PREPARATION OF ^{125}I-LABELED OLIGOSACCHARIDE DERIVATIVES WITH THE AID OF 3-(4-HYDROXYPHENYL)PROPIONIC ACID *N*-HYDROXYSUCCINIMIDE ESTER+

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Received 26 November 1976

1. Introduction

The study of the binding of oligosaccharides to antibodies has been greatly facilitated by the availability of methods for the tritiation of sugars [1–3] which has enabled their use in a radiolabeled state. No such methods have been described for radio-iodinating oligosaccharides, though both ^{125}I and ^{131}I offer some distinct advantages over tritium, such as easier measurement and the higher specific activities attainable.

In the present work various oligosaccharides were converted into ^{125}I-labeled derivatives. The procedure was based on reductive amination of the oligosaccharide to its 1-amino-1-deoxy alditol, as described by Wiegandt and Ziegler [4] followed by attachment of a phenolic group by acylation with 3-(4-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester (HPPE). This reagent has been introduced by Bolton and Hunter [5] in a novel technique of protein radioiodination. In our procedure acylation and iodination were combined in one preparative step by successive addition of the ester and $^{125}I^-$/chloramine-T to the aminoalditol. The products are obtained by chromatographic separation. This method has proved to be convenient and successful in producing ^{125}I-labeled oligosaccharide derivatives, whose utility for binding measurements has been demonstrated in binding assays with adequate antibodies.

+ This paper is considered as part I of a series: Preparation and immunological uses of radio-iodinated oligosaccharide derivatives

2. Materials and methods

2.1. *Materials*

Isomaltotetraose (IM4) was obtained from dextran as described by Taylor and Whelan [6]. 1,3-α-Glucan and its subunit α-nigerosyl-1,3-nigerose (N 4) were prepared by the methods of Johnston [7]. The capsular polysaccharide of *Klebsiella pneumoniae* B 5055 (01 : K 2) was obtained according to Jann et al. [8]. Conversion into the octameric and tetrameric subunits Kl 8 and Kl 4 was effected by phage degradation [9] as by Thurow et al. [10]. MOPC 104E IgM was produced in tumor bearing Balb/c mice and purified according to Hiramoto et al. [11]. The tumor was kindly donated by Dr M. Potter, National Cancer Institute, Bethesda, Maryland. Anti-dextran serum was obtained by immunizing rabbits with dextran coupled to edestin as described earlier [12]. Anti-Kl 4/Kl 8 serum was taken from rabbits immunized with a Kl 4–edestin conjugate [9].

2.2. *Preparation of 1-amino-1deoxy alditols*

The oligosaccharide (10 μmol), 2.5 mg of sodium cyanoborohydride (EGA-Chemie, Steinheim/Albuch, FRG) and 25 mg of ammonium acetate were dissolved in 750 μl of methanol/water (2:1, v/v). The mixture was refluxed for 6 h (IM 4, N 4) or stirred at 37°C for 6 days (Kl 4, Kl 8). After evaporation to dryness the residues were subjected to preparative high-voltage electrophoresis on Whatman 3 MM paper in pyridine/acetic acid/water (10:4:86, v/v/v, pH 5.3). The aminoalditols were localized by staining with ninhydrin [13] and periodate/alkaline silver nitrate [14], eluted and the eluates lyophilized. The yields were about 30% of the theoretical.

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2.3. *Acylation and radioiodination*

To 100 μl of an aqueous solution of the aminoalditol (0.15 μmol), 100 μl of borate buffer, pH 8.5 [15] and 50 μl of a solution of HPPE (from ICN-Pharmaceuticals, Cleveland, Ohio, 1 mg of HPPE was dissolved in a few drops of ethanol and made up to 5 ml with water) were added. The mixture was kept at 0°C for 15 min, 3 μl of carrier free $Na^{125}I$ solution (100 mCi/ml, Amersham-Buchler) were added, followed by 50 μl of a solution of chloramine-T (100 μg) in water. The reaction was allowed to proceed at room temperature. After 5 min it was terminated by addition of 240 μg of sodium metabisulfite dissolved in 100 μl of water. The mixture was then chromatographed on Sephadex G-25 (for details see legend of fig.1). In the case of Kl 4, in which Sephadex had failed to separate the labeled conjugate from excess $^{125}I^-$ (fig.1), separation was achieved by absorbing both components on Dowex AG 1 X 2 chloride (1 X 10 cm column) and subsequent elution of the conjugate with 50% acetic acid. The eluate was then neutralized with NaOH. The efficiencies of radioiodination ranged from 75% (IM 4, N 4) to about 30% (Kl 8, Kl 4). The chromatographic fractions containing the radiolabeled oligosaccharide derivatives were pooled and the pools used for the binding measurements as decribed below.

