Morales, M. F., Borejdo, J., Botts, J., Cooke, R., Mendelson, R. A., & Takashi, R. (1982) *Annu. Rev. Phys. Chem. 33*, 319–351.
Mornet, D., Bertrand, R., Pantel, P., Audemard, E., & Kassab, R. (1979) *Biochem. Biophys. Res. Commun. 89*, 925–932.
Mulrad, A., & Morales, M. E. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1003–1007.
Reisler, E. (1982) *Methods Enzymol. 85*, 84–93.
Rosenfeld, S. S., & Taylor, E. W. (1984) *J. Biol. Chem. 259*, 11920–11929.
Sekine, T., & Kielley, W. (1964) *Biochim. Biophys. Acta 81*, 336–345.
Shriver, J. W., & Sykes, B. D. (1982) *Biochemistry 21*, 3022–3028.
Spudich, J. A., & Watts, S. (1971) *J. Biol. Chem. 246*, 4866–4871.
Stern, O., & Volmer, M. (1919) *Phys. Z. 20*, 183–188.
Wagner, P. D., & Weeds, A. G. (1977) *J. Mol. Biol. 109*, 455–473.
Weeds, A. G., & Taylor, R. S. (1975) *Nature (London) 257*, 54–56.
Wells, C., & Bagshaw, C. R. (1984) *J. Muscle Res. Cell Motil. 5*, 97–112.
Wells, J. A., & Yount, R. G. (1982) *Methods Enzymol. 85*, 93–116.
White, H. D. (1977) *Biophys. J. 17*, 40a.
Wiedner, H., Wetzel, R., & Eckstein, F. (1978) *J. Biol. Chem. 253*, 2763–2768.

Preparation of a High-Affinity Photolabeling Reagent for the Gal/GalNAc Lectin of Mammalian Liver: Demonstration of Galactose-Combining Sites on Each Subunit of Rabbit Hepatic Lectin†

Reiko T. Lee* and Yuan Chuan Lee
Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218
Received June 17, 1986

ABSTRACT: On the basis of the knowledge that the D-galactose/*N*-acetyl-D-galactosamine-specific lectin of rabbit liver can tolerate a large group on the C-6 hydroxyl group of a galactoside [Lee, R. T. (1982) *Biochemistry 21*, 1045–1050], we prepared a high-affinity photolabeling reagent for this lectin from a triantennary glycopeptide fraction of asialofetuin. The C-6 hydroxyl group of a D-galactopyranoside was converted, under mild conditions, into a primary amino group. The procedure involves conversion of the hydroxyl group to an oxo group with galactose oxidase, followed by reductive amination using benzylamine and sodium cyanoborohydride. Catalytic hydrogenolysis of the benzylamino derivative yielded the desired 6-amino-6-deoxy-D-galactoside. A 4-azidobenzoyl group was attached to the newly produced amino group to yield a photoactivatable affinity-labeling reagent. The reagent labeled the Triton-solubilized, purified hepatic lectins of rabbit and rat in a *photo-* and *affinity*-dependent manner. All the polypeptide subunits of the lectins were labeled, indicating that each subunit contains at least one D-galactose-combining site. In the case of the rabbit hepatic lectin, the minor subunit (46 kDa) was labeled more efficiently than the major one (40 kDa).

The Gal/GalNAc-specific[1] lectin of mammalian livers (also known as the "asialoglycoprotein receptor") is a transmembrane protein present on the cell surface and the internal membranes of hepatocytes (Pricer & Ashwell, 1976; Harford & Ashwell, 1981; Chiacchia & Drickamer, 1984). The lectin has been purified in a detergent-solubilized form by affinity chromatography from livers of several mammalian species (Hudgin et al., 1974; Pricer & Ashwell, 1976; Baenziger & Maynard, 1980; Bezouska et al., 1985). The Triton-solubilized lectins from different species all exist as large molecular species (>200 kDa), composed of subunits of 40–60 kDa in size that are not held together by disulfide bonds. Although the lectin is capable of binding monovalent galactosides[2] ($K_d = 10^{-3}$–10^{-4} M), proper clustering of galactosyl residues within the ligand structure increases the affinity to the lectin enormously (Baenziger & Fiete, 1980; Connolly et al., 1982; Lee et al., 1983; Lee, R. T., et al., 1984). For instance, the Gal-terminated triantennary and tetraantennary oligosaccharides that exist in desialylated serum glycoproteins, such as asialoorosomucoid and asialofetuin, manifest up to a millionfold higher affinity toward this lectin on the hepatocyte surface than a simple galactoside, although the actual Gal concentration is increased by only 3–4-fold (Lee et al., 1983). The dramatic increase in affinity by clustering of sugars seems to be a unique property of the hepatic Gal/GalNAc lectin and possibly other animal lectins (Hoppe & Lee, 1983; Kuhlenschmidt & Lee, 1984).

†Supported by USPHS NIH Research Grants AM9970 and CA21901. Publication No. 1333 from the McCollum-Pratt Institute, The Johns Hopkins University.

[1] Abbreviations: ASOR, asialoorosomucoid; BOC, *tert*-butyloxycarbonyl; BSA, bovine serum albumin; Me_2SO, dimethyl sulfoxide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

[2] Unless otherwise specified, all sugars are of the D configuration and in pyranose form, and all amino acids are of the L configuration.

0006-2960/86/0425-6835$01.50/0

Photoaffinity labeling can be a powerful tool for probing the ligand–lectin interaction. In order to photoaffinity label the Gal/GalNAc-specific lectin in a purified state as well as in a complex environment, such as on the hepatocyte membrane, we needed to prepare a high-affinity reagent from a multivalent ligand. Baenziger and Fiete (1982) have prepared a photoaffinity reagent from one such high-affinity glycopeptide (desialylated fetuin triantennary glycopeptide) by attaching a photolyzable group on the peptide backbone of the glycopeptide. Though this reagent photolabeled the *Ricinus communis* lectin and the human hepatic lectin, labeling would occur at considerable distances from the Gal-combining site of the lectins. For the purpose of specific labeling, it is more desirable to place the labeling group near or on the Gal residues.

