The Capsular Polysaccharide of Pneumococcus Type XII, SXII*

J. A. Cifonelli, P. Rebers,† M. B. Perry,‡ and J. K. N. Jones§

ABSTRACT: Crude type XII polysaccharide was found after chromatography on Dowex 1 columns to contain appreciable amounts of C-polysaccharide as well as chondroitin 4-sulfate, the latter presumably arising from the culture medium. Final purification by precipitation of SXII with type-specific antiserum, concanavalin A, or ammonium sulfate eliminated these contaminants. Purified SXII consists of two parts of hexosamine and three parts of neutral sugars. The hexosamine fraction contained equimolar amounts of D-galactosamine and L-fucosamine and the neutral sugars, D-galactose and D-glucose, similarly were present in equal concentrations. Periodate oxidation of purified SXII destroyed only the neutral sugars, supporting previous suggestions that these may be 1→2 linked.

The specific polysaccharide (SXII) from type XII pneumococcus has been reported to contain galactose, glucose, and unidentified amino sugars (Heidelberger *et al.*, 1954) as well as uronic acid and phosphate (Brown, 1939). Nothing appears to be known regarding the structural features of this polysaccharide although tests with dextrans in type XII antisera indicate that those with 1→2 linkages precipitate best (Goodman and Kabat, 1960; Suzuki and Hehre, 1964). Support for this linkage type is found in the increased precipitation of SXII (J. A. Cifonelli, unpublished data) and dextrans containing α-D-1,2 linkages (Goldstein *et al.*, 1965) with concanavalin A (Cifonelli and Smith, 1955).

The presence of hexosamine in SXII is of interest because of the finding of new types of amino sugars in certain of the pneumococcal polysaccharides (Barker *et al.*, 1961). The present study describes the isolation of both L-fucosamine and D-galactosamine from SXII. Crude SXII is shown to contain, in addition to the specific polysaccharide, contaminants consisting of C substance and chondroitin 4-sulfate.

Experimental Procedures

Materials. Antipneumococcal horse sera were supplied by the Division of Laboratories and Research, Department of Health, State of New York, through the kindness of Miss Jessie L. Hendry. The amounts of antibody per milliliter of sera to the specific polysaccharide, S, and C substance are given for the two sera used: type VII (no. 1074) 428 μg of anti-SVII and 580 μg of anti-C, type XII (no. 625) 1420 μg of anti-SXII and 400 μg of anti-C. Crude type XII pneumococcal polysaccharide was generously supplied by Dr. M. Heidelberger, New York University School of Medicine.

Methods

Immunodiffusion. Ouchterlony plates were prepared with 0.6% ionagar no. 2 in 0.05 M "Tris" buffer, pH 7.2, containing 0.10 M NaCl. Holes were punched with Feinberg cutter no. 1083, Consolidated Laboratories, Inc., Chicago Heights, Ill. Plates were stored at 22° and observed daily for periods up to 1 week.

Quantitative Precipitin Reaction. The purity of different fractions of SXII was estimated by precipitation with C-absorbed horse antiserum at 0° in the antibody excess region according to the procedure of Heidelberger and Kendall (1935).

Fractionation of Crude Polysaccharide. Polysaccharide (3 g) dissolved in 50 ml of H_2O was passed over a 4.5 × 35 cm column of Dowex 1-2X, chloride form (200–400 mesh). The column then was eluted with 300 ml of H_2O followed successively by 0.2, 1.0, and 2.0 M NaCl solutions, each being continued as long as a positive Molisch reaction was obtained from the eluates. An elution rate of approximately 20 ml/hr was maintained.

The eluates were separately dialyzed against tap water, concentrated, and precipitated by the addition of 3 volumes of ethanol. Per cent recoveries and analytical results for the various fractions are shown in Table I.

Purification of Fraction A. A. PRECIPITATION WITH AMMONIUM SULFATE. Fraction A (20 mg) was dissolved in 4.0 ml of 0.85% NaCl and 1.5 g of ammonium sulfate was added (Estrada-Parra *et al.*, 1962). The

* From the La Rabida-University of Chicago Institute and Department of Biochemistry, University of Chicago, Chicago, Illinois 60649. *Received May 18, 1966.* Work supported by grants from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service (No. AM-05996), the National Foundation, and the Chicago Heart Association.

† Department of Agriculture, National Animal Disease Laboratory, Ames, Iowa.

‡ Division of Biosciences, National Research Council, Canada, National Research Council No. 9002.

§ Chemistry Department, Queens University, Kingston, Ontario, Canada.

TABLE I: Analytical Composition of SXII and Fractions Isolated from Dowex 1, Chloride.[a]

Fraction	NaCl (M)	% Yield[b]	Uronic Acid	Carbohydrate[c]	Sulfate	Phosphate	$[\alpha]_D^{24}$ (deg)
A	0.2	52	0.10[d]	1.7	0	0.10	−2 (H_2O)
B	1.0	40	0.22	1.6	0.10	0.67	
C	2.0	8	0.95	0.68	0.74	0.20	−26 (H_2O)
Original SXII			0.23	0.98	0.12		−2 (H_2O)

[a] Composition given as molar ratios to hexosamine content. [b] Calculated on basis of hexosamine content. [c] Determined as glucose by the Phenol–H_2SO_4 method. [d] Carbazole reaction showