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Note

An improved method for the preparation of derivatives of reducing oligosaccharide with 2-(4-aminophenyl)ethylamine*

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INTRODUCTION

Oligosaccharides alone, or as components of various glycoconjugates, participate in a wide variety of biological processes[1–3]. Isolation and characterization of oligosaccharides is often facilitated by generating or isolating an oligosaccharide-specific antibody. Because of their small size, oligosaccharides behave as simple haptens and must be linked either to carrier proteins[4] or to a solid support (NBr-activated Sepharose) in order to raise and isolate a specific antibody[5].

Many methods have been developed for linking a carbohydrate to carriers and solid supports[4–10]. Of these, the method of Zopf *et al.*[4], which utilizes 2-(4-aminophenyl)ethylamine as a linker, offers special advantages. In this method, the carbonyl atom of the terminal reducing monosaccharide residue reacts with the primary alkylamine group of the linker to form an unstable intermediate, which is reduced with sodium borohydride to yield a stable secondary amine. The derivatized sugar may now easily and efficiently be coupled *via* the terminal arylamino group of the linker[4] by a diazo bridge to lysine residues of a protein, or to cyanogen bromide-activated Sepharose to form an isourea linkage[5].

The procedure of Zopf *et al.*[4] is especially useful as it can be used with sugar haptens that are available only in small quantities or contain labile linkages (or both). However, this procedure has two serious limitations, *i.e.*, (a) although it has been reported that, in some cases, the yield of derivative may be quantitative, in other cases, the yield may be considerably lower[5]; and (b) the oligosaccharide being derivatized must be soluble in the linker 2-(4-aminophenyl)ethylamine[5]. In our experience using oligosaccharides isolated from cell membrane glycolipids, we observed that, although di- to tetra-saccharides may be soluble enough in 2-(4-amino-

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phenyl)ethylamine for some reaction to occur, higher mol.-wt. oligosaccharides were very poorly soluble, even at elevated temperatures, in the linker which resulted in the formation of little or no derivatized product. As most of the oligosaccharides that we isolated from the glycolipids of *Leishmania* sp. had generally a higher mol. wt. than tetrasaccharide, the method described herein was developed in order to use 2-(4-aminophenyl)ethylamine with all the advantages previously described[4], but with higher potential yields and no limitation on the size of oligosaccharide derivatized.

EXPERIMENTAL

Materials. — Oligosaccharides used in these experiments were isolated from the glycolipids of *Leishmania mexicana amazonensis* by the procedure described below. The promastigote form of the organism was harvested from spent culture medium by centrifugation (4000*g* for 30 min at 4°) and rinsed in Hanks' balanced salt solution (without glucose) by repeated suspension and centrifugation (4 times at 4000*g*, for 10 min at 4°).

Methods. — The promastigotes were first extracted with 2:3 2-propanol–hexane (25 mL of solvent/1 mL of packed promastigote)[11], followed by an equal volume of 15:15:5:1:0.017 water–ethanol ethyl ether–pyridine–NH_4OH (solvent *E*)[12]. The material extracted by solvent *E* is a glycolipid which was hydrolyzed by trifluoroacetolysis for the release of oligosaccharide residues[13]. The carbohydrate

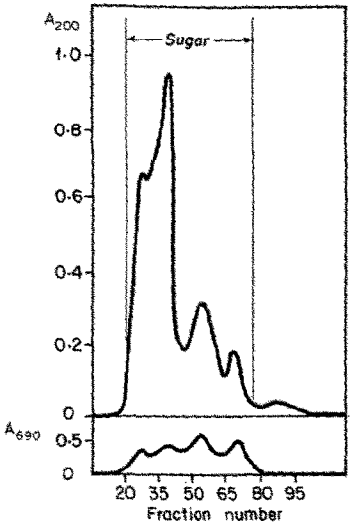


Fig. 1. Isolation of products by gel filtration chromatography from ether-extracted, water phase of trifluoroacetolysis reaction mixture. Chromatography on a Bio-Gel P-2 column (2.5 × 35 cm) heated at 65° and eluted with distilled water. The eluant was continuously monitored at 200 nm and collected in 2.2-mL fractions. Area between arrows (labeled "sugar") includes fractions found to contain sugar by t.l.c. analysis. Lower curve (A_{690}), represents the reducing power.