We have conducted detailed studies on the structural requirements for a galactose-containing ligand to be bound effectively by this lectin (Stowell et al., 1980; Lee, 1982; Lee et al., 1982). On the basis of these results, we deduced that a photolyzable group can be attached to C-6 of Gal residues without seriously compromising the binding affinity. To facilitate attachment of a photolyzable group, the 6-OH on Gal was converted to a 6-NH_2 group. Although such a conversion has been accomplished in the past (Lee et al., 1982), the conditions are too drastic for use with glycopeptides. In this paper we describe a relatively mild modification scheme to accomplish this goal. Once the amino group is generated, it can be used to attach a variety of groups, such as chemoactive or fluorescent groups, in addition to photolabeling groups. The reaction scheme was first tested on a model compound, methyl β-D-galactopyranoside, and then applied to a Gal-terminated triantennary glycopeptide for preparation of a high-affinity reagent. In this paper, we show that this reagent covalently labeled the isolated rat and rabbit hapatic lectins by a specific photoaffinity mechanism.

Materials and Methods

The following materials were obtained from the indicated sources: galactose oxidase (EC 1.1.3.9), catalase (EC 1.11.1.6), 2,4,6-trichlorophenyl *N*-BOC-L-tyrosinate,[1] and Triton X-100 (Sigma Chemical Co.); benzylamine hydrochloride, triethylamine, sodium cyanoborohydride, and palladium catalyst (10% on carbon) (Aldrich); methyl β-D-galactopyranoside (Pfanstiehl Labs, Inc.); succinimido 4-azidobenzoate (Pierce Chemical Co.); HEPES (Research Organics, Inc.); BSA (CRG-7 grade, Armour Pharmaceutical Co.); fetuin (CalBiochem); leucine aminopeptidase (EC 3.4.1.1) and carboxypeptidase Y (EC 3.4.16.1) (Boehringer-Mannheim Biochemicals); and carrier-free $Na^{125}I$ (Amersham). Neuraminidase from *Arthrobacter ureafaciens* was a gift from Dr. Y. Uchida (Uji, Japan).

Triton X-100 was purified according to Ashani and Catravas (1980) and stored in the dark. Gal_{44}-BSA is a modified BSA preparation containing, on the average, 44 residues of thiogalactoside per molecule of BSA (Lee et al., 1976).

Carbohydrate concentration was measured by using a phenol–sulfuric acid method (McKelvy & Lee, 1969). Concentration of 6-oxo-D-galactopyranoside was determined by the carbazole method (Avigad et al., 1962). All evaporations were done under reduced pressure with a rotary evaporator. TLC was carried out on precoated layers of silica gel F-254 (E. Merck) with solvent A (ethyl acetate–acetone, 4:1 v/v), solvent B (ethyl acetate–pyridine–water, 9:4:2 v/v), or solvent C (ethyl acetate–pyridine–acetic acid–water, 5:5:1:3 v/v). Carbohydrates were visualized by charring (ca. 140 °C) after spraying with 15% sulfuric acid in 50% ethanol. Amino-containing compounds were detected by spraying with 0.2% ninhydrin in 95% ethanol and heating the plates briefly. The trifluoroacetamido group was detected by the ninhydrin reagent after the plates were first sprayed with 1 M NaOH to expose the amino group by brief (1–2-min) standing at room temperature and then neutralized by spraying with glacial acetic acid. The carbonyl function was detected by spraying with 0.4% 2,4-dinitrophenylhydrazine in 2 M HCl. Glycopeptide derivatives were quantified by determining the mannose content after hydrolyzing samples in 4 M trifluoroacetic acid at 100 °C for 4 h and then analyzing the evaporated samples with an automated sugar analyzer basically as described previously (Lee, 1972). Elemental analyses were performed by Galbraith Labs, Inc. (Knoxville, TN). ^1H NMR spectra were obtained with a Varian 80-MHz Spectrometer.

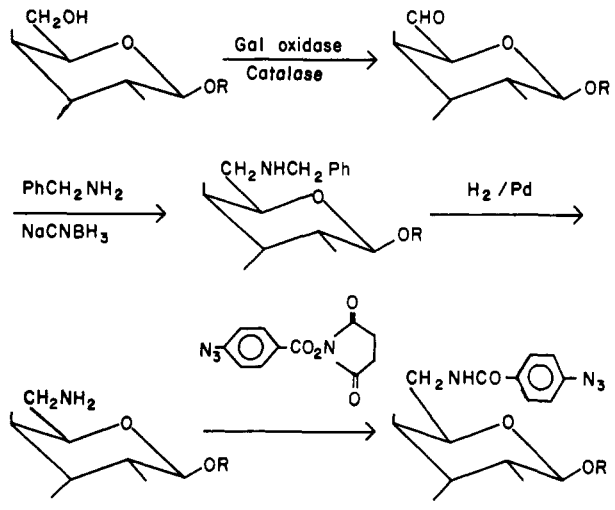
The Gal/GalNAc-specific lectin was isolated from rabbit or rat livers according to Hudgin et al. (1974). The affinity medium was prepared by coupling asialofetuin to partially hydrolyzed Sepharose 4B (Stults et al., 1983). The inhibition assay was carried out according to Connolly et al. (1982) using ^{125}I-ASOR as a reference ligand for estimation of the affinity of the glycopeptide derivatives for the lectin.

