merase, is a very weak inhibitor of RT. A similar, but slightly different order of inhibition of RT's by individual streptovaricins has been obtained with RT systems in other laboratories (Carter *et al.*, 1972). In view of the relatively low activity of the compounds as RT inhibitors, these conclusions should be regarded as tentative. The somewhat greater RT inhibitory activity of the streptovaricin acetates (SvG triacetate, SvC tri- and tetraacetates) *vis-à-vis* the unacetylated compounds is encouraging, since acetates tested are derived in one step from the major components of the complex, SvC, SvG, and SvA, and are thus available in larger quantity than SvD, a minor component of the complex.

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Interaction of Concanavalin A with the Capsular Polysaccharide of Pneumococcus Type XII and Isolation of Kojibiose from the Polysaccharide†

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ABSTRACT: The disaccharide kojibiose (2-*O*-α-D-glucopyranosyl-D-glucose) was isolated from a partial acid hydrolysate of the capsular polysaccharide of type XII pneumococcus. Acetolysis of the polysaccharide provided α-kojibiose octaacetate. These findings confirm several decades of immunochemical support for the presence of kojibiosyl residues in type XII pneumococcal polysaccharide. A precipitin curve is generated when the Jack bean lectin, concanavalin A, interacts with SXII, but no precipitate forms with periodate oxidized, borohydride-reduced SXII. Since oxidation by periodate destroys kojibiosyl residues and this oligosaccharide was shown to be a good inhibitor of the reaction of SXII with concanavalin A, it is probable that this disaccharide forms the basis for the interaction of SXII with concanavalin A.

A previous paper (Cifonelli *et al.*, 1966) described the purification of the specific polysaccharide (SXII) of type XII pneumococcus. Hydrolysis of the polysaccharide gave rise to neutral (D-glucose and D-galactose) and amino (D-galactosamine and L-fucosamine) sugars. One of the procedures employed for the purification of SXII involved complex formation with concanavalin A (*cf.* Goldstein and Iyer, 1966), the phytohemagglutinin of the Jack bean (Sumner and Howell, 1936). Of the component sugars present in SXII, only D-glucose is capable of interacting with the combining sites of concanavalin A (Goldstein *et al.*, 1965; Smith and Goldstein, 1967; So and Goldstein, 1967b; Poretz and Goldstein, 1970). Furthermore, there are substantial immunochemical data indicating the occurrence of kojibiosyl residues (*O*-α-D-glucopyranosyl-(1→2)-D-glucose) in SXII (Goodman and Kabat, 1960; Suzuki and Hehre, 1964). This paper reports the interaction of concanavalin A with SXII and the isolation of kojibiose from a controlled acid hydrolysis of the polysaccharide.

Experimental Section

SXII polysaccharide and periodate-oxidized, borohydride-reduced SXII were prepared as described earlier (Cifonelli

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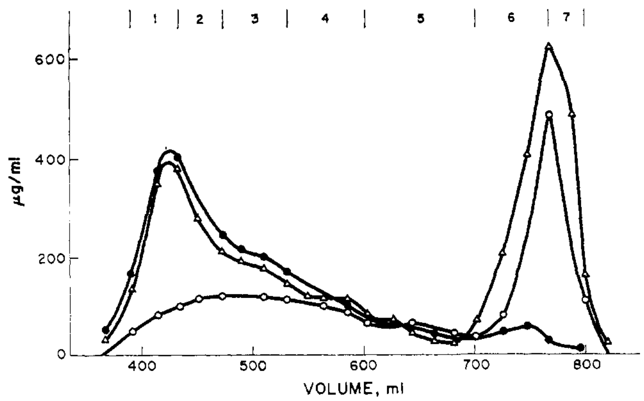

FIGURE 1: Gel filtration pattern from Sephadex G-25 of hydrolysis products from SXII. Elution was with 0.2 M sodium acetate in 10% ethanol. Analyses: (△) neutral sugars; (●) hexosamines: (○) reducing sugars.

et al., 1966). Concanavalin A was prepared by the procedure of Agrawal and Goldstein (1967).