content of each extract by *E* was determined by the method of Dubois *et al.*[14]. A portion of extract containing 10 mg of sugar) was placed in the Teflon cup of a Parr bomb (Parr Instrument Co., Moline, IL). The extract was dried under a stream of dry N_2, trifluoroacetic anhydride (4.0 mL) and trifluoroacetic acid (40 μL) were added, and N_2 replaced by dry Ar. The cup was sealed, the bomb incubated for 48 h at 100°, and then cooled. The oligosaccharides were isolated by ether–water partition extraction[15]. The water phase was taken to dryness by flash evaporation, the residue dissolved in distilled water (5.0 mL), and the solution chromatographed on a Bio-Gel P-2 column (Bio-Rad, Rockville Centre, NY; 2.5 × 35 cm; 65°) with distilled water as eluant[15,16]. An oligosaccharide profile was established by continuous monitoring of the column effluent at 200 nm with a V4 variable wavelength detector (ISCO, Lincoln, NB) (Fig. 1). Sugars were detected by spotting 50 μL of each 1.5-mL fraction on a scored Silica Gel G plate (Analtech, Inc., Newark, DE) and treating with orcinol[17]. All carbohydrate material eluted from the column was found to be reducing[18] and did not absorb light appreciably at 285 nm. The oligosaccharides eluted at, or near the void volume (fractions 20–40; Fig. 1) were pooled, and 5.0 μg of this material was used in subsequent experiments. This material was selected for use in the present experiments because, in the procedure of Zopf *et al.*[13], these oligosaccharides (~8–14 saccharide units[19]) failed to dissolve in 2-(4-aminophenyl)ethylamine and little if any was derivatized.

Preparation of derivatives. — The anhydrous oligosaccharide (5 mg) was placed into a 250-mL round-bottomed flask and dissolved in anhydrous dimethyl sulfoxide (5 mL). To this was added 2-(4-aminophenyl)ethylamine (0.5 mL) and a magnetic stirring bar. A 3.0-mL reactivial (Pierce Chemical Co., Rockford, IL) with P_2O_5 (40 mg) was suspended by a steel wire (above the solution) within the round-bottomed flask. The flask was flushed with Ar and sealed with a double layer

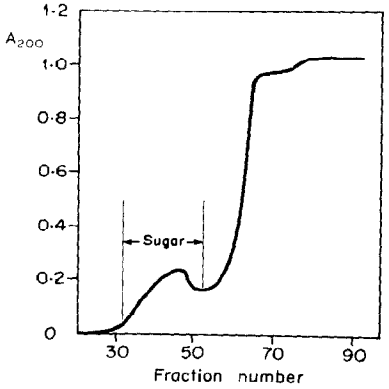


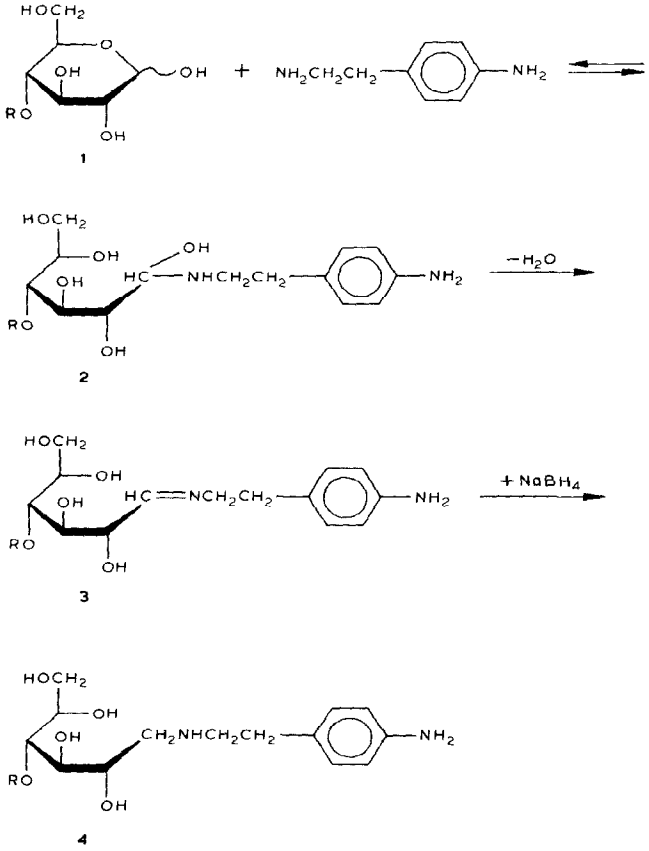
Fig. 2. Isolation of products by gel-filtration chromatography after treatment of oligosaccharides with 2-(4-aminophenyl)ethylamine in dimethyl sulfoxide and reduction with sodium borohydride. Chromatography on a 2.5 × 120 cm column of Sephadex G-100, eluted with mM ammonium acetate, pH 5.6. The area between arrows (labeled "sugar") includes fractions found to contain sugar by t.l.c. analysis.

