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Polysaccharide Intermediates Formed during Intracellular Transport of a Carbohydrate-Containing, Secreted Immunoglobulin Light Chain†

Paul M. Knopf,* Eric Sasso,‡ Antonia Destree,§ and Fritz Melchers#

ABSTRACT: Intracellular and secreted forms of the carbohydrate-containing light chain synthesized by MOPC-46 murine myeloma are heterogeneous in their single, carbohydrate moiety. To determine the number of different polysaccharide species contained in these forms of light chain, a technique was developed to separate and qualitatively analyze glycopeptides differing in carbohydrate composition. The glycopeptides were prepared by protease digestion of serologically precipitated, sugar radiolabeled light chain. Separation of glycopeptides was accomplished by column chromatography on Bio-Gel P6 polyacrylamide and paper electrophoresis. The carbohydrate moiety of secreted light chain contains *N*-acetylglucosamine, mannose, galactose, fucose, and variable amounts of *N*-glycolylneuraminic acid. Glycopeptides from secreted light chain were resolved into three species, differing in their content of *N*-glycolylneuraminic acid (0, 1, or 2 residues). Intracellular light chain glycopeptides were resolved into four species: the major glycopeptide species contained only "core" sugars, *N*-acetylglucosamine and mannose; another glycopeptide species contained core sugars and galactose; two glycopeptide species contained core sugars, galactose and one or two residues of *N*-glycolylneuraminic acid. Glycopeptides of intracellular light chain contained too few residues of fucose to be detected by incorporation of radioactive fucose. These findings corroborate the previous conclusion that carbohydrate attachment occurs in several steps to molecules destined to be secreted. Since a significant pool of light chain with core sugars, galactose, and neuraminic acid was found inside cells, attachment of fucose can now be designated as the final step in carbohydrate assembly, occurring close to or at the time of light chain secretion.

The light chain secreted by MOPC-46 myeloma cells possesses a polysaccharide moiety attached covalently to a single amino acid residue (Melchers, 1969). Previous studies have established certain features of the intracellular pathway leading to light chain secretion, and of the concomitant addition of sugar residues to the intracellular protein. The light chain polypeptide is synthesized on membrane-bound polyribosomes (Cioli and Lennox, 1973b). Pulse-chase kinetic studies, using radiolabeled leucine, show that light chain resides initially in the rough membrane fraction, is transported to the smooth membrane fraction, and is then secreted (Choi et al., 1971b; Melchers, 1971). The carbohydrate composition of secreted light chain (isolated from urine of tumor-bearing mice) is: 3 GlcNAc,[1] 4 Man, 4 Gal, 2 Fuc, and 0, 1, or 2 NGNA (Melchers, 1969). The polysaccharide composition of light chain isolated from either the rough membrane or smooth membrane fractions shows a deficiency in Gal and Fuc; NGNA was not analyzed (Choi et al., 1971b; Melchers, 1971). It was concluded that addition of sugar residues to light chain occurs in stages during the intracellular transport phase of secretion;

† From the Armand Hammer Center for Cancer Research, The Salk Institute, San Diego, California 92112 (P.M.K., E.S., and A.D.), and the Max-Planck Institut für Molekulare Genetik, Berlin, West Germany (F.M.). *Received April 18, 1975.* We thank Dr. E. S. Lennox for supporting a portion of the research (USPHS-AI-06544).

* Recipient of a U.S. Public Health Service Career Development Award, K3-CA-08591, and supported by Research Grants RR-05664 and AI-11169. Present address: Division of Biological and Medical Sciences, Brown University, Providence, Rhode Island 02912.

‡ Student under a National Science Foundation Student Science Training Program (precollege), Grant No. GW-5234.

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Present address: Basel Institute for Immunology, Basel, Switzerland.

[1] Abbreviations used are: GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; Gal, D-galactose; Fuc, L-fucose; NGNA, *N*-glycolylneuraminic acid; NANA, *N*-acetylneuraminic acid; NP40, Nonidet P.40 (Shell Chem. Co., London, U.K.); RM, rough membrane; SM, smooth membrane.

Table I: Polysaccharide Residues of Intracellular and Secreted Light Chain Labeled by Radioactive Sugars.[a]

Radioactive Precursor Sugars	Polysaccharide Residues Labeled									
	Intracellular Light Chain					Secreted Light Chain				
	Glc-NAc	Man	Gal	Fuc	NG-NA[b]	Glc-NAc	Man	Gal	Fuc	NG-NA[b]
[^{3}H]Glucosamine	+	0	0	0	+	+	0	0	0	+
[^{3}H]Mannose	+	+	0	Tr[c]	0	+	+	0	+	0
[^{3}H]Galactose	0	0	+	0	0	0	0	+	0	0
[^{3}H]Fucose	0	0	0	Tr[c]	0	0	0	0	+	0
[^{3}H]Glucose	+	+	+	Tr[c]	+	+	+	+	+	+

[a] References: Melchers (1969, 1970). [b] Result established in current study. [c] Trace amount.

GlcNAc and Man (core sugars) are attached to light chain in rough membrane and the remaining sugars attached later. Since intracellular light chain was not analyzed for NGNA, the pattern of terminal carbohydrate addition was not established.

To further analyze the pathway of carbohydrate assembly, fractionation of potentially complex varieties of intermediates is required. As a first step toward accomplishing this fractionation, we developed a technique which separates glycopeptides (released from light chain by extensive protease digestion) according to carbohydrate composition, in particular through differences in size and charge. Filtration of glycopeptides over a Bio-Gel P6 polyacrylamide column, followed by paper electrophoresis, resolved intracellular light chain into four species, differing in their contents of Gal and NGNA.

The results confirm our previous supposition that attachment of sugars occurs in stages. We reestablished the existence of a very small pool of Fuc-containing intracellular light chain; however, a measurable pool of light chain containing NGNA on the carbohydrate moiety was found. Thus addition of Fuc is the terminal stage of carbohydrate attachment, occurring closest to the time of light chain secretion.