2.4. *Binding assays*

2.4.1. Equilibrium dialysis

Dianorm equipment, from Bachofer, Reutlingen (FRG) with teflon microcells, was used. Dilutions were made in Dulbecco's PBS [16] for measurements of the binding of N 4 to MOPC 104E IgM and in 0.05 M phosphate buffered saline, pH 8.0, for measurements of the binding of IM 4 to antidextran antibodies. Hapten solutions: to a series of dilutions

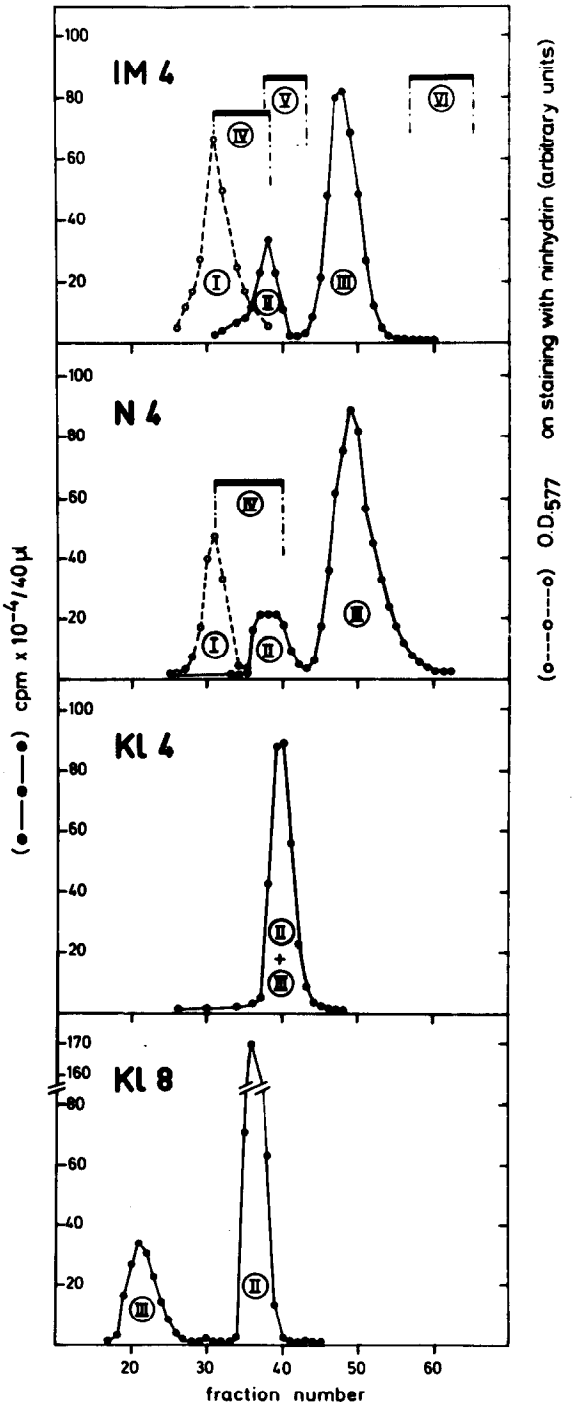


Fig.1. Chromatography of iodination mixtures on Sephadex G-25 (column 2.7 X 35 cm, eluent water containing 1% *n*-butanol, fraction size 4.7 ml). Elution patterns: (I) unreacted aminoalditols, (II) excess $^{125}I^-$, (III) iodinated HPP–oligosaccharide conjugates, (IV) non-iodinated HPP–oligosaccharide conjugates, (V) $^{125}I^-$–HPP, (VI) HPP. The identity of the products (III) was verified by binding to adequate antibodies (figs 2 and 3). (V) Was localized by iodination of HPP in absence of sugar and passage through the G-25 column. The detection of primary amino groups was effected with ninhydrin [18]. Sugar moieties were detected subsequent to spotting fraction samples on paper by staining with periodate/alkaline silver nitrate [14]. Phenol groups were analogously detected by staining with diazotized sulfanilic acid [19].