All handling of photoactive reagents was carried out under subdued light or in the dark. Tyrosylated 6-amino-6-deoxy-Gal glycopeptide (40–70 nmol) was iodinated with 2 or 5 mCi carrier-free $Na^{125}I$ by using a modification of the Chloramine T method (Greenwood et al., 1963) and then fractionated on a Sephadex G-10 column (0.6 × 18 cm) with water as eluant. The first radioactive peak (iodinated glycopeptide reagent) was evaporated to dryness and suspended in 0.2 mL of Me_2SO; to this was added 0.12 mL of 0.19 M succinimido 4-azidobenzoate in Me_2SO, and the solution was stored overnight at room temperature in the dark. The reaction mixture was fractionated on a second column of Sephadex G-10 (0.9 × 21 cm). The fractions of the first radioactive peak were combined and used as the stock solution of the photolabeling reagent. Photolysis was done in a quartz tube (0.6 × 7 cm) placed in the center of three photoflashes (without the diffuser) (Ji, 1979) which were synchronously controlled by slave units from Spiratone (New York, NY). Four synchronous flashes were sufficient to photolyze the reagent totally, as determined by the decrease of the UV absorption (270 nm) attributable to the reagent. Polyacrylamide gel electrophoresis (PAGE) of SDS-dissociated protein samples was carried out according to Laemmli (1970). ^{125}I-Labeled samples were generally applied to 1–3 lanes of a slab gel (7.5% acrylamide) with molecular weight markers and isolated rabbit or rat lectin applied on either side. After electrophoresis, gels were stained with Coomassie Blue and either dried and autoradiographed or cut horizontally in 2-mm- or $^1/_{16}$-in.-wide slices and counted for radioactivity. All radioactive samples were counted with a Packard PDG γ counter.

Results

Preparation of Photoaffinity Reagents. Prior to the preparation of the photoaffinity reagent, the 6-OH groups of the galactosyl residues on the glycopeptide were converted to amino groups by a series of reactions as shown in Scheme I. The 6-amino groups were then reacted with succinimido 4-azidobenzoate in the dark. The reaction sequence was first carried out with methyl β-D-galactopyranoside as the starting material, in order to establish the feasibility of the reaction scheme and to verify the formation of methyl 6-amino-6-deoxy-β-D-galactoside as the end product. The actual photoaffinity reagent was prepared from a triantennary glycopeptide fraction isolated from asialofetuin.[3]

Scheme I



Methyl 6-Deoxy-6-oxo-β-D-galactopyranoside. Methyl β-D-galactopyranoside was oxidized with a mixture of galactose oxidase and catalase by using conditions similar to those of Avigad et al. (1962). A mixture containing the galactoside (0.4 g, 2.06 mmol), galactose oxidase (450 units), catalase (1 mg), and a drop of toluene in 60 mL of 0.01 M sodium phosphate buffer, pH 7, was incubated for 5 days at room temperature. The yield of the 6-oxo derivative was estimated to be about 88% on the basis of the carbazole assay. TLC examination of the reaction mixture (solvent B) showed the appearance of a new spot that moved slower (R_f 0.40) than the starting material (R_f 0.49), and that could be charred as well as stained by the dinitrophenylhydrazine reagent. The reaction mixture was concentrated to ca. 5 mL, and the enzymes were removed by gel filtration using a column (2 × 33 cm) of Sephadex G-25 with 0.01 M sodium phosphate buffer, pH 7, as the eluant. Fractions containing the carbohydrate material were combined and evaporated to a syrup that contained ca. 10% of the unreacted starting material as well as the desired product. This material was used without further purification in the next step.

Methyl 6-(Benzylamino)-6-deoxy-β-D-galactopyranoside. The carbohydrate material obtained in the above step was dissolved in 6 mL of the phosphate buffer, pH 7. Benzylamine hydrochloride (2.37 g, 15.5 mmol) and $NaCNBH_3$ (0.3 g, 5 mmol) were added to this solution. This mixture was left overnight at room temperature and then fractionated on a column (5 × 190 cm) of Sephadex G-25 with 0.1 M NH_4OH as the eluant. The elution profile showed two well-separated peaks by the phenol–sulfuric acid assay. The TLC (solvent B) indicated the first peak to be methyl β-D-galactopyranoside (R_f 0.49), while the second peak, which was eluted even later than inorganic phosphate, contained mostly one component with an R_f of 0.30. The fractions of the second peak were combined and evaporated to yield ca. 0.5 g of solid. This product exhibited a UV absorption spectrum characteristic of a benzylamine derivative (maxima at 252, 258, 262, and 268 nm; ϵ_{258nm} = ~200).

Methyl 6-Amino-6-deoxy-β-D-galactopyranoside. The 6-benzylamino derivative obtained above was dissolved in 60% acetic acid (7 mL) and hydrogenated at room temperature overnight with 10% palladium on carbon (50 mg) in a micro-Brown hydrogenator (Brown & Brown, 1962). The mixture was filtered, evaporated to dryness, and fractionally crystallized from water–95% ethanol. The first crop of crystals did not char on a TLC plate after spraying with sulfuric acid and heating and was apparently inorganic phosphate. The second and the third crops of crystals had one identical spot on TLC (R_f 0.50 in solvent C) that gave a positive response to both charring and the ninhydrin reagent spray. This product comigrated with methyl 6-amino-6-deoxy-α-D-galactopyranoside, which was prepared by an entirely different procedure for converting 6-OH to 6-NH_2 via 6-*O*-tosyl and 6-azido derivatives (Lee et al., 1982).