Materials and Methods

Incubation of Cells in Vitro with Radioactive Sugars. Cell suspensions of transplantable MOPC-46 tumors (M. Potter, National Institutes of Health) were prepared as described previously (Choi et al., 1971a) and modified (Cioli and Lennox, 1973a). Cells were incubated at 2×10^6 live cells/ml in Dulbecco's modified Eagle's medium minus dextrose and 3% fetal calf serum. Incubations were conducted for 3 hr at 37°, a time sufficient to label all sugars (Melchers, 1970).

The following radioactive sugars were used in separate in vitro incubations at the concentrations indicated: 100 μCi/ml of D-[6-^{3}H]glucosamine (New England Nuclear, 3 Ci/mmol); 100 μCi/ml of D-[1-^{3}H]mannose (Amersham/Searle, 0.7 Ci/mmol); 200 μCi/ml of D-[6-^{3}H]galactose (New England Nuclear, 7 Ci/mmol); 50 μCi/ml of L-[1-^{3}H]fucose (Amersham/Searle, 0.9 Ci/mmol); 50 μCi/ml of D-[6-^{3}H]glucose (New England Nuclear, 9.5 Ci/mmol); 5 μCi/ml of D-[^{14}C(U)]glucose (New England Nuclear, 0.2 Ci/mmol). The light chain polysaccharide residues labeled by these sugars are given in Table I.

Isolation of Light Chain. Light chain in urine of BALB/c mice carrying MOPC-46 tumor, generations of 42–48, was purified as described perviously (Melchers et al., 1966).

Radioactive light chain was isolated from cell suspensions labeled in vitro with radioactive monosaccharides (Choi et al., 1971a). Following incubation, the suspension was centrifuged, yielding S_1 (the supernatant fraction containing secreted light chain) and P_1 (the cell pellet). The cell pellet was washed once in phosphate-buffered saline and the cell contents released by lysis with nonionic detergent (NP40). Following detergent treatment, the lysate was centrifuged, yielding S_2 (the supernatant fraction containing total intracellular light chain) and P_2 (the nuclei and insoluble material).

Radioactive fractions containing light chain were incubated with specific antisera to precipitate this protein. Rabbit anti-(MOPC-46) light chain was added to form complexes with the radioactive light chain; complexes were precipitated by addition of sheep anti-(rabbit) IgG. Controls, containing rabbit anti-ovalbumin in place of anti-light chain, measured the amount of nonspecifically precipitated radioactivity. Serological precipitates were collected by centrifugation and washed with phosphate-buffered saline (Choi et al., 1971a).

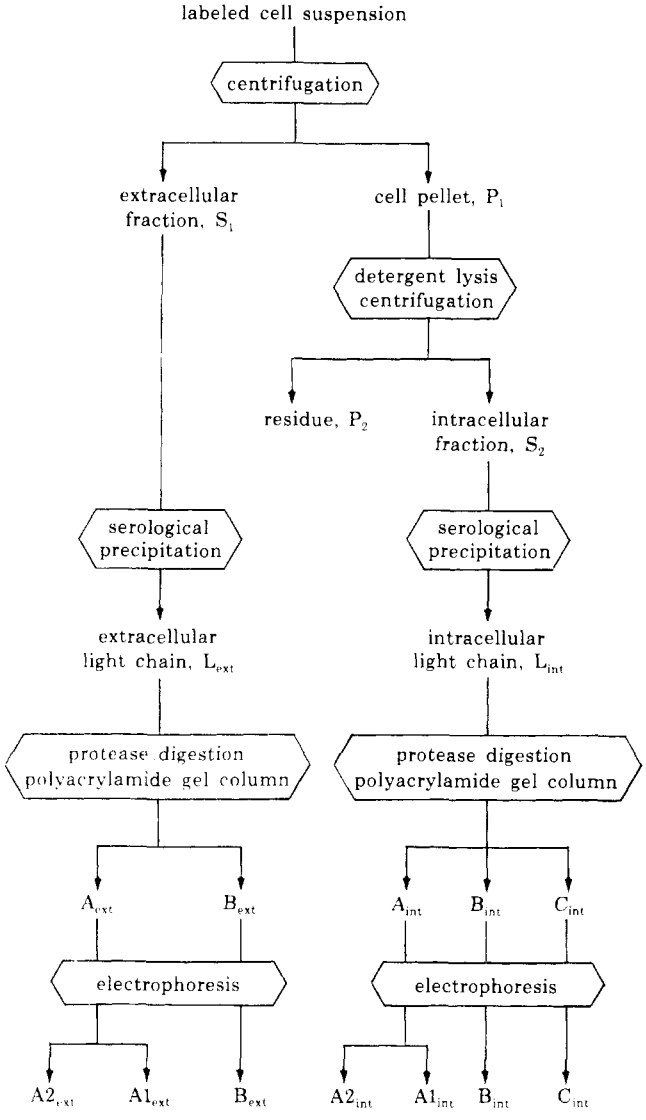
Proteolytic Digestion of the Serological Precipitate. Serological precipitates of radioactive light chain (20–30 mg of protein) were dissolved in a solution containing 8 *M* deionized urea, 0.4 *M* Tris (pH 8.5), and 0.2 *M* β-mercaptoethanol (10 mg of protein/ml). When the precipitates were completely dissolved (2 hr, 37°), ethylenimine (Pierce Chemical Co.) was added with swirling to a concentration of 0.4 *M*, converting cysteine to aminoethylcysteine. After 30 min at room temperature, β-mercaptoethanol was added to 0.8 *M*; the solution was then exhaustively dialyzed against 0.2 *M* ammonium formate (pH 8.3). The protein precipitated during dialysis.