of parafilm held firmly in place by several rubber bands. The sealed flask was placed in an incubator at 37° and the contents stirred slowly for 48 h. The flask was unsealed, $NaBH_4$ (12 mg) added, and P_2O_5 renewed. Again the flask was flushed with Ar and the seal reestablished. After 24 h, the content of the flask was removed into a 20-mL beaker, ice-cold, glass-distilled water (5.0 mL) added, and the pH adjusted to 5.5 with glacial acetic acid. After bubble formation had ceased (a few min), the solution was chromatographed on a Sephadex G-10 column (Pharmacia, Piscataway, NJ; 2.5 × 120 cm) and eluted with 0.1M ammonium acetate (pH 5.5). The column effluent was monitored continuously at 200 nm and the sugar content determined as previously described (see Fig. 2). Some unreacted 2-(4-aminophenyl)ethylamine may be present as a contaminant and this may be removed by ion exchange chromatography (see ref. 5). The fractions containing sugar were pooled and the content determined[14]. The sugar quantity (~4.9 mg) was essentially the same as that added to the reaction flask, suggesting that little or no oligosaccharide was lost during the process. An aliquot of the solution was chromatographed on the heated P-2 column, to established that little or no change in size or distribution of oligosaccharides had occurred. Evidence for successful derivatization of all size classes of oligosaccharides was provided by the strong absorption at 285 nm now shown by all sugar-containing fractions.

Since the exact molecular weight of the oligosaccharides was not precisely known, the extinction coefficient of the linker could not be used to assess spectrophotometrically the proportion of oligosaccharide successfully derivatized. Therefore, this value was established by determining what proportion of the processed oligosaccharides would anneal to bovine serum albumin. The latter procedure was accomplished by use of a modification of the method of Zopf *et al.*[4]. The reaction components were kept in the previously described ratios, but the volume of the reaction mixture was reduced two-fold and the coupling reaction conducted at pH 9.5 instead of 11.5. In control experiments, underivatized oligosaccharides were processed in a similar manner. The products of the annealing procedure were chromatographed on a Sephadex G-150 column (1.5 × 100 cm; Pharmacia) in 0.1M ammonium acetate, pH 9.5, and the amount of sugar in the peak associated with the protein and that found in the post-void volume fractions quantitated[14].

RESULTS AND DISCUSSION

The aforementioned results showed that, without derivatization, no sugar became associated with the protein, whereas after derivatization, all of the oligosaccharides, regardless of size became associated with the protein. The derivatization procedure, as outlined in Scheme 1, involves three steps. In the first, reaction of the carbonyl groups of the reducing oligosaccharide **1** with the alkylamine group of 2-(4-aminophenyl)ethylamine forms a carbinolamine intermediate **2**. This reaction is a nucleophilic addition and it may occur in the presence of polar aprotic solvents, such as dimethyl sulfoxide. The second and rate-limiting step in the pro-