Since trace inorganic contaminants could not be removed from the product by repeated recrystallization, it was peracetylated for the purpose of identification. A small amount (150 mg) of the product was stirred for a few hours with 4 mL each of pyridine and acetic anhydride. The mixture was concentrated to about half of the original volume and partitioned between chloroform and water. The chloroform layer was washed successively with cold solutions of 1.2 N sulfuric acid, saturated sodium bicarbonate, and 1 M NaCl. The chloroform layer was dried (anhydrous sodium sulfate), filtered, and evaporated to a small volume. The crystals that formed upon addition of diethyl ether and petroleum ether (bp 35–75 °C) were harvested: yield, 60 mg (29%); mp 108–110 °C; homogeneous by TLC in solvent A (R_f 0.33); ^1H NMR ($CDCl_3$) δ 1.69 (s, 6 H, $COCH_3$), 1.76 (s, 3 H, $COCH_3$), 1.88 (s, 3 H, $COCH_3$), 3.22 (s, 3 H, OCH_3). Anal. Calcd: C, 49.86; H, 6.42; N, 3.88. Found: C, 49.68; H, 6.63; N, 3.76.

Preparation of N-BOC-L-Tyrosylated Triantennary Glycopeptide. Bovine fetuin was digested for 3 days with Pronase under sterile conditions according to the method used for digestion of fibrinogen (Townsend et al., 1982). The digest was fractionated on a Bio-Gel P-2 column, and the carbohydrate-containing fraction was digested again in a similar fashion. The redigested material was then fractionated on a column (2.5 × 200 cm) of Sephadex G-50, and the carbohydrate-containing material was completely desialylated with *A. ureafaciens* neuraminidase at pH 5. After additional digestion with leucine aminopeptidase and carboxypeptidase Y, glycopeptide fractions from the Sephadex G-50 column were combined and lyophilized.

An *N*-BOC-L-tyrosyl group was attached to the existing amino terminus of the peptide portion of the glycopeptide in order to mask the primary amino group as well as to introduce a radioiodinatable group. The glycopeptide (63 mg, ca. 28 μmol) and 2,4,6-trichlorophenyl *N*-BOC-L-tyrosinate (57.5 mg, 125 μmol) were suspended in 2 mL of Me_2SO and stirred until a clear solution was obtained. After overnight incubation at room temperature, toluene (15 mL) was added to the reaction mixture, and the syrupy precipitate that formed was collected by decantation. The precipitate was washed twice with diethyl ether (by decantation) and dried. A neutral solution of this material showed a typical UV spectrum of tyrosine indicating 82% yield of the tyrosylated glycopeptide (ϵ_{277nm} = 1300). An estimate of the carbohydrate concentration by the phenol–sulfuric acid method (using a standard containing mannose and galactose in a 3:2 ratio) indicated the yield of the tyrosylated glycopeptide to be 85%. The yield calculated from the mannose content was 82% (see Materials and Methods).

Conversion of Galactosyl Residues of the Glycopeptide to 6-Amino-6-deoxygalactosyl Residues. The reaction sequence as described for methyl β-D-galactopyranoside (Scheme I) was carried out on the glycopeptide with some modification. The

[3] Although the triantennary glycopeptide from bovine fetuin was thought to be pure and to possess a structure as reported by Nilsson et al. (1979), our recent studies showed that a variant triantennary structure of ca. 100-fold inferior affinity was also present. Due to its greatly inferior affinity, this variant glycopeptide can be effectively ignored in all the affinity experiments (Townsend et al., 1986).

Table I: Synthetic Yield and Binding Affinity of Glycopeptide Derivatives

stages of Gal modification	yield per step (%)	K_i for purified rabbit lectin (μM)	K_i for rat hepatocytes (nM)
none	82	2	90
6-oxo	70	120	not inhibitory at 100 μM
6-(benzylamino)-6-deoxy	81	0.9	280
6-amino-6-deoxy	101	0.9	270
6-(4-azidobenzamido)-6-deoxy	73	1.4	430

tyrosylated glycopeptide (ca. 28 μmol) was treated with galactose oxidase (250 units) and catalase ($\sim$1 mg) for 3 days at room temperature, at which time, on the average, ca. 2 Gal residues had been oxidized per molecule of the glycopeptide. The enzymes were removed from the glycopeptide by gel filtration through a column (2 × 30 cm) of Sephadex G-50. The reductive amination was carried out overnight in a 4-mL solution containing an 8-fold excess of benzylamine hydrochloride over Gal residues and 0.25 M $NaCNBH_3$. The reaction mixture was purified on a column (2 × 143 cm) of Sephadex G-25 in 0.1 M ammonium hydroxide. Unlike the benzylaminated methyl glycoside, the glycopeptide derivative eluted well ahead of inorganic phosphate.

The benzylaminated glycopeptide was hydrogenolyzed in 18% (v/v) acetic acid with 10 mg of 10% palladium on carbon overnight at room temperature. The final product (45 mg) was dissolved in 0.9 mL of water and stored frozen. A small aliquot (25 μL) of this solution was evaporated to dryness, dissolved in Me_2SO (0.2 mL), and reacted with succinimido 4-azidobenzoate in the presence of triethylamine ($\sim$1 μL) for a few hours at room temperature. The product was purified by passing through a column (0.8 × 15 cm) of Sephadex G-10 in 0.1 M ammonium hydroxide. The carbohydrate-containing material was eluted well ahead of the excess reagent and byproducts.

A portion (ca. 5%) of glycopeptide at each step of modification was reserved for determination of concentration and binding affinity. The concentration of the glycopeptide derivatives was determined by measuring their mannose content after acid hydrolysis. The UV absorption spectra of all the derivatives, except the glycopeptide containing 6-(4-azidobenzamido)-6-deoxy-Gal, were similar to that of tyrosine with a molar absorbance at 277 nm in the range of 1600–1850. The glycopeptide containing a 4-azidobenzoyl group had an absorption maximum at 268 nm with a molar absorbance greater than 10 000 (the exact value could not be accurately determined due to its light sensitivity). The binding affinity of these glycopeptide derivatives to the Gal/GalNAc-specific lectin of mammalian livers was estimated by an inhibition assay using Triton-solubilized, purified lectin as well as isolated hepatocytes (Connolly et al., 1982). The synthetic yield and inhibition data for these glycopeptide derivatives are summarized in Table I.