To the dialyzed, reduced, and aminoethylated sample, $CaCl_2$ was added to 5 m*M* and trypsin (Worthington Biochemicals) to a weight ratio of protein/enzyme of 100:1. After 30 min, at 37°, Pronase (Calbiochem Corp.) was added to a weight ratio of 100:1. Equal amounts of Pronase were added 2 and 4 hr later, and the suspension was incubated overnight at 37°. The pH of the suspension was maintained at 8.3 by the periodic addition of 1 *M* ammonium formate. A few drops of toluene was added to the overnight incubation mixture as a bacteriocidal agent. The digest was centrifuged to remove a slight amount of insoluble material and lyophilized.

Fractionation of Proteolytic Digests. A gel chromatographic procedure, which separated different hexoses as well as oligosaccharides of the same hexose subunit (Trenel et al., 1968), was successfully adapted for separating light chain glycopeptides. The digest was dissolved in 0.5 ml of water and passed through a Bio-Gel P6 (200–400 mesh, Bio-Rad Laboratories) column (0.9 cm × 150 cm) in water, maintained at 50°, and 1.5-ml fractions were collected. The optical density was measured at 280 mμ and radioactivity in aliquots of fractions was measured in a liquid scintillation counter (Choi et al., 1971a). Fractions of resolved components were pooled, lyophilized, resuspended in 1 ml of water, and stored frozen.

To further separate glycopeptides differing in charge, paper electrophoresis of aliquots of the P6 column components was conducted at pH 6, 4 kV/meter, 3 hr, in electrophoresis tanks containing Varsol-1. Radioactive glucose was

Scheme I: Fractionation Scheme.



added to some samples and ϵ-Dnp-lysine was spotted at the edges prior to electrophoresis, as indicators of migration due to electroendosmosis. After electrophoresis, papers were dried, and panels were cut in the direction of migration and sectioned into 1-cm segments. The segments were placed in counting vials, 1 ml of water was added, they were incubated 30 min, 37°, to elute the glycopeptides, and assayed for radioactivity after adding 10 ml of scintillation fluid. A flow diagram of these procedures is given in Scheme I.

Preparation of Glycopeptides from Purified Light Chain. Light chain, purified from urine, was reduced and aminoethylated, then dialyzed, as described above. The protein was digested with trypsin for 18 hr, and tryptic glycopeptides were prepared following chromatography and electrophoresis on paper (Melchers, 1969). Three tryptic glycopeptides (located on the papers by their fluorescence under uv light) were obtained, differing in their neuraminic acid content. Their amino acid compositions were verified by analysis. Each tryptic glycopeptide was then digested with Pronase, as described above. The amount of Pronase added was proportional to the weight of light chain from which the tryptic glycopeptide was derived. The digests were lyophilized.

Pronase digests of each tryptic glycopeptide were subjected to chromatography on the Bio-Gel P6 column, after mixing with radioactive marker glycopeptides. Following chromatography, the location of fractions containing the Pronase-digested tryptic glycopeptides was determined using the phenol–sulfuric acid colorimetric assay for carbohydrate (Dubois et al., 1956). Neither the Pronase nor the marker glycopeptides, in the amounts used, yielded any color in this carbohydrate assay. Thus, their presence does not interfere with interpretation of results. The positions of the radioactive marker glycopeptides were determined by counting aliquots from column fractions prior to the carbohydrate assay.

Neuraminidase Digestion of Glycopeptides. Glycopeptide components from the P6 column were treated with neuraminidase (Vibrio cholera, Nutritional Biochemical Co.) to determine the presence of neuraminic acid on the glycopeptides. Aliquots of glycopeptides were suspended in 5 m*M* acetate buffer (pH 5.25)–1 m*M* $CaCl_2$. Neuraminidase was added to 80 units/ml and the solutions were incubated at 37°; an equal dose of enzyme was added after 4 hr and digestion allowed to proceed overnight. Control samples received acetate buffer instead of enzyme. The digests were lyophilized and subjected to analysis by electrophoresis at pH 6. Panels were assayed for radioactivity, as described above.

Identification of NGNA as one of the products of digestion was carried out as follows. Prior to electrophoresis, NGNA and NANA were spotted on the paper, adjacent to the neuraminidase digested, radioactive glycopeptides. Following electrophoresis, positions of the reference markers were determined on eluates of sectioned panels, by the thiobarbituric acid assay for neuraminic acid (Aminoff, 1961). Electrophoresis at pH 6 did not resolve NGNA and NANA. Thus, sections of the paper adjacent to the markers, containing the presumptive neuraminic acid product of the digest, were sewn into new papers and subjected to descending paper chromatography in 0.1 *N* HCl–1-propanol–1-butanol (1:2:1, v/v) (Svennerholm and Svennerholm, 1958). NGNA and NANA were again spotted alongside and located after chromatography by the thiobarbituric acid assay. Panels containing the neuraminidase digestion products were assayed for radioactivity as before.

Results

A. Secreted Light Chain

1. Glycopeptides from in Vitro Carbohydrate-Labeled Secreted Light Chain. Initial studies were conducted on carbohydrate-labeled secreted light chain. It had been previously shown that exhaustive digestion of purified light chain with Pronase yielded glycopeptides (containing 13–15 sugars and 1–2 amino acids) and very low molecular weight products (amino acids, peptides), which could be separated by gel filtration (Melchers, 1969). We assumed that a serological precipitate containing radioactive light chain upon digestion would yield labeled glycopeptides from light chain, unlabeled glycopeptides from antisera, and smaller, nonlabeled products. To promote the formation of such products, the precipitates were denatured in 8 *M* urea, disulfide bridges ruptured, and additional sites for tryptic cleavage introduced by aminoethylation.