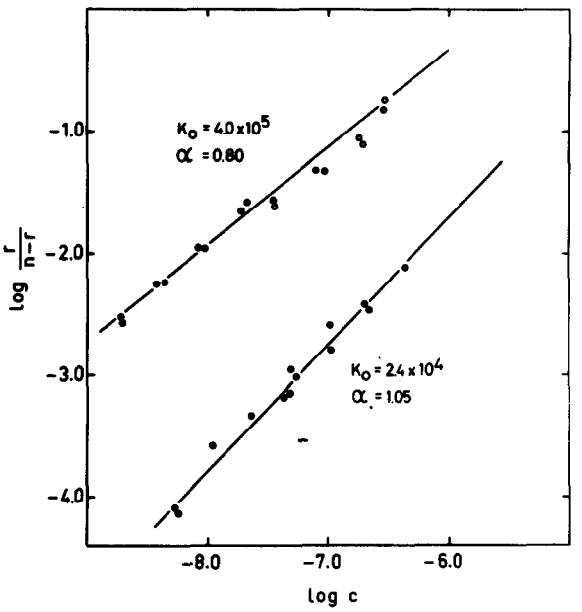


Fig.2. Sips plot for the binding of N 4–HPP–^{125}I to MOPC 104E IgM (•) and of IM 4–HPP–^{125}I to antibodies in rabbit antidextran serum (○) as determined by equilibrium dialysis, with c = free hapten concentration, r = bound hapten/total antibody and n = valence. The IgM was 11.1 X 10^{-6} M (by *OD*, calc. for molecular weight per site = 90 000). Antidextran antibodies were present at a concentration of 2.8 X 10^{-6} M (sites, by hapten saturation [23]) corresponding to a 10-fold dilution of initial antiserum. The ligand concentrations ranged from 10^{-6}–10^{-8} M, the solutions being mixtures of labeled HPP–^{125}I oligosaccharide conjugate and the respective non-iodinated aminoalditol.

(1 X 10^{-6} M – 1 X 10^{-8} M) of unlabeled aminoalditols an amount of labeled derivative was added such that a final activity of 20 000 cpm/ml was achieved. Considering that the added solutions of labeled derivatives were free of unlabeled carrier (cf. Results and Discussion) their actual molarity could be neglected. Antibodies were used as specified in the legend of fig.2. The dialysis chambers were loaded each with 150 μl of antibody or hapten solution and the cells rotated overnight in a thermostated room at 22°C. Samples were counted in a Packard autogamma spectrometer (Tri-carb model 3002).

2.4.2. Farr method

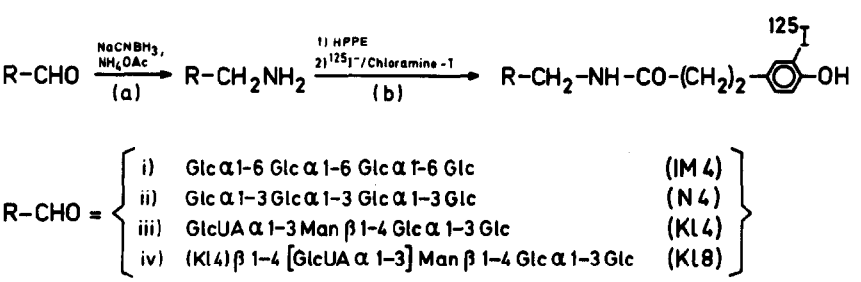
The tests were performed as described by Meyer-Delius et al. [17] using the labeled conjugates derived from IM 4, Kl 8 and Kl 4 as haptens. Prior to use their solutions (from chromatography) were adjusted to approximately 40 000 cpm/100 μl by dilution with borate buffer, pH 8.5 [15]. Sample counting was effected as indicated above.

3. Results and discussion

3.1. *Chemical preparations*

Isomaltotetraose (IM 4), α-nigerosyl-1,3-nigerose (N 4) and the monomeric and dimeric repeating units of *Klebsiella pneumoniae* B 5055 (01 : K 2) capsular polysaccharide (Kl 4 and Kl 8, respectively) were converted into ^{125}I-labeled derivatives by the procedure formulated in scheme 1. In step (a) reductive amination as described by Wiegandt and Ziegler [4] was used for conversion of the oligosaccharides into their 1-amino-1-deoxy alditols.