Photoaffinity Labeling of Mammalian Liver Lectin. The binding affinity of the glycopeptide containing 6-(4-azidobenzamido)-6-deoxy-Gal, estimated by the inhibition assay, is about the same or slightly lower than that of the parent glycopeptide and is sufficiently high for affinity labeling. Most of the photoaffinity labeling experiments were carried out with the rabbit lectin. The lectin (ca. 25 μg) and the ^{125}I-labeled affinity reagent (0.1–0.5 μM) were incubated at 25 °C for 45 min in the dark in 0.5 mL of 0.05 M Tris-HCl buffer, pH 7.8, containing 1 M NaCl, 0.05 M $CaCl_2$, and 0.5% Triton X-100. The mixture was flashed 6 times, and noncovalently bound ^{125}I-labeled ligand was dissociated by adding 0.3 mL of 0.27 M EDTA (pH 7.8). After it was allowed to stand for at least $^1/_2$ h, the mixture was passed through a column (1 × 26 cm) of Sephadex G-150 with 5 mM HEPES buffer at pH 7.8 containing 0.2 M NaCl, 0.02% BSA, and 0.1% Triton X-100 in order to separate the labeled lectin from small, ^{125}I-containing compounds. Two kinds of blank experiments were carried out in parallel. For the photo blank, the sample was treated as above except flashing was omitted. For the afffinity blanks, competitive inhibitors, such as GalNAc (20 mM) or Gal_{44}-BSA (1.4 μM), were included in the incubation mixture, or the incubation was carried out in the absence of calcium ion and in the presence of EDTA (10 mM). The experimental conditions are tabulated in Table II, and elution profiles are shown in Figure 1. Figure 1 shows that the incorporation of radiolabel in the void volume peak was much reduced in all

Table II: Yield of Labeled Lectin under Various Conditions

expt	reaction mixture	flash	amount labeled (%)
A	buffer A	+	100[a]
B	buffer A (photo blank)	–	15–20
C	buffer A + 0.2 M GalNAc (affinity blank)	+	12
D	buffer A + 1.4 μM Gal_{44}-BSA (affinity blank)	+	13–20
E	buffer A + 10 mM EDTA–$CaCl_2$ (affinity blank)	+	4–14

[a] Total cpm in the void volume peak (Sephadex G-150) of experiment A (ligand binding + photolysis) was set to 100%.

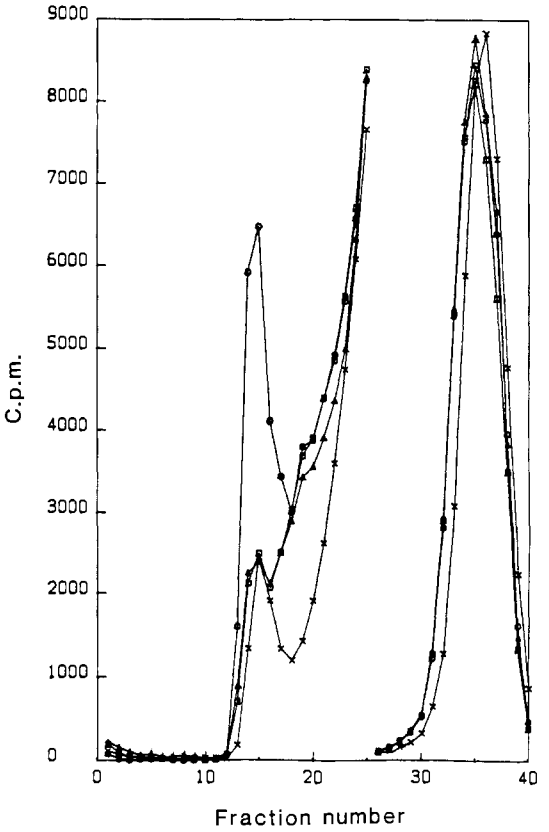


FIGURE 1: Elution profiles of the photolysis mixture on Sephadex G-150. The void volume is at fraction 15. (○) Experiment A, photolyzed, complete mixture; (×) experiment B, nonphotolyzed, complete mixture; (□) experiment C, photolyzed mixture containing 0.2 M GalNAc; (△) experiment E, photolyzed mixture containing 10 mM EDTA and no $CaCl_2$. The ordinate was scaled down 100-fold after fraction 26.

Table III: Effect of Calcium Chloride Concentration on Radiolabeling and ASOR-Binding Activity

calcium concn (mM)	^{125}I incorporation in the void volume peak[a] (10^3 cpm)	^{125}I-ASOR bound[b] (10^3 cpm)
0	0.6	1.5
0.16	0.2	0
0.8	0.8	1.6
4	1.7	4.0
20	2.4	14.0
50	2.5	16.0

[a] After subtracting appropriate blank values obtained without photolysis. [b] Assayed as described by Connolly et al. (1981), except that the calcium concentration was varied.

the blank experiments. Interestingly, all the affinity blanks (experiments C–E, Table II) had very similar elution profiles, which also resembled that of the photolyzed mixture in which the lectin was omitted (not shown). The efficiency of photolabeling in experiment A (Table II) ranged from 10% to 30%, if one assumes full recovery of the lectin at the void volume region and the binding stoichiometry of 1 molecule of ligand per 500 kDa (Kawasaki & Ashwell, 1976; Connolly et al., 1981). The incorporation of radioactivity in the void volume peak under blank conditions (experiments B–E) was calculated as the percent of incorporation in experiment A, and results from three sets of such experiments are summarized in Table II.