Light chain in the S_1 fraction (Scheme I) of cell suspensions incubated in vitro with radioactive monosaccharides was prepared by serological precipitation. Proteolytic di-

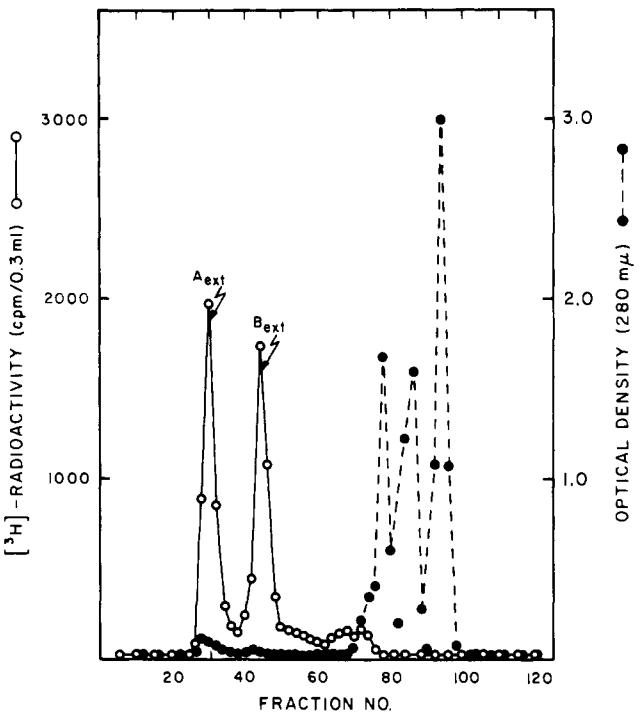


FIGURE 1: Elution profile of glycopeptides. The Pronase digest of secreted light chain from [^{3}H]mannose-labeled myeloma cells was filtered through a P6-polyacrylamide gel column. Fractions collected were assayed for radioactivity and optical absorbance.

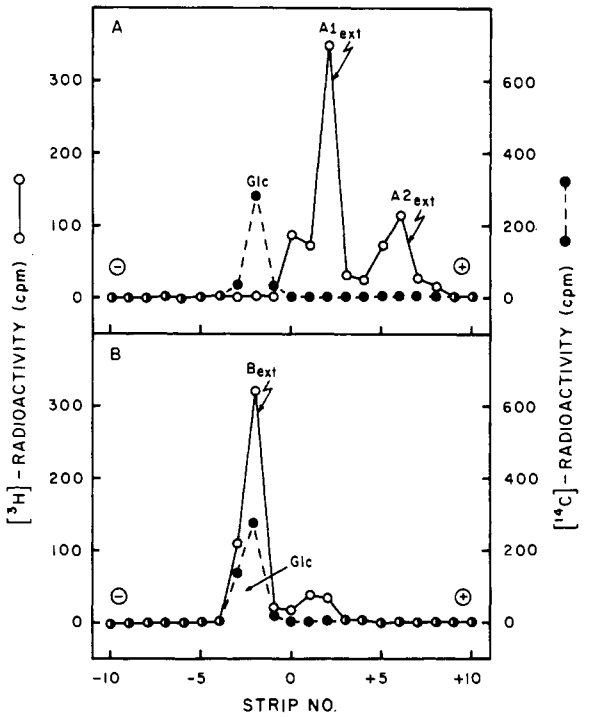


FIGURE 2: Electrophoretic patterns of glycopeptides. The glycopeptide fractions from the P6 column (Figure 1) were subjected to paper electrophoresis at pH 6. Segments of the paper were eluted and assayed for radioactivity. Glc is the [^{14}C]glucose electrophoretic marker. (A) Peak A_{ext}, [^{3}H]mannose-labeled cells; (B) peak B_{ext}, [^{3}H]mannose-labeled cells.

gests of the precipitates were chromatographed on a P6 polyacrylamide gel column. Altogether, five analyses were performed, differing from each other in the species of radioactive monosaccharide used during in vitro incubation, viz., [^{3}H]glucosamine, [^{3}H]mannose, [^{3}H]galactose, [^{3}H]fucose, and [^{3}H]glucose. A typical profile, from [^{3}H]mannose-labeled cells, is shown in Figure 1.

The results in each case were quite similar. There were two major peaks of radioactivity, the first (peak A_{ext}) eluting between fractions 25 and 35 and the second (peak B_{ext}) eluting between fractions 40 and 50. There was usually some radioactivity eluting in the vicinity of the column volume (fraction 65) as well as a small amount eluting even later. The total recovery of radioactivity was greater than 90% of the input, with most of this being found in peaks A_{ext} and B_{ext}. There was always about 30% more radioactivity in peak A_{ext}, compared to peak B_{ext}.

Optical density profiles in each case were also similar. There were three major peaks, eluting between fractions 70 and 100. A very small peak was also found to precede radioactive peak A_{ext}, between fractions 20 and 25, i.e., at the void volume of the column. The optical density profile between this minor front peak and the three later peaks was always very low and without any distinguishing feature.

Peaks A_{ext} and B_{ext} were further characterized by electrophoresis at pH 6. The results of this operation are shown in Figure 2. Radioactivity in peak A_{ext} separated into two negatively charged fractions, $A2_{ext}$ and $A1_{ext}$; radioactivity in peak B_{ext} migrated as a single neutral fraction. The same results were obtained for all the labeled preparations. There was always more radioactivity in $A1_{ext}$ than in $A2_{ext}$ and the amount of radioactivity in B_{ext} was slightly greater than that in $A1_{ext}$.

We tentatively identified $A2_{ext}$, $A1_{ext}$, and B_{ext} as the three glycopeptides of secreted light chain which differ from each other in their content of neuraminic acid (2, 1, and 0 residues, respectively). This assignment was verified, both by comparison to Pronase-digested tryptic glycopeptides isolated from purified light chain and by neuraminidase digestion of the radioactive fractions.

2. Glycopeptides from Purified Light Chain. Tryptic digestion of purified urinary light chain yields three glycopeptides, composed of identical amino acid sequences. These glycopeptides each contain 3 GlcNAc, 4 Man, 4 Gal, and 2 Fuc residues; they differ only in the amount of NGNA present in the carbohydrate portion—0, 1, or 2 residues of NGNA, respectively (Melchers, 1969). We prepared these three tryptic glycopeptides and used Pronase digests of them as markers to establish the identity of radioactive components $A2_{ext}$, $A1_{ext}$, and B_{ext}. The results of this study, described under Materials and Methods, are given in Figure 3.