Methyl α-D-glucopyranoside, methyl α-D-mannopyranoside, and maltose were purchased from Pfanstiehl Laboratories, Waukegan, Ill. Kojibiose was prepared in this laboratory by acetolysis of dextran B-1299-S (Duke *et al.*, 1973). Methyl α-kojibioside was synthesized by Dr. R. Iyer. 2-*O*-α-D-Galactopyranosyl-D-glucose was a gift from Professor B. Helferich. Sephadexes were purchased from Pharmacia, Piscataway, N. J.

Analytical methods used for hexosamine, total carbohydrates by the phenol-sulfuric technique, and carbohydrates by the Technicon autoanalyzer have been reported previously (Cifonelli and King, 1970). Paper chromatography was performed with Whatman No. 1 filter paper using as irrigant *tert*-pentyl alcohol–isopropyl alcohol–H_2O (8:2:3, v/v/v) and components were visualized with a silver stain (Trevelyan *et al.*, 1950). For gas–liquid chromatography, of silyl derivatives, an F & M Model 400 chromatograph was used with a 6-ft column containing 3% SE-30 on Chromosorb W (80–100 mesh) at 220°.

Isolation of Oligosaccharide Fraction. SXII (400 mg) was dissolved in 80 ml of 0.1 N hydrochloric acid and heated on a boiling water bath for 1.0 hr. After cooling and neutralization with 1.0 N sodium hydroxide, the solution was concentrated to 4 ml for application to a Sephadex G-25 column (superfine, 2.5 × 200 cm). Ten-milliliter fractions were collected every 40 min and pooled as indicated in Figure 1. The volume collected between 705 and 765 ml (fraction 6, Figure 1) contained the major portion of the disaccharide produced by hydrolysis and was used for isolation of kojibiose.

The pooled eluate (50 ml) was passed through a column (2.2 × 8 cm) of Norit-Celite (1:1) and washed with 25 ml of H_2O. Oligosaccharides were eluted with 200 ml each of 2 and 10% ethanol (v/v). The latter eluate was concentrated eightfold and analyzed for carbohydrates.

Acetolysis of SXII and Isolation of α-Kojibiose Octaacetate. Purified SXII (47 mg) was acetolyzed using the conditions which liberated α-kojibiose octaacetate from dextran B-1299-S (Duke *et al.*, 1973). Thin-layer chromatography (tlc) was performed on laboratory prepared silica gel coated glass plates using benzene–ethanol (95:5, v/v) as developing solvent. Sugar components were visualized by spraying with 50% ethanol–sulfuric acid (v/v) and charring in an oven at 120°.

Deacetylation of α-kojibiose octaacetate was carried out by treatment with sodium methoxide. The kojibiose so obtained was methylated by a modification of the Hakamori procedure (Sandford and Conrad, 1966). After methanolysis of the methylated kojibiose (3.6% methanolic hydrogen chloride in a sealed tube at 100° for 5 hr), the *O*-methyl sugars were identified by gas–liquid chromatography (glc) in an F & M Scientific 402 high efficiency gas chromatograph (Hewlett-Packard) using a 15% butanediol succinate column at 175° by the procedure of Aspinall (1963).

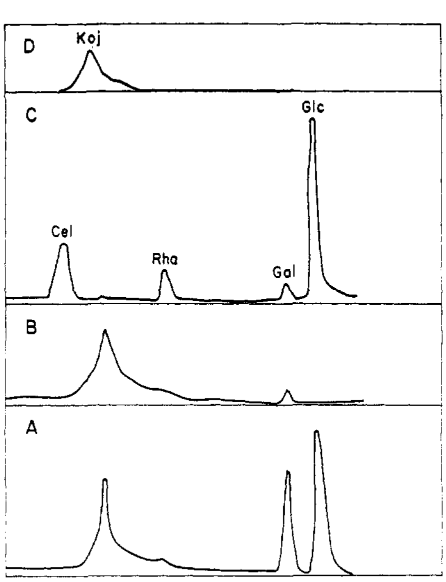

FIGURE 2: Technicon carbohydrate autoanalyses of SXII, fraction 6 showing: (A) the components present in the original fraction; (B) after fractionation from charcoal, as described in the Experimental Section; (C) after hydrolysis of the purified material; internal standards of cellobiose and rhamnose are shown; (D) authentic kojibiose.