In another experiment, incubation of the photoaffinity reagent and lectin was carried out at various calcium ion concentrations, and the extent of ^{125}I incorporation into the void volume peak was compared with the binding activity at the corresponding calcium concentrations. As shown in Table III, ^{125}I incorporation increases as the binding activity increases.

To confirm that the radioactive material in the void volume peak was indeed the lectin covalently modified with the glycopeptide derivative, the radioactive material was treated with 2.9% SDS at 100 °C for 7 min and subjected to SDS–PAGE. Both autoradiography and direct counting of gel slices indicated that both (40- and 46-kDa) subunits of the rabbit lectin were labeled to a similar extent (Figure 2A,C). Since the ratio of the two polypeptides (46 kDa:40 kDa) is approximately 1:2 (Kawasaki & Ashwell, 1976), the 46-kDa band was apparently labeled more efficiently than the 40-kDa band under the conditions used. In addition to the monomeric bands, the radiolabel was often associated with dimeric and oligomeric bands. This is probably due to incompelte dissociation of the lectin by the SDS treatment, since Coomassie Blue staining of the nonphotolabeled lectins also indicated the presence of these bands. As expected, no labeled bands corresponding to the lectin were visible in the blank lane (Figure 2C). A limited number of photoaffinity labeling experiments carried out with the rat lectin showed that it was labeled as efficiently as the rabbit lectin under comparable conditions. Direct counting of gel slices suggested that all three polypeptide components (43, 52, and 60 kDa) of the rat lectin were labeled (Figure 2B). Since the rat lectin has an unusual subunit distribution of one major (43 kDa, ca. 75%) and two minor polypeptides (52 and 60 kDa, each 10–15%), it is difficult to assess the relative labeling efficiencies of the three bands.

Discussion

One of the unique properties of the Gal/GalNAc-specific hepatic lectins of mammalian species is their enormously enhanced affinity for branched oligosaccharides (Lee et al., 1983)

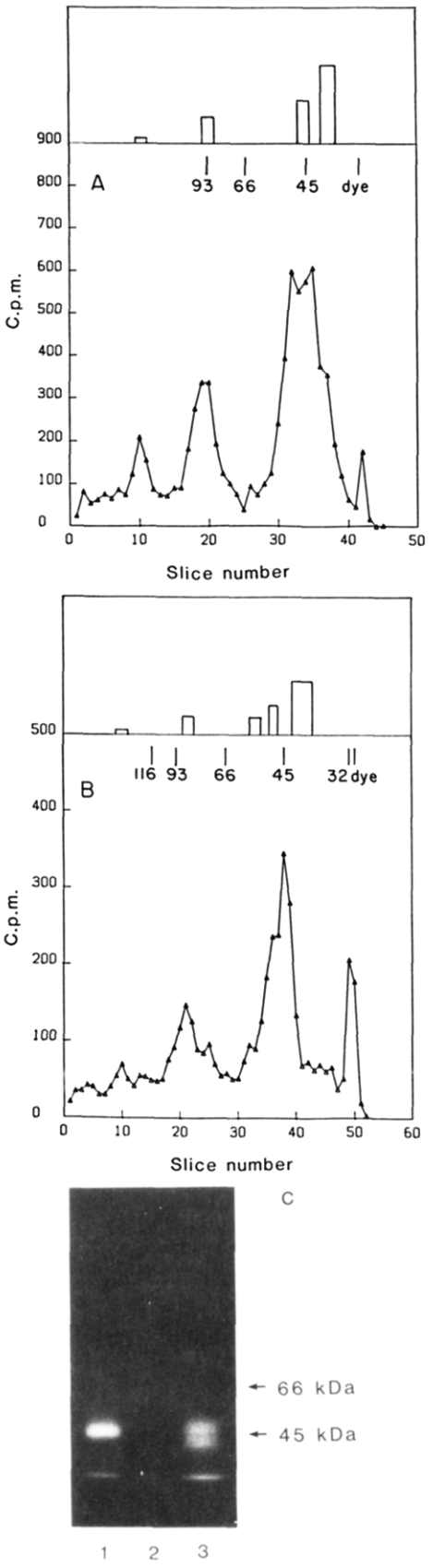


FIGURE 2: SDS–PAGE patterns of photoaffinity-labeled lectins. Gel slices were numbered starting from the top of the separating gel. (A) Rabbit lectin, 2-mm slices; (B) rat lectin, $^1/_{16}$-in. slices. The position and the molecular mass (kDa) of the protein standards are shown. The bar graphs (top panels) show the position and the relative Coomassie Blue stain intensity of the unlabeled lectin bands. (C) Autoradiograph of the photoaffinity-labeled lectins. Lanes: (1) Rat lectin, complete mixture; (2) rabbit lectin, mixture containing 10 mM EDTA and no $CaCl_2$; (3) rabbit lectin, complete mixture.

and cluster glycosides (Connolly et al., 1982; Lee R. T., et al., 1984) compared to that of simple galactosides. In designing a high-affinity reagent that can label not only the purified lectin but also the lectin in a complex environment, such as cell surfaces and membranes, it is highly desirable to prepare the reagent from such a high-affinity ligand. We chose the triantennary glycopeptide of asialofetuin as the starting material because of its high affinity and its relative ease of preparation (Baenziger & Fiete, 1980). A photoaffinity reagent for this lectin has in fact been prepared from such a glycopeptide by attaching a photolyzable group on the peptide portion of the molecule (Baenziger & Fiete, 1982). Though this reagent appeared to affinity label the human hepatic lectin, the site of labeling may be considerably removed (30–40 Å) from the Gal/GalNAc-combining site.