The Pronase-digestion product of the tryptic glycopeptide containing 2 NGNA residues yielded a major component eluting from the P6 column over the leading edge of peak A_{ext} (Figure 3A). The Pronase-digestion product of the tryptic glycopeptide containing 1 NGNA residue yielded a major component eluting from the column in a position coincident with peak A_{ext} (Figure 3B). The tryptic glycopeptide containing no NGNA yielded a mixture of Pronase-digestion products, separating into three components eluting in the vicinity of peak B_{ext} (Figure 3C).

Peak A_{ext} contains those glycopeptides with 1 or 2 NGNA residues. In an additional, separate experiment we subjected individual column fractions of peak A_{ext} to electrophoresis at pH 6. The leading edge of peak A_{ext} was rich in component $A2_{ext}$; the trailing edge of peak A_{ext} was rich in component $A1_{ext}$. Thus, the more negatively charged component, $A2_{ext}$, is the glycopeptide containing 2 NGNA

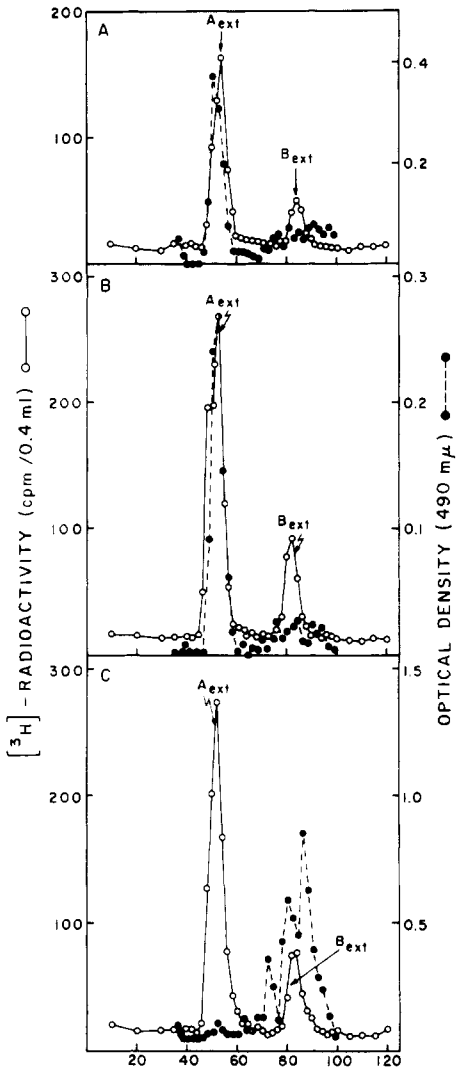


FIGURE 3: Elution profiles of glycopeptides from purified light chain. The Pronase-digested, tryptic glycopeptides prepared from light chain (purified from urine of tumor-bearing mice) were filtered through the P6-polyacrylamide gel column. Fractions A_{ext} and B_{ext} from [^{3}H]galactose-labeled cells were added to each digest. Fractions collected were assayed for purified light chain glycopeptides by the phenol–sulfuric acid colorimetric assay (optical absorbance at 490 mμ) and for radioactivity. (A) Tryptic glycopeptide with 2 NGNA residues; (B) tryptic glycopeptide with 1 NGNA residue; (C) tryptic glycopeptide with 0 NGNA residues.

residues, while $A1_{ext}$ is the glycopeptide containing 1 NGNA. Consistent with this conclusion is the greater electrophoretic mobility of $A2_{ext}$ toward the anode, compared with $A1_{ext}$.

The assignment of component B_{ext} as the 0 NGNA glycopeptide is complicated by the finding of three carbohydrate-positive peaks in the Pronase digest of the 0 NGNA-tryptic glycopeptide. The in vitro secreted light chain yields a homogeneous glycopeptide while the in vivo secreted light chain, subsequently isolated from the urine, gives rise to a heterogeneous glycopeptide. Lack of material limited our determining the basis of this in vivo produced heterogeneity. We conclude that component B_{ext} is a 0 NGNA glycopeptide but that this glycopeptide may become modified in vivo.

3. Presence of NGNA in Negatively Charged Glycopeptides. We have concluded that the glycopeptides $A2_{ext}$ and $A1_{ext}$ contained neuraminic acid, which accounted for their negativity at pH 6. To verify this, we digested the radioactive glycopeptides with neuraminidase and examined the products by electrophoresis. We analyzed glycopeptides from S_1 light chains labeled with each of the ^{3}H-labeled monosaccharides. Fractions from the P6 column were incubated with or without neuraminidase, as described under Materials and Methods. Following neuraminidase digestion and lyophilization, the samples were electrophoresed and the radioactivity was measured. The results are given in Figure 4. The positions of NGNA and NANA in electrophoresis are also shown; they were identical (Figure 4D).

The results may be summarized as follows. For [^{3}H]mannose, [^{3}H]galactose, or [^{3}H]fucose labeled peak A glycopeptides, over 80% of the radioactivity migrated as a neutral component following neuraminidase digestion; the remainder migrated like component $A1_{ext}$ (Figure 4A). In the case of [^{3}H]glucosamine or [^{3}H]glucose-labeled peak A_{ext}, there was a third component, which migrated toward the anode with a mobility close to that of free neuraminic acid (Figure 4B). For peak B_{ext} glycopeptides and the controls, no change in charge or yield occurred following neuraminidase digestion (Figure 4C).

The identification of the radioactive component migrating close to neuraminic acid (fractions 17–20, Figure 4B) was accomplished by subjecting it to chromatography, as described under Materials and Methods. The radioactivity profile showed one peak (R_f 0.44). The positions of NGNA and NANA were R_f 0.45 and 0.56, respectively. We conclude that this major radioactive component is NGNA. That NGNA was labeled only when [^{3}H]glucosamine or [^{3}H]glucose was used during the in vitro incubation of cells is consistent with known pathways of conversion of these sugars to neuraminic acid (Table I).