These were then (b) subjected to acylation by HPPE and, by subsequent addition of ^{125}I/chloramine-T to the reaction mixture, radio-iodinated. The iodination mixtures were then passed through Sephadex G-25. Figure 1, representing the elution



Scheme 1

patterns obtained, shows that only in the case of Kl 4 did this chromatographic method fail to separate the labeled oligosaccharide conjugates from excess free iodide. Separation from non-iodinated uncoupled 3-(4-hydroxyphenyl)propionic acid (HPP) was effected in all instances. Moreover, for IM 4 and N 4 it could be demonstrated that both the unreacted amino-alditols and their non-iodinated HPP conjugates were eluted prior to the iodinated HPP–oligosaccharide conjugates. This means that these were obtained in a virtually carrier-free state and consequently at extremely high specific activity, which was calculated to be in the order of 2 mCi/μg, assuming an isotopic abundance of nearly 100% for the $^{125}I^-$ employed. Similar separations of iodinated and non-iodinated low molecular weight compounds (hormones) have been reported recently [20,21]. Figure 1 also indicates that the effectiveness of Sephadex G-25 chromatography, as a means of purification of the labeled oligosaccharide derivatives, largely depends on the molecular size and structure of their carbohydrate moieties. Thus for Kl 4 the peaks of labeled product and excess free iodide were found to coincide. Separation of the two components was then achieved with the aid of Dowex anion-exchange resin (cf. Materials and methods).

3.2. *Binding assays*

The immunoreactivity of the labeled oligosaccharide derivatives was assessed using equilibrium dialysis and the Farr assay. Thus, the derivative of IM 4 was tested against dextran-specific antibodies from rabbits and the labeled conjugate of N 4 against MOPC 104E IgM with specificity for 1,3-α-Glucans.

Equilibrium dialysis resulted in the Sips curves [22] shown in fig.2. Whereas binding is indicated in both instances, the lower of the two curves is of special interest, since N 4/MOPC 104E IgM may be considered as a standardized system for which an association constant of $K_0 = 2.7 \times 10^4$ was reported by Young et al. [24]. Our finding of $K_0 = 2.4 \times 10^4$ agrees well with this value and the heterogeneity index $\alpha = 1.05$ meets the expectations for homogeneous binding.

Binding curves obtained with the IM 4, Kl 4 and Kl 8 derived radio-iodinated conjugates in the Farr assay are depicted in fig.3. In this figure the antiserum used for the IM 4 conjugate is the same as in fig.2, whereas the Kl–haptens are measured against serum obtained by immunizing rabbits with a Kl 4–edestin conjugate. The Farr assay, in spite of various efforts, cannot be considered to be on an equally solid basis for affinity determinations as is equilibrium dialysis [25]. However, its value as a rapid and economical means to obtaining preferentially comparative binding data is generally appreciated. In the present study the curves of fig.3 demonstrate the binding of all three haptens to their antibodies. A detailed report of binding studies with Kl-type haptens will be given separately [9]. Altogether the results presented seem to indicate a more general applicability of the described procedure, both with respect to other oligosaccharides as potential substrates and to methods used for estimating the extent of binding.

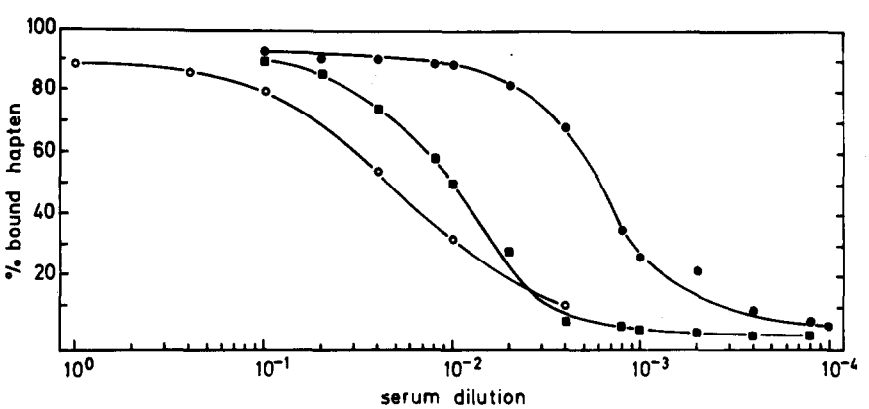


Fig.3. Plot of binding data obtained by the Farr method of ammonium sulfate precipitation: (o) IM 4–HPP–^{125}I tested against rabbit antidextran serum, (▪) Kl 8–HPP–^{125}I and (•) Kl 4–HPP–^{125}I, both tested against rabbit anti-Kl 4 serum.

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