In order to study the distribution of Gal-combining sites among the subunits as well as subunit arrangement, we wanted to accomplish the labeling in the immediate vicinity of the Gal-combining site. On the basis of previously acquired information that modification of a galactosyl residue at the C-6 position with a bulky group, such as a tosyl group or a monosaccharide, only slightly lowers its binding affinity (Lee, 1982; Lee et al., 1982), we derivatized the fetuin triantennary glycopeptide by the reaction scheme shown in Scheme I. Estimation of the binding affinity by an inhibition assay confirmed that the product indeed retained its binding potency. Interestingly, the binding affinity of the 6-oxo-Gal derivative was at least 2 orders of magnitude weaker, which is in agreement with the earlier results of clearance studies of the galactose oxidase modified, desialylated serum glycoproteins (Morell et al., 1971). The low affinity of the 6-oxo-Gal derivative may seem contradictory to the proven dispensability of the 6-OH (Stowell et al., 1980), but it is likely that the 6-oxo group is engaged in either an intramolecular hemiacetal linkage with the 3-OH or an intermolecular hemiacetal linkage (Theander, 1962; Maradufu & Perlin, 1974), resulting in nonbinding entities.

As is evident from Table II and Figure 1, labeling of the lectin by the described reagent meets the criteria of a photoaffinity mechanism. Under a variety of conditions, the "nonspecific labeling" could be as low as 4% (in the presence of EDTA) and was never higher than 20% (in the presence of Gal_{44}-BSA) of the total radioactivity incorporated into the macromolecular fractions. The relatively inefficient blocking of labeling by Gal_{44}-BSA (at a concentration 1000-fold higher than its binding constant) compared to GalNAc or EDTA can be explained by the concept of a combining site lattice (Hardy et al., 1985). A smaller ligand such as the photoaffinity labeling reagent can occupy combining sites not available to a macromolecular ligand such as Gal_{44}-BSA.

Buffer A of Hudgin et al. (1974), which is used in the ligand-binding assay of the isolated lectin (by ammonium sulfate precipitation of the ^{125}I-labeled ligand–lectin complex followed by filtration through glass fiber filters), contains 0.6% BSA for the purpose of minimizing nonspecific binding of the ^{125}I-labeled ligand to the filter. When photolabeling of the lectin was carried out in the presence of 0.6% BSA, a third radioactive peak about the size of the void volume peak seen in Figure 1, experiment A, was apparent. This peak, which eluted between the void volume and the included peaks of the Sephadex G-150 profile (not shown), made the estimation of ^{125}I incorporation into the lectin peak inaccurate. For this reason BSA was usually excluded in the photolysis experiments. The observation that BSA and the lectin were labeled to a similar extent under the described conditions indicates that the efficiency of the BSA labeling is less than 0.1% of the lectin labeling, since the mixture contained more than a 1000-fold higher concentration of BSA than the lectin (on a weight basis). This result further supports the specificity of the lectin labeling, in addition to the greatly reduced labeling of the lectin in the presence of a competitive inhibitor or in the absence of calcium ion.

The photoaffinity labeling experiments reported here were carried out primarily with the solubilized, purified lectin of rabbit liver rather than that of rat liver, since more is known about the binding characteristics of the former (Kawasaki & Ashwell, 1976; Sarkar et al., 1979; Anderson et al., 1982; Lee, 1982; Lee et al., 1983). A limited number of experiments with the rat liver lectin gave very similar results overall. Since the molecular mass of the photolabeling reagent is ca. 2500 Da, the labeled subunit bands in the SDS–PAGE appeared about 2–3 kDa larger than usual. The fact that all of the Coomassie Blue bands of the rabbit (40 and 46 kDa) and the rat (43, 52, and 60 kDa) lectins were labeled under affinity conditions suggests that each polypeptide contains at least one Gal/GalNAc-combining site. To the best of our knowledge, this is the first demonstration that both subunits of the rabbit hepatic lectin contain a carbohydrate-combining site. Recently, Hsueh et al. (1986) demonstrated that the carbohydrate-binding site resides in a 17-kDa C-terminal portion of the major polypeptide (43 kDa) of the rat hepatic lectin. However, their experiment does not address whether a similar site exists in the minor polypeptide subunits (52 and 60 kDa). In the case of the rabbit lectin, more label was incorporated into the 46-kDa band compared to the 40-kDa band on a weight basis. This may be due to the presence of more than one Gal-combining site on the 46-kDa subunit or the stronger ligand-binding affinity of the 46-kDa subunit.

It has been postulated (Lee, Y. C., et al., 1984) that the Gal-combining sites of the lectin on the hepatocyte cell surface are organized in a special manner and this spatial arrangement is responsible for the enormously enhanced binding affinity for certain Gal cluster ligands. It is likely, then, that subunits of the lectin are also clustered tightly in an orderly fashion on the cell surface. As shown earlier (Lee, R. T., et al., 1984) and as illustrated in Table I, this cluster effect is considerably diminished when the Triton-solubilized, purified lectin is used. We recently reported (Lee & Lee, 1985) that, in contrast to the solubilized lectin, the photoaffinity labeling of the lectin on rat hepatocytes resulted in a disproportionately large incorportion of the radiolabel into one of the minor subunits. We speculate that the arrangement of subunits in the solubilized lectin is somewhat different from that which exists on cell membranes and is responsible for the diminished cluster effect of the soluble lectin as well as the dissimilar subunit labeling pattern observed for the cell-associated and soluble lectins. This intriguing phenomenon of asymmetric photolabeling is currently under investigation.

ACKNOWLEDGMENTS

We thank Dr. Reid Townsend and Michael Rosen for the generous supply of the fetuin glycopeptide and Dr. June K. Bronfenbrenner for performing ^{1}H NMR spectroscopy.