B. Studies on Intracellular Light Chain

1. Glycopeptides from in Vitro Carbohydrate-Labeled Light Chain. The intracellular light chain, precursor to the secreted light chain, has been previously shown to be a mixture of glycoproteins containing less carbohydrate than the secreted product (Choi et al., 1971b; Melchers, 1971). Attachment of different sugars occurs while the precursors are in transit through different intracellular compartments. We analyzed the Pronase digestion products of the total intracellular light chain.

Intracellular light chain was isolated by serological precipitation from the S_2 fraction (Scheme I) of MOPC-46 cells labeled in vitro with [^{3}H]glucosamine, [^{3}H]mannose, or [^{3}H]galactose. The S_1 fraction of cells labeled with [^{14}C]glucose was added, prior to serological precipitation, as a marker of peaks A_{ext} and B_{ext}. The serological precipitates were reduced, aminoethylated, and digested with trypsin and Pronase. The digests were applied to the P6 column; the results for [^{3}H]mannose and for [^{3}H]galactose are given in Figure 5.

The elution profiles for [^{3}H]mannose- and [^{3}H]glucosamine-labeled products were very similar; profiles for [^{3}H]galactose-labeled products differed from these. For the digests of [^{3}H]mannose labeled light chain, the elution profile of radioactivity yielded three components, A_{int}, B_{int}, and C_{int} (Figure 5A). The elution profile of radioactivity for [^{3}H]galactose light chain digests yielded only two components, A_{int} and B_{int} (Figure 5B). The marker [^{14}C]glucose labeled, secreted light chain digest yielded an elution profile of two peaks, A_{ext} and B_{ext}, as found before. The positions of peaks A_{int} and A_{ext} were indistinguishable; likewise, the

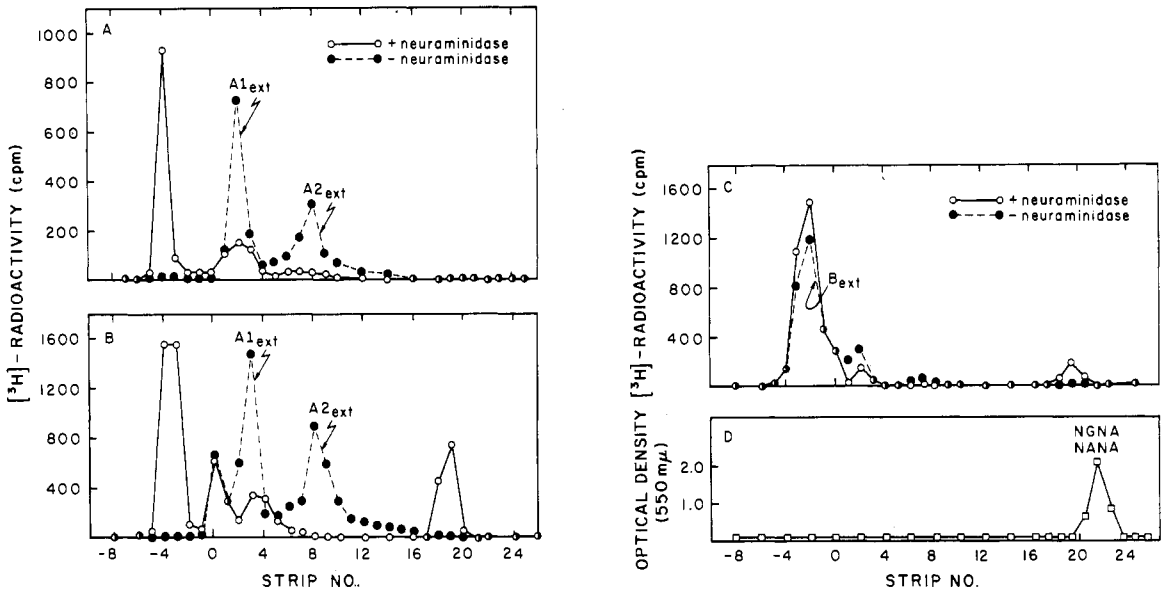


FIGURE 4: Electrophoretic patterns of neuraminidase-digested glycopeptides. Glycopeptide fractions from P6 columns were incubated with or without neuraminidase and then subjected to paper electrophoresis at pH 6. Segments of the paper were eluted and assayed for radioactivity. The same glycopeptide fractions incubated with or without neuraminidase were placed at adjacent locations of the same paper; data for each are presented in a composite display. (A) Peak A_{ext}, [^{3}H]galactose-labeled cells; (B) peak A_{ext}, [^{3}H]glucosamine-labeled cells; (C) peak B_{ext}, [^{3}H]glucosamine-labeled cells; (D) NGNA and NANA mixture (thiobarbituric acid assay, 550 mμ).

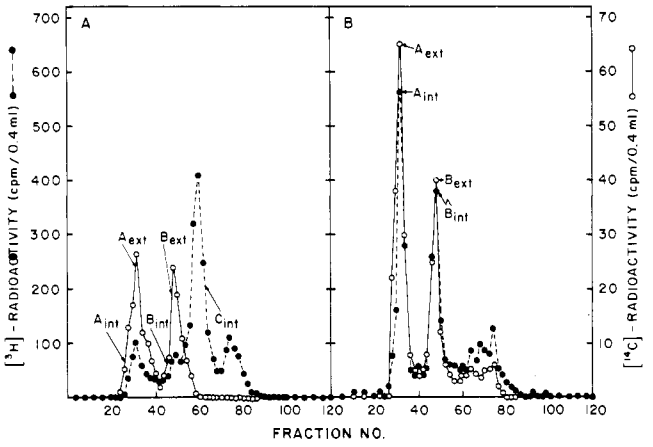


FIGURE 5: Elution profiles of glycopeptides. The Pronase digests of intracellular light chain prepared from (A) [^{3}H]mannose- or (B) [^{3}H]galactose-labeled cells were filtered through the P6-polyacrylamide gel column. Secreted fraction from [^{14}C]glucose-labeled cells was added to each sample prior to serological precipitation. Fractions collected were assayed for radioactivity.