cess involves dehydration of the carbinolamine **2** to yield a Schiff base[19] (**3**). This step is favored and driven to completion by maintaining anhydrous conditions through the use of a strong drying agent (phosphorus pentaoxide). The final step involves reduction of the Schiff base **3** to a secondary amine **4** with sodium borohydride. If quantitative yields of end product are to be realized, step 3 may not be initiated until the reactions in steps 1 and 2 have gone to completion, because sodium borohydride will reduce the aldehyde or ketone groups of free oligosaccharides to alcohols, making them poorly reactive with alkylamines. In the procedure described herein, the conditions favor the completion of steps 1 and 2 and, therefore, little or no reducing oligosaccharide is present to be reduced by sodium borohydride. On the other hand, in older procedures[5], unreacted oligosaccharide may have been present and this may not only explain the failure to obtain quantitative yields but also the presence of nonreactive byproducts. Thus, the high-mol. wt. oligosaccharides from *L. mexicana amazonensis*, which were resistant to phenethylamine derivatization by the older procedure, were quantitatively derivatized and

coupled to protein. In synthesizing neoglycoproteins from various oligosaccharides, it may be necessary, for optimum activity, to vary the molar ratios of oligosaccharide to protein. Under these conditions, the reaction will generally not be quantitative although, in this investigator's experience with maltoheptaose, the coupling efficiency remained high over a wide range of oligosaccharide-to-protein ratio.

The present procedure has been used to quantitatively derivatize oligosaccharides of all sizes from such divergent organisms as *Leishmania tropica, Leishmania donovani, Trichomonas vaginalis, Schistosoma mansoni,* and *Nematospiroides dubius*. The results have been extremely consistent and the neoantigens produced by coupling the derivatized oligosaccharides to protein reacted strongly with a preformed, specific anti-oligosaccharide antibody. These findings suggest that the present procedure, which is conducted under very mild conditions, has broad applicability and conserves the chemical integrity of the oligosaccharide haptens.

REFERENCES

1 B. K. BRANDLEY AND R. L. SCHNAAR, *J. Leukocyte Biol.*, 40 (1986) 97–111.
2 T. FEIZI, *Nature (London)*, 312 (1985) 53–57.
3 R. HARRISON AND G. G. LUNT, *Biological Membranes, their Structure and Function*, 2nd edn., Blackie & Son, Glasgow, 1980, pp. 130–165.
4 D. A. ZOPF, C.-M. TSAI, AND V. GINSBURG, *Methods Enzymol.*, 50 (1978) 163–169.
5 D. A. ZOPF, D. F. SMITH, S. DRZENIEK, C.-M. TSAI, AND V. GINSBURG, *Methods Enzymol.*, 50 (1978) 171–175.
6 C. P. STOWELL AND Y. C. LEE, *Methods Enzymol.*, 83 (1982) 278–288.
7 R. T. LEE AND Y. C. LEE, *Methods Enzymol.*, 83 (1982) 289–294.
8 K. HIMMELSPACH AND G. KLEINHAMMER, *Methods Enzymol.*, 50 (1978) 222–231.
9 J. LÖNNGREN AND I. J. GOLDSTEIN, *Methods Enzymol.*, 50 (1978) 160–162.
10 G. R. GRAY, *Methods Enzymol.*, 50 (1978) 155–159.
11 N. S. RADIN, *Methods Enzymol.*, 72 (1981) 5–7.
12 S. J. TURCO, M. A. WILKERSON, AND D. R. CLOWSON, *J. Biol. Chem.*, 259 (1984) 3883–3889.
13 A. GUNNARSSON, J. LUNDSTEN, AND S. SVENSSON, *Acta Chem. Scand., Ser., B*, 38 (1984) 603–609.
14 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
15 M. JOHN AND H. DELLWEG, *Sep. Purif. Methods*, 2 (1973) 231–257.
16 K. YAMASHITA, T. MIZUOCHI, AND A. KOBATA, *Methods Enzymol.*, 83 (1982) 105–126.
17 W. W. CHRISTIE, *Lipid Analysis: Isolation, Separation, Identification, and Structural Analysis of Lipids*, 2nd edn., Pergamon Press, New York, 1982, p. 120.
18 J. T. PARK AND M. J. JOHNSON, *J. Biol. Chem.*, 181 (1949) 149–151.
19 G. M. LOUDON, *Organic Chemistry*, Addison–Wesley, Reading, Massachusetts, 1984, pp. 904–905.