Interaction of SXII with Concanavalin A. Precipitin tubes were set up according to the procedure of So and Goldstein (1967a). Each tube contained concanavalin A (111.5 µg of nitrogen) and increasing quantities of SXII (5–450 µg) in 1.0 ml. The mixtures were 1 M in NaCl and 0.018 M in sodium phosphate buffer (pH 7.0). Digestions, nitrogen determinations, and solubility studies of precipitates were done according to So and Goldstein (1967a).

The interaction of periodate-oxidized, KBH_4-reduced SXII (100–500 µg) with concanavalin A was carried out as above except that concanavalin A was employed at levels of 100, 150 and 200 µg of nitrogen.

The concentration of SXII polysaccharide was determined by the phenol-H_2SO_4 reagent with melibiose as a standard with the following assumptions: (1) only D-glucose and D-galactose contribute to the value, since amino sugars do not react with this reagent; (2) the above neutral sugars constitute 60% of the polysaccharide so that the concentration of the SXII polymer may be computed on this basis.

Quantitative hapten inhibition analysis was conducted on the SXII–concanavalin A system by the procedure of So and Goldstein (1967b).

Results

Heating the SXII polysaccharide in 0.1 N hydrochloric acid for 1.0 hr resulted in more extensive cleavage of the neutral sugar units than of the hexosamine bonds, giving a peak containing mono- and disaccharides comprising approximately half of the neutral sugars present in SXII (Figure 1) and less than 10% of the hexosamine. The hexosamine–oligosaccharide

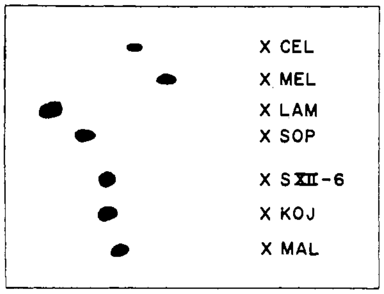

FIGURE 3: Paper chromatographic results of SXII, fraction 6, purified from charcoal, compared with the following glucose-containing disaccharides: cel, cellobiose; mel, melibiose: lam, laminaribiose; sop, sophorose; koj, kojibiose; mal, maltose.

fraction was eluted mainly as a broad peak which was retarded only slightly and was composed of material with a 1:1 ratio of hexosamine and neutral sugars, suggesting that kojibiose may be attached as short branches to a hexosamine-containing backbone. This proposal has support from the lack of appreciable amounts of intermediate-sized fragments after mild conditions of hydrolysis (Figure 1). The disaccharide fraction, as described below, appeared to be the major type of oligosaccharide produced under the conditions used.

Isolation of Kojibiose. Fraction 6 (Figure 1) contained galactose, glucose, and a component with an elution volume similar to that for kojibiose by borate column chromatography (Figure 2). Fractions 5 and 7 contained a similar fast-moving component, but in amounts 10 or 20% of that present in fraction 6. After adsorption on a charcoal–Celite column and elution with 10% ethanol, fraction 6 gave the following results (μg/ml): carbohydrate, 480; reducing sugar, 170. Reduction with borohydride showed a decrease in carbohydrate value to 236 μg, indicating a disaccharide. Approximately 90% of the ethanol-eluted fraction appeared at the position for kojibiose in a yield of approximately 10% of the neutral carbohydrate recovered from the Sephadex G-25 column. This result, together with the smaller amounts of similar material found in fractions 5 and 7, indicates that the overall yield approached at least 13 or 14% of the total neutral sugars in SXII implying that kojibiose is a major structural component of SXII.

Further evidence that the oligosaccharide isolated from SXII is kojibiose was obtained from paper chromatographic data, the SXII disaccharide having a mobility similar to authentic kojibiose (Figure 3), from glc results of the trimethylsilyl derivative (Figure 4), and from the relatively low reducing sugar value found which is consistent for a (1→2) linked product.