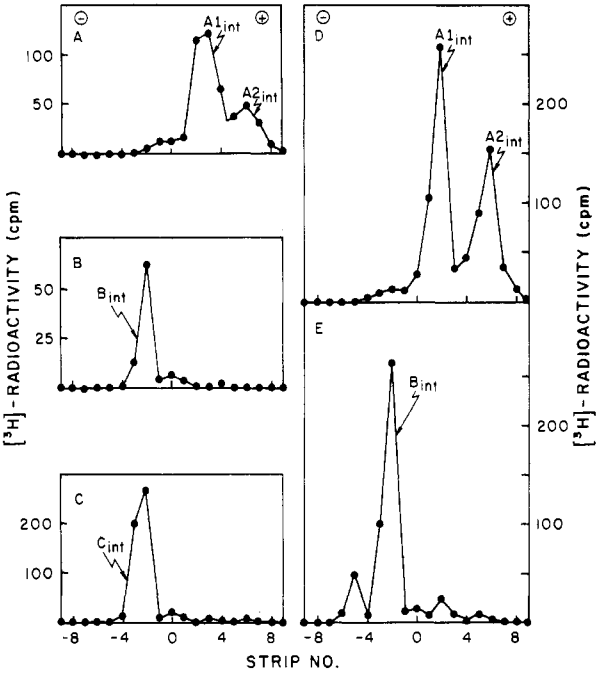


FIGURE 6: Electrophoretic patterns of glycopeptides. The glycopeptide fractions from the P6 columns (Figure 5) were subjected to paper electrophoresis at pH 6. Segments of the papers were eluted and assayed for radioactivity. (A) A_{int}, [^{3}H]mannose-labeled cells; (B) B_{int}, [^{3}H]mannose-labeled cells; (C) C_{int}, [^{3}H]mannose-labeled cells; (D) A_{int}, [^{3}H]galactose-labeled cells; (E) B_{int}, [^{3}H]galactose-labeled cells.

positions of peaks B_{int} and B_{ext} were coincident. For the [^{3}H]mannose-labeled components, the amount of peak A_{int} was 17%; peak B_{int} was 16%; and peak C_{int} was 67% of the total radioactivity in these three components. Small amounts of radioactivity were found to elute behind these components.

The intracellular components were further characterized by electrophoresis at pH 6. The results are given in Figure 6. The radioactivity in peak A_{int} migrated as two negatively charged components, called $A2_{int}$ and $A1_{int}$. Their electrophoretic mobilities were indistinguishable from $A2_{ext}$ and $A1_{ext}$. The radioactivity in peak B_{int} migrated as a neutral component, indistinguishable from B_{ext}. Likewise, peak C_{int} yielded a single, neutral radioactive component upon electrophoresis at pH 6.

The presence of neuraminic acid in the glycopeptides of intracellular light chain was assessed by the neuraminidase digestion. We analyzed [^{3}H]glucosamine-labeled glycopeptides and the results were similar to those obtained for secreted protein (Figure 4B). For peak A_{int} glycopeptides, 60–70% of the radioactivity migrated as a neutral component in electrophoresis following neuraminidase digestion; the remainder of the radioactivity was distributed between peak $A1_{int}$ and neuraminic acid. Neither peaks B_{int} or C_{int}

Table II: Radioactive Sugar Residues in Light Chain Glycopeptides.

Sugars	$A2_{ext}$	$A1_{ext}$	B_{ext}	$A2_{int}$	$A1_{int}$	B_{int}	C_{int}
GlcNAc	+	+	+	+	+	+	+
Man	+	+	+	+	+	+	+
Gal	+	+	+	+	+	+	0
Fuc	+	+	+	(?)	(?)	(?)	(?)
NGNA	+	+	0	+	+	0	0
Peak position on P6 gel	A	A	B	A	A	B	C

changed in their mobility following digestion, nor did they yield other radioactive species. The radioactive component migrating like neuraminic acid on electrophoresis was shown to be primarily NGNA by paper chromatography.

The glycopeptide profile of intracellular light chain from fucose-labeled cells was not determined, owing to the low specific activity of radioactive light chain present in these cells. Since incorporation of [^{3}H]fucose into secreted light chain occurs without a detectable lag, the low amounts of intracellular fucose labeled light chain cannot be attributed to a failure to activate [^{3}H]fucose (Melchers, 1970). Low amounts of fucose labeled light chain were expected from previous studies showing that total intracellular light chain contains less than 0.1 residue of Fuc per chain, compared with 2 residues of Fuc per chain in secreted light chain. The coincidence on P6 gel chromatography and paper electrophoresis of peaks A_{int} and A_{ext} and of peaks B_{int} and B_{ext} suggests that our procedures do not separate glycopeptides differing in their content of Fuc.

We draw the following conclusions on the sugar compositions of intracellular light chain glycopeptides. Fraction C_{int} becomes labeled upon incubation with [^{3}H]glucosamine and [^{3}H]mannose but not [^{3}H]galactose; it is neutral in electrophoresis and yields no radioactive NGNA after neuraminidase treatment. Thus C_{int} contains residues of GlcNAc and Man, but not Gal or NGNA. Fraction B_{int} becomes labeled upon incubation with [^{3}H]glucosamine, [^{3}H]mannose, and [^{3}H]galactose; it is neutral in electrophoresis and yields no radioactive NGNA after neuraminidase treatment. Thus B_{int} contains residues of GlcNAc, Man, and Gal, but not NGNA. Fraction A_{int} becomes labeled with [^{3}H]glucosamine, [^{3}H]mannose, and [^{3}H]galactose; it yields two negatively charged species in electrophoresis and radioactive NGNA after neuraminidase. Thus both species, $A2_{int}$ and $A1_{int}$, contain residues of GlcNAc, Man, Gal, and NGNA but differ in their content of NGNA. These conclusions are summarized in Table II.

Discussion

We will first review how P6 polyacrylamide column chromatography and paper electrophoresis separate glycopeptides of known composition. Then we will discuss the analyses of intracellular light chain glycopeptides in view of the question of possible intermediates in carbohydrate assembly of glycoproteins (Gottschalk, 1969).

The P6 polyacrylamide gel column resolved some glycopeptides differing in their size and carbohydrate composition. The species containing 1 or 2 NGNA residues (A_{ext}) separate from the species containing no NGNA (B_{ext}). Species containing only GlcNAc and Man (C_{int}) separate from species which contain GlcNAc, Man, and Gal (B_{int}). Resolution exceeds that expected by differences in molecular weights of these glycopeptides. Thus, differences in the chromatographic properties of individual sugars, as shown by Trenel et al. (1968), influence chromatography of mixed polysaccharides (Table II). Glycopeptide fractionation on P6 polyacrylamide gel is dependent upon carbohydrate composition.

Other glycopeptides, differing in size and carbohydrate composition, are not resolved on the P6 column. The capacity to separate the two NGNA-containing species is limited (Figure 3A). This resolution has been improved by using a P10 polyacrylamide gel column.[2] Glycopeptides differing in their content of Fuc (and possibly Gal, also, as discussed below) are not resolved by the P6 gel (Figure 5). Thus, A_{int} and A_{ext} are indistinguishable, as are B_{int} and B_{ext} (Table II).

The separations of glycopeptides achieved by filtration over the P6 gel may be influenced by differences in amino acid composition. Digestion of light chain with trypsin plus Pronase results in almost complete removal of amino acids other than the aspartic acid or asparagine to which the GlcNAc is attached (Melchers, 1969). With our technique, it was not possible to perform a meaningful amino acid analysis of the labeled glycopeptides, owing to the presence of unlabeled glycopeptides, derived from the antisera immunoglobulins. This could be overcome either by using immunoabsorbents and eluting the bound, radioactive molecules, or by preparing glycoprotein in quantities sufficient for analyzing glycopeptides by chemical methods. In an effort to reveal amino acid heterogeneity in glycopeptides, we subjected all labeled glycopeptides to electrophoresis at pH 2, an acid concentration expected to introduce charge differences into peptide chains of variable compositions. Each of the glycopeptides migrated as a single species under this condition.[3] These results do not preclude the possibility that differences in the amino acid portion of glycopeptides influence their separation on polyacrylamide gel columns. We limit our conclusion, that differences in amino acid composition are not a factor influencing the separation, to the MOPC-46 light chain glycopeptides.

The glycopeptides from total intracellular light chain have been assigned the following qualitative sugar compositions (Table II): C_{int} (GlcNAc, Man); B_{int} (GlcNAc, Man, Gal); $A1_{int}$ (GlcNAc, Man, Gal, $NGNA_1$); $A2_{int}$ (GlcNAc, Man, Gal, $NGNA_2$). These assignments are based upon labeling experiments (Table I) and neuraminidase digestion studies.

The number of residues of GlcNAc, Man, and Gal have not been specified above. Previous studies indicated that GlcNAc and Man are present throughout intracellular light chain in amounts close to or identical with those of secreted light chain, 3 and 4 residues, respectively (Choi et al., 1971b; Melchers, 1971). It has recently been shown that GlcNAc and Man are preassembled, stepwise, as a unit attached to dolichol molecules in the membrane (Hsu et al., 1974). This preassembled polysaccharide is subsequently attached to the light chain, which is present as an acceptor molecule in some yet to be defined form. Thus, only one light chain bound intermediate in assembly of the core sugars is expected and our results are compatible with this. There is one interesting difference between the results of Hsu et al. (1974) and our results. Their in vitro synthesized core sugar light chain product contains Man and GlcNAc in a ratio of 5:2. Our core sugar light chain product, isolated from intact cells, contains the respective core sugars in a

[2] F. Melchers, unpublished experiments.

[3] P. M. Knopf, unpublished experiments.

ratio of 4:3. Further experimentation is required to resolve among the several possible explanations accounting for this difference.

Intracellular light chain lacks a full complement of Gal; analyses showed that there were an average of about 2 Gal residues per light chain (Choi et al., 1971b; Melchers, 1971). It is not possible at this time to conclude what are the average number of Gal residues present in B_{int}, $A1_{int}$, and $A2_{int}$. Species of these three glycopeptides with different quantities of Gal (1, 2, 3, or 4 residues) may exist but are not resolved by present techniques. It should be appreciated that cells were incubated for 3 hr, a time sufficient to achieve steady-state incorporation of radioactive sugars. Thus, short-lived intermediates would not be present as a high percentage of the total radioactivity in glycopeptide species.

Kinetic studies show that light chain in the RM is the precursor of that in the SM, which in turn is the source of secreted light chain (Choi et al., 1971b; Melchers, 1971). The distribution of different glycopeptides among the subcellular fractions should therefore be representative of the sequence of events occurring during intracellular transport. We performed one experiment in which the distribution of glycopeptides ($A2_{int}$, $A1_{int}$, B_{int}, and C_{int}) in light chain isolated from different subcellular fractions was analyzed (unpublished). The results were complex. The most significant finding was that glycopeptide C_{int} was the major species in light chain present in the RM fraction. To resolve the complexities of glycopeptide distribution in subcellular fractions will require kinetic analysis of cells pulse labeled with radioactive sugars.

We detected a significant pool of intracellular light chain with GlcNAc, Man, Gal, and NGNA residues in the carbohydrate moiety. Thus, we provide good evidence for attachment of NGNA to light chain inside the cell. There is no evidence from these data to account for the existence of different amounts of NGNA (0, 1, or 2 residues) on light chain polysaccharide, in both the intracellular and secreted fractions. Light chain molecules containing a full complement of sugars in the carbohydrate moiety are a negligible fraction of the intracellular pool. From the fucose-labeling experiments, which corroborate our previous chemical analyses, the attachment of Fuc must occur as the terminal event in carbohydrate assembly, close to the time when light chain is released from the cell.

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