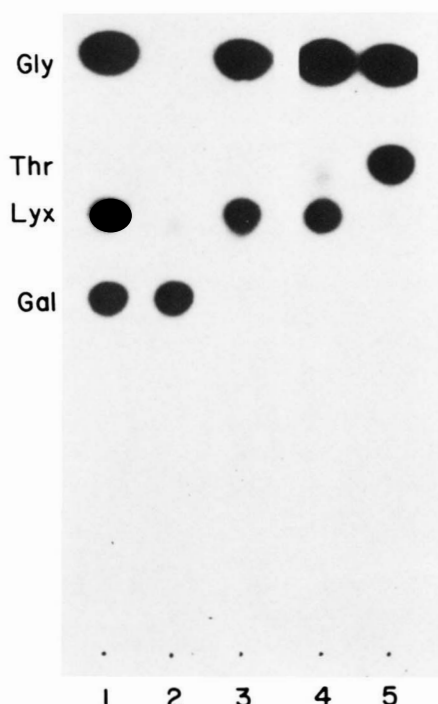


FIG. 9. Effect of periodate oxidation on calf thyroglobulin oligosaccharide B as evaluated by thin-layer chromatography of the  $[^3\text{H}]\text{NaBH}_4$ -reduced product after hydrolysis with acid. The unreduced form of oligosaccharide B was treated with  $[^3\text{H}]\text{NaBH}_4$  and acid hydrolyzed with (lane 3) or without (lane 2) a previous periodate oxidation as described under "Experimental Procedures." Chromatography of the products of the reactions was carried out on a cellulose-coated plate for 6 h, and the components were detected by fluorography. For comparison, standard  $\alpha$ -D-Gal-(1 $\rightarrow$ 3)-D-Gal (lane 4) and  $\alpha$ -D-Gal-(1 $\rightarrow$ 4)-D-Gal (lane 5) were oxidized with periodate and applied to the thin-layer plate after  $[^3\text{H}]\text{NaBH}_4$  reduction and acid hydrolysis. Lane 1 contains  $[^3\text{H}]\text{NaBH}_4$ -reduced galactose and lyxose as well as  $^{14}\text{C}$ -labeled glycerol. Gly, glycerol; Thr, threitol; Lyx, lyxitol; Gal, galactitol.

lectin column, and indeed chromatography of the material in the void volume peak (peak 1) demonstrated their absence from this fraction (not shown). When the human oligosaccharide fraction or the  $\alpha$ -galactosidase-treated calf oligosaccharides were chromatographed on *B. simplicifolia* I-agarose, all of the radioactivity emerged in the void volume of the column (not shown).

#### DISCUSSION

The present study indicates that the thyroglobulins from several mammalian species contain terminal  $\alpha$ -D-galactosyl residues in their complex type (unit B) carbohydrate units. The occurrence of  $\alpha$ -linked galactose end groups was first observed in glycoconjugates with blood group B activity (17) and was subsequently noted in a number of defined glycoproteins including several cell-surface components (18–20) as well as mouse laminin (9); annelid cuticle collagens moreover are known to contain short *O*-glycosidically linked sugar chains made up entirely of  $\alpha$ -D-galactosyl residues (21, 22).

In our study of thyroglobulins, a remarkable species influence on the content of  $\alpha$ -D-galactosyl groups was observed, which ranged from 11 mol/mol of protein in calf to a complete absence in man. Such an effect of species may not be unique to thyroglobulin as Peters and Goldstein (23) found that the fluorescein-conjugated *B. simplicifolia* I labeling of basement-membrane  $\alpha$ -D-galactosyl residues which they observed in various rodent tissues did not occur in sections from human