Kojibiose was also isolated as its peracetate, α-kojibiose octaacetate, by acetolysis of SXII polysaccharide. After 8 days at 25°, tlc analysis of the acetolysate showed formation of components cochromatographing with α-kojibiose octaacetate and D-glucose pentaacetate. Preparative tlc in benzene–ethanol (95:5, v/v) separated the acetolysis products into fractions, one of which cochromatographed with D-glucose pentaacetate and one with α-kojibiose octaacetate. Crystalline α-kojibiose octaacetate (1.5 mg) was obtained from a purified fraction, mp 169–170°, $[\alpha]^{27}_{D}$ +147 ± 5° (*c* 0.068, chloroform) (lit. mp 166°, $[\alpha]_D$ +152° (*c* 2.4, chloroform) (*cf.* Duke *et al.*, 1973; Barker *et al.*, 1959; Matsuda *et al.*, 1961; Helferich and Zirner, 1962). Deacetylation of a small portion (*ca.* 1 mg) gave the free sugar, mp and mmp with authentic kojibiose, 187–188° (lit. mp 188°, Yamauchi and Aso, 1961).

Methylation of the remainder of the disaccharide (*ca.* 0.5 mg) followed by methanolysis and glc gave peaks corresponding in their retention times to the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-glucose and 3,4,6-tri-*O*-methyl-D-glucose.

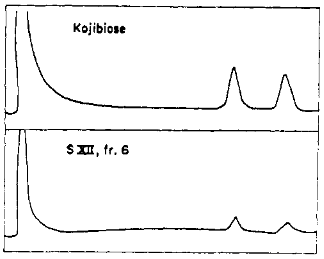

FIGURE 4: Glc patterns for purified SXII, fraction 6, and authentic kojibiose.

SXII–Concanavalin A Interaction. The precipitin curve of concanavalin A–SXII interaction (Figure 5) was similar to curves obtained for most dextrans and mannans but unlike that given by glycogen (So and Goldstein, 1967a, 1968, 1969; Goldstein *et al.*, 1968). The SXII polysaccharide precipitated 79% of the 111.5 μg of concanavalin A nitrogen present. The solubility of the SXII–concanavalin A precipitate was found to be 8 μg of nitrogen/ml. Incubation of concanavalin A with SXII which had been oxidized by periodate and reduced with borohydride gave no precipitate despite the much higher levels of concanavalin A used (100, 150, and 200 μg of concanavalin A nitrogen).

Quantitative hapten inhibition analysis employing a series of sugars is presented in Figure 6. The inhibitive order and the ratios of the various sugars producing 50% inhibition are similar to all other systems investigated, indicating that SXII is almost certainly interacting with the same sites on concanavalin A as dextrans, glycogens, levans, etc. (Goldstein *et al.*, 1965; So and Goldstein, 1967a,b, 1968, 1969; Poretz and Goldstein, 1970).

The sugars of special interest to this study are kojibiose, methyl α-kojibioside, and 2-*O*-α-D-galactosyl-D-glucose. Although both D-glucosyl moieties of kojibiose are potentially available for interaction with concanavalin A (unsubstituted C-3, C-4, and C-6 hydroxyl groups), the protein appears to interact with the *nonreducing* α-D-glucopyranosyl moiety of the *free* sugar. This is perhaps indicated by the fact that 2-

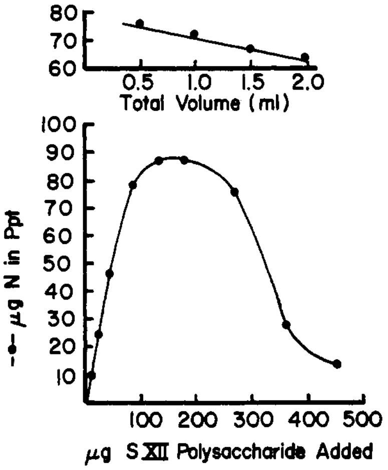

FIGURE 5: Quantitative precipitin curve of SXII with concanavalin A. Each tube contained concanavalin A (111.5 μg of nitrogen) and increasing quantitites of SXII in a total volume of 1.0 ml. The solubility curve for SXII–concanavalin precipitation is also shown; each tube contained concanavalin A (111.5 μg of nitrogen), SXII (175 μg), in varying volumes as indicated.

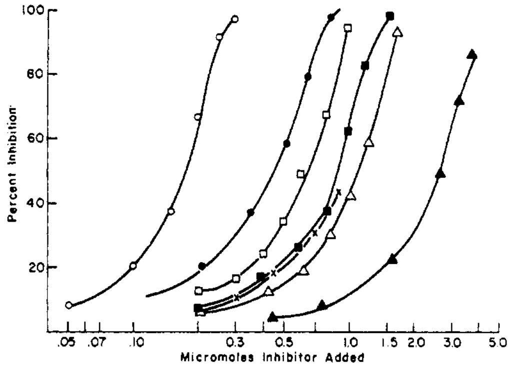

FIGURE 6: Inhibition by saccharides of SXII–concanavalin A precipitation. Each tube contained concanavalin A (100 μg of nitrogen), SXII (150 μg), and inhibitor as noted in a total volume of 1.0 ml: (○) methyl α-D-mannopyranoside; (●) methyl α-kojibioside; (□) methyl α-D-glucopyranoside; (■) kojibiose (Glc(1→2)Glc); (×) kojibiose isolated from SXII; (△) maltose; (▲) Gal(1→2)Glc.

O-α-D-galactopyranosyl-D-glucose is only one-third as potent as kojibiose. However, methyl α-kojibioside is twice as potent as free kojibiose suggesting that concanavalin A interacts selectively with the *reducing* α-methyl glucoside moiety of the disaccharide glycoside.

The kojibiose isolated from SXII was tested as an inhibitor of the SXII–concanavalin A system with the results presented in Figure 6. It will be noted that the inhibition of the kojibiose from SXII parallels closely the inhibition curve of authentic kojibiose. This coupled with the glc identification of the glucobiose as kojibiose is good evidence for the occurrence of kojibiosyl residues in type XII pneumococcal polysaccharide.

Discussion

The isolation of kojibiose both from a partial acid hydrolysate of SXII and from an acetolysate of the polysaccharide caps more than 20 years of speculation regarding the presence of a glucosyl residue which reduced 1 mol of periodate (Hehre, 1956) as a structural feature of pneumococcal type XII capsular polysaccharide.

In an early investigation, Hehre (1943) found that the dextrans produced by several strains of *Leuconostoc* could be divided into two groups (designated serotypes A and B) on the basis of their capacity to form a precipitate with type XII antipneumococcus sera. Dextrans of serotype A, which precipitated with type XII antiserum, were shown by Suzuki and Hehre (1964) to contain kojibiose as a component of their molecular structure (*cf.* Matsuda *et al.*, 1961).

The ability of a series of dextrans to cross-react with horse anti-XII sera was shown by Goodman and Kabat (1960) to be related to the content of α-(1→2)-glucosidic linkages present in the dextrans. The latter investigators also reported that kojibiose was the best inhibitor of the precipitin reaction of anti-type XII with dextran B-1299-S-3 which contains a high proportion of kojibiosyl residues.

The present isolation of kojibiose, then, supports these observations and lends further credence to the immunochemical approach as a powerful tool in studies of polysaccharide structure.

The precise molecular basis for the interaction of SXII with concanavalin A cannot be assigned at this time inasmuch as the structure of this polysaccharide has not been elucidated. Glucose is the only sugar in the polysaccharide which reacts with concanavalin A and a large proportion of the glucosyl residues are destroyed by periodate oxidation, including those residues possessing receptor sites for concanavalin A. It has now been established that kojibiosyl residues are present in SXII and inhibition data (Figure 6) establish the reactivity of concanavalin A with this disaccharide (*cf.* Goldstein *et al.*, 1965). The inhibition data indicate that both the reducing and nonreducing moieties of this disaccharide possess the receptor sites necessary for interaction with the combining sites of concanavalin A (unmodified hydroxyl groups at C-3, C-4, and C-6), thus distinguishing this disaccharide from sophorose (2-*O*-β-D-glucopyranosyl-D-glucose) in which only the *reducing* D-glucose unit is capable of reacting (Goldstein *et al.*, 1967). Although the location of the kojibiosyl residues within the polysaccharide is unknown at this time the hydrolysis data suggest that the disaccharide may occur as terminal units. Preliminary methylation data (J. Duke, unpublished results) indicate the presence of 2,3,4,6-tetra-*O*-methyl- and 3,4,6-tri-*O*-methyl-D-glucose among the hydrolysis products of methylated SXII. This also supports the occurrence of kojibiosyl side chains in SXII.

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