Registry No. Methyl β-D-galactopyranoside, 1824-94-8; methyl 6-deoxy-6-oxo-β-D-galactopyranoside, 52571-76-3; methyl 6-(benzylamino)-6-deoxy-β-D-galactopyranoside, 104324-31-4; methyl 6-amino-6-deoxy-β-D-galactopyranoside, 83377-36-0.

REFERENCES

Anderson, T. T., Freytag, J. W., & Hill, R. L. (1982) *J. Biol. Chem. 257*, 8036–8041.

Ashani, Y., & Catravas, G. N. (1980) *Anal. Biochem. 109*, 55–62.
Avigad, G., Amaral, D., Asensio, C., & Horecker, B. L. (1962) *J. Biol. Chem. 237*, 2736–2743.
Baenziger, J. U., & Fiete, D. (1980) *Cell (Cambridge, Mass.) 22*, 611–620.
Baenziger, J. U., & Maynard, Y. (1980) *J. Biol. Chem. 255*, 4607–4613.
Baenziger, J. U., & Fiete, D. (1982) *J. Biol. Chem. 257*, 4421–4425.
Bezouska, K., Karaskova, H., Taborsky, O., Kofronova, O., Vorisek, J., Kubrycht, J., & Kocourek, J. (1985) *Lectins: Biol., Biochem., Clin. Biochem. 4*, 353–367.
Brown, C. A., & Brown, H. C. (1966) *J. Org. Chem. 31*, 3989–3995.
Chiacchia, K. B., & Drickamer, K. (1984) *J. Biol. Chem. 259*, 15440–15446.
Connolly, D. T., Hoppe, C. A., Hobish, M. K., & Lee, Y. C. (1981) *J. Biol. Chem. 256*, 12940–12948.
Connolly, D. T., Townsend, R. R., Kawaguchi, K., Bell, W. R., & Lee, Y. C. (1982) *J. Biol. Chem. 257*, 939–945.
Greenwood, F. C., Hunter, N. M., & Glover, J. S. (1963) *Biochem. J. 89*, 114–123.
Hardy, M. R., Townsend, R. R., Parkhurst, S. M., & Lee, Y. C. (1985) *Biochemistry 24*, 22–28.
Harford, J., & Ashwell, G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1557–1561.
Hoppe, C. A., & Lee, Y. C. (1983) *J. Biol. Chem. 258*, 14193–14199.
Hsueh, E. C., Holland, E. C., Carrera, G. M., & Drickamer, K. (1986) *J. Biol. Chem. 261*, 4940–4947.
Hudgin, R. L., Pricer, W. E., Ashwell, G., Stockert, R. J., & Morell, A. (1974) *J. Biol. Chem. 249*, 5536–5543.
Ji, T. H. (1979) *Biochim. Biophys. Acta 559*, 39–69.
Kawasaki, T., & Ashwell, G. (1976) *J. Biol. Chem. 251*, 1296–1302.
Kuhlenschmidt, T. B., & Lee, Y. C. (1984) *Biochemistry 23*, 3569–3575.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lee, R. T. (1982) *Biochemistry 21*, 1045–1050.
Lee, R. T., & Lee, Y. C. (1985) in *Glycoconjugates, Proceedings of the VIIIth International Symposium* (Davidson, E. A., Williams, J. C., & DiFerrante, N. M., Eds.) p 477, Praeger, New York.
Lee, R. T., Myers, R. W., & Lee, Y. C. (1982) *Biochemistry 21*, 6292–6298.
Lee, R. T., Lin, P., & Lee, Y. C. (1984) *Biochemistry 23*, 4255–4261.
Lee, Y. C. (1972) *Methods Enzymol. 28*, 63–73.
Lee, Y. C., Stowell, C. P., & Krantz, M. J. (1976) *Biochemistry 15*, 3956–3962.
Lee, Y. C., Townsend, R. R., Hardy, M. R., Lönngren, J., Arnarp, J., Haraldsson, M., & Lönn, H. (1983) *J. Biol. Chem. 258*, 199–202.
Lee, Y. C., Townsend, R. R., Hardy, M. R., Lönngren, J., & Bock, K. (1984) in *Biochemical and Biophysical Studies of Proteins and Nucleic Acids* (Lo, T. B., Liu, T. Y., & Li, C. H., Eds.) pp 349–360, Elsevier, New York.
McKelvy, J., & Lee, Y. C. (1969) *Arch. Biochem. Biophys. 132*, 99–110.
Morell, A., Gregoriadis, G., Scheinberg, I. H., Hickman, J., & Ashwell, G. (1971) *J. Biol. Chem. 246*, 1461–1467.
Muradufu, A., & Perlin, A. (1974) *Carbohydr. Res. 32*, 127–136.
Nilsson, B., Norden, N. E., & Svensson, S. (1979) *J. Biol. Chem. 254*, 4545–4553.
Pricer, W. E., Jr., & Ashwell, G. (1976) *J. Biol. Chem. 251*, 7539–7544.
Sarkar, M., Liao, J., Kabat, F. A., Tanabe, T., & Ashwell, G. (1979) *J. Biol. Chem. 254*, 3170–3174.
Stowell, C. P., Lee, R. T., & Lee, Y. C. (1980) *Biochemistry 19*, 4904–4908.
Stults, N. L., Lin, P., Hardy, M., Lee, Y. C., Uchida, Y., Tsukada, Y., & Sugimori, T. (1983) *Anal. Biochem. 135*, 392–400.
Townsend, R. R., Hilliker, E., Li, Y.-T., Laine, R. A., Bell, W. R., & Lee, Y. C. (1982) *J. Biol. Chem. 257*, 9704–9710.
Townsend, R. R., Hardy, M. R., Wong, T. C., & Lee, Y. C. (1986) *Biochemistry 25*, 5716–5725.