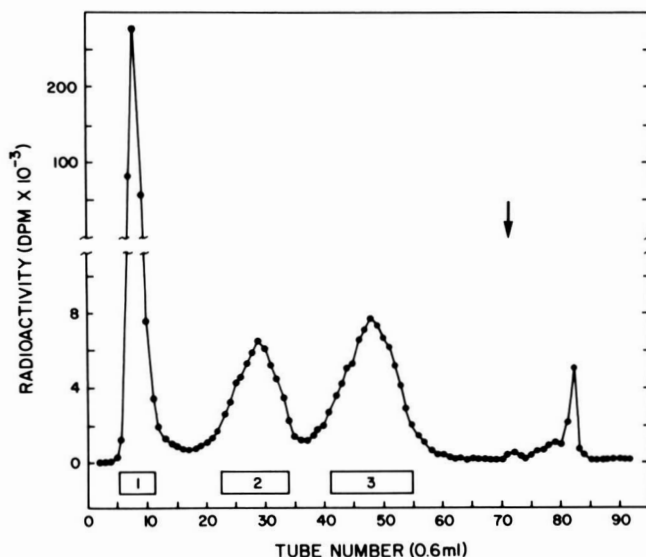


FIG. 10. Resolution by affinity chromatography on *B. simplicifolia* I-agarose of  $\alpha$ -D-galactosyl-containing oligosaccharides released by partial acid hydrolysis of calf thyroglobulin unit B glycopeptides. The column (0.7  $\times$  13 cm) was loaded with the charcoal-Celite-fractionated,  $[^3\text{H}]\text{NaBH}_4$ -reduced saccharides obtained by partial acid hydrolysis (0.1 N  $\text{H}_2\text{SO}_4$ , 1 h, 100  $^\circ\text{C}$ ) of glycopeptides originating from 1.05 mg of thyroglobulin (see "Experimental Procedures"). Chromatography was carried out under similar conditions as described in the legend to Fig. 3; the arrow designates the beginning of elution with 10 mM methyl- $\alpha$ -D-galactoside. Aliquots of the collected fractions were taken for the measurement of radioactivity, and numbered areas designate tubes which were pooled for further study. Recovery of radioactivity from the column was 93%.

tissue, and more recently, work from our laboratory (24) indicated that the thyrotropin receptor from calf, but not human thyroids, bound to a column of immobilized *B. simplicifolia* I lectin.

The structural studies on unit B glycopeptides from calf thyroglobulin which we report indicate that the  $\alpha$ -D-galactosyl groups are situated in nonreducing terminal positions and are linked to C-3 of  $\beta$ -D-galactosyl residues which are components of the well-known  $\beta$ -D-Gal $\rightarrow$  $\beta$ -D-GlcNAc $\rightarrow$ Man branches of complex carbohydrate units (25). The localization of  $\alpha$ -D-galactose was achieved by sequential glycosidase digestions as well as the isolation from partial acid hydrolysates of the disaccharide  $\alpha$ -D-Gal-(1 $\rightarrow$ 3)-D-Gal and the trisaccharide  $\alpha$ -D-Gal-(1 $\rightarrow$ 3)- $\beta$ -D-Gal-(1 $\rightarrow$ 4)-GlcNAc. In contrast to the  $\alpha$ -D-galactosyl groups which together with the sialic acid residues occupy capping positions in the unit B oligosaccharide branches, the  $\beta$ -D-galactosyl residues were primarily found in penultimate locations being only susceptible to enzymatic release after prior removal of the two types of terminal sugar components. The isolation of the *N*-acetylglucosamine as well as of a trisaccharide apparently not previously obtained with a  $\beta$ -D-Gal $\rightarrow$  $\beta$ -D-GlcNAc $\rightarrow$ Man sequence helped to position the  $\beta$ -D-galactosyl groups in the saccharide chains.

The occurrence of about 20% of the calf thyroglobulin galactose in the form of  $\alpha$ -linked residues explains previous findings from this laboratory (1, 2) which indicated that the complex carbohydrate units of this protein contain a greater amount of galactose than is consistent with its occurrence in only one position in each of the unit B branches. This extra content of galactose was not observed in the carbohydrate units B from human thyroglobulin (2), and consistent with this, the human glycopeptides upon partial acid hydrolysis failed to yield the  $\alpha$ -D-galactosyl-containing di- and trisaccharides.

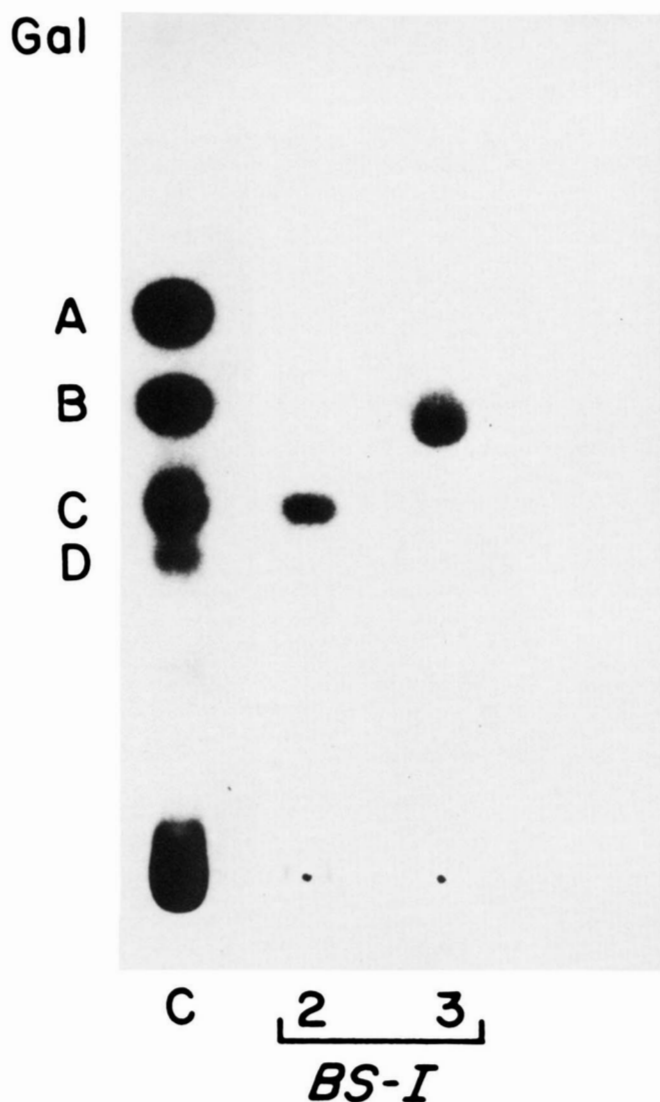


FIG. 11. Migration on thin-layer chromatography of calf thyroglobulin [ $^3\text{H}$ ]NaBH $_4$ -reduced oligosaccharides resolved by *B. simplicifolia* I-agarose chromatography in relation to the components in the initial saccharide fraction. Material from the *B. simplicifolia* I column (Fig. 10) peak 2 (8,400 dpm) and peak 3 (12,100 dpm) as well as the initial saccharide fraction (C) originating from 0.15 mg of calf thyroglobulin was applied to a cellulose-coated plate and chromatographed for 13 h as described under "Experimental Procedures." A-D designate oligosaccharides, while Gal refers to galactitol; the components were visualized by fluorography.

Since calf thyroglobulin is known to contain 13–14 complex carbohydrate units/molecule (2), a high degree of variation in their structure, particularly pertaining to the peripheral sugar residues, might be expected. Indeed, our analyses of various glycopeptide fractions indicated that the  $\alpha$ -D-galactosyl residues of this protein are unevenly distributed and seem to be preferentially located in carbohydrate units with a relatively low sialic acid content.

The *B. simplicifolia* I lectin (26) which has been shown to be a valuable tool for identifying  $\alpha$ -D-galactosyl-containing glycoconjugates (9, 18–20, 23, 27) also proved to be very useful in our studies of thyroglobulin. While the extent of binding of various thyroglobulins to *B. simplicifolia* I-agarose was in general related to their  $\alpha$ -D-galactosyl content, this correlation was not a strict one as exemplified by the relatively low interaction of the pig and dog proteins. It is likely that the distribution of the  $\alpha$ -D-galactosyl residues among the numer-

ous thyroglobulin carbohydrate units as well as the location of the units on the polypeptide chains play a major role in determining the binding of the protein to the immobilized lectin.

The solid-phase lectin binding radioassay which we employed afforded an alternative sensitive technique for evaluating the interaction of thyroglobulin with *B. simplicifolia* I. As noted in assays of the calf and pig thyroglobulins, the solid-phase procedure provided a better correlation between  $\alpha$ -D-galactose content and *B. simplicifolia* I binding than did the *B. simplicifolia* I-agarose affinity chromatography; moreover, the extent of  $^{125}\text{I}$ -labeled *B. simplicifolia* I binding to the microwells coated with mouse laminin and calf thyroglobulin, respectively, was found to closely reflect their content of  $\alpha$ -D-galactosyl groups.

The weak interaction between *B. simplicifolia* I-agarose and the  $\alpha$ -D-galactose-containing oligosaccharides from thyroglobulin was consistent with a study of low-molecular-weight ligands carried out by Blake and Goldstein (28). The trisaccharide  $\alpha$ -D-Gal-(1 $\rightarrow$ 3)- $\beta$ -D-Gal-(1 $\rightarrow$ 4)-D-GlcNAcH $_2$  as well as the disaccharide  $\alpha$ -D-Gal-(1 $\rightarrow$ 3)-D-GalH $_2$  were substantially retarded on our column of immobilized *B. simplicifolia* I, emerging after 3 and 5 column volumes, respectively. Affinity chromatography on *B. simplicifolia* I-agarose of calf thyroglobulin unit B glycopeptides indicated that the material which was retarded by the column demonstrated for the most part only a weak interaction with the lectin, which was consistent with the occurrence of a single  $\alpha$ -D-galactosyl residue/carbohydrate unit; firm binding, requiring elution with methyl- $\alpha$ -D-galactoside, was noted only in glycopeptide fraction 2 in which multiple  $\alpha$ -D-galactosyl groups appear to occur.

Although the *B. simplicifolia* I employed in the present study constitutes a mixture of isolectins which could potentially interact with terminal  $\alpha$ -D-N-acetylgalactosamine as well as  $\alpha$ -D-galactosyl groups (26), this property did not hinder the interpretation of our binding data. Our current analyses as well as previous studies (29) indicate that galactosamine is absent from all the thyroglobulins investigated with the exception of the human (2, 30) and guinea pig (3) proteins, and it was indeed the latter two which demonstrated minimal or no reaction with the lectin. The  $\alpha$ -galactosidase-susceptible nature of the interaction with *B. simplicifolia* I as demonstrated for calf thyroglobulin as well as glycopeptides and oligosaccharides prepared from this protein further helped to clarify the specificity of the lectin-sugar interaction.

The occurrence of  $\alpha$ -linked galactose residues in thyroglobulins from several species indicates that the enzymatic machinery for the biosynthesis of the multiple asparagine-linked carbohydrate units of this protein needs to be even more extensive than previously appreciated (31). Studies with Ehrlich ascites cells (32) and rabbit stomach mucosa (6) have shown the presence in each instance of two distinct  $\alpha$ -3-D-galactosyltransferases; both enzymes attach the galactose to a terminal nonreducing  $\beta$ -D-galactosyl group which in one case must be unsubstituted and in the other requires an  $\alpha$ -L-fucosyl residue on C-2. Since our glycosidase digestion studies indicated that the  $\alpha$ -D-galactosyl groups of calf thyroglobulin are linked to unsubstituted  $\beta$ -D-galactose, it appears likely that the first mentioned  $\alpha$ -D-galactosyltransferase is involved in their attachment; indeed the  $\alpha$ -D-Gal(1 $\rightarrow$ 3)- $\beta$ -D-Gal(1 $\rightarrow$ 4)-D-GlcNAc sequence which occurs in the thyroglobulin oligosaccharide branches has been reported to represent the preferential biosynthetic product of this enzyme (6, 32). The existence of this sugar sequence in calf thyroglobulin as well as in several calf thyroid plasma membrane glycoproteins (33)

suggests that thyroids from bovine sources are well endowed with such an  $\alpha$ -D-galactosyltransferase; the lack of  $\alpha$ -D-galactosyl groups in human thyroglobulin as well as in other glycoconjugates from man (23, 24) most likely reflects a species-dictated absence of an enzyme with the appropriate specificity.

## REFERENCES

1. Spiro, R. G. (1965) *J. Biol. Chem.* **240**, 1603-1610
2. Arima, T., Spiro, M. J., and Spiro, R. G. (1972) *J. Biol. Chem.* **247**, 1825-1835
3. Spiro, M. J. (1977) *Biochem. Biophys. Res. Commun.* **77**, 874-882
4. Spiro, M. J. (1961) *J. Biol. Chem.* **236**, 2901-2907
5. Jentoft, N., and Dearborn, D. G. (1979) *J. Biol. Chem.* **254**, 4359-4365
6. Betteridge, A., and Watkins, W. M. (1983) *Eur. J. Biochem.* **132**, 29-35
7. Spiro, R. G. (1962) *J. Biol. Chem.* **237**, 646-652
8. Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963) *Biochem. J.* **89**, 114-123
9. Shibata, S., Peters, B. P., Roberts, D. D., Goldstein, I. J., and Liotta, L. A. (1982) *FEBS Lett.* **142**, 194-198
10. Trevelyan, W. E., Procter, D. P., and Harrison, J. S. (1950) *Nature (Lond.)* **166**, 444-445
11. Spiro, R. G., Spiro, M. J., and Bhoyroo, V. D. (1976) *J. Biol. Chem.* **251**, 6409-6419
12. Kawai, Y., and Spiro, R. G. (1977) *J. Biol. Chem.* **252**, 6229-6235
13. Bray, G. A. (1960) *Anal. Biochem.* **1**, 279-285
14. Perlin, A. S. (1980) in *The Carbohydrates, Chemistry and Biochemistry* (Pigman, W., and Horton, D., eds) 2nd Ed., Vol. IB, pp. 1167-1215, Academic Press, New York
15. Spiro, R. G. (1972) *Methods Enzymol.* **28**, 3-43
16. Spiro, R. G., and Bhoyroo, V. D. (1974) *J. Biol. Chem.* **249**, 5704-5717
17. Watkins, W. M. (1966) *Science (Wash. D. C.)* **152**, 172-181
18. Eckhardt, A. E., and Goldstein, I. J. (1979) in *Glycoconjugate Research* (Gregory, J. D., and Jeanloz, R. W., eds) Vol. 2, pp. 1043-1045, Academic Press, New York
19. Okada, Y., and Spiro, R. G. (1980) *J. Biol. Chem.* **255**, 8865-8872
20. Eckhardt, A. E., and Goldstein, I. J. (1983) *Biochemistry* **22**, 5280-5289
21. Muir, L., and Lee, Y. G. (1969) *J. Biol. Chem.* **244**, 2343-2349
22. Spiro, R. G., and Bhoyroo, V. D. (1980) *J. Biol. Chem.* **255**, 5347-5354
23. Peters, B. P., and Goldstein, I. J. (1979) *Exp. Cell Res.* **120**, 321-334
24. Kress, B. C., and Spiro, R. G. (1984) *Fed. Proc.* **43**, 1706
25. Spiro, R. G. (1973) *Adv. Protein Chem.* **27**, 349-467
26. Murphy, L. A., and Goldstein, I. J. (1977) *J. Biol. Chem.* **252**, 4739-4742
27. McCoy, J. P., Jr., Varani, J., and Goldstein, I. J. (1983) *Anal. Biochem.* **130**, 437-444
28. Blake, D. A., and Goldstein, I. J. (1980) *Anal. Biochem.* **102**, 103-109
29. Spiro, R. G., and Spiro, M. J. (1965) *J. Biol. Chem.* **240**, 997-1001
30. Spiro, M. J. (1977) *J. Biol. Chem.* **252**, 5424-5430
31. Spiro, R. G., Spiro, M. J., and Adamany, A. M. (1974) *Biochem. Soc. Symp.* **40**, 37-55
32. Blake, D. A., and Goldstein, I. J. (1981) *J. Biol. Chem.* **256**, 5387-5393
33. Edge, A. S. B., and Spiro, R. G. (1983) *Fed. Proc.* **42**, 2100

**Occurrence of alpha-D-galactosyl residues  
in the thyroglobulins from several species.  
Localization in the saccharide chains of the  
complex carbohydrate units.**

R G Spiro and V D Bhoyroo  
*J. Biol. Chem.* 1984, 259:9858-9866.

Access the most updated version of this article at <http://www.jbc.org/content/259/15/9858>

Find articles, minireviews, Reflections and Classics on similar topics on the [JBC Affinity Sites](#).

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at  
<http://www.jbc.org/content/259/15/9858.full.html#ref-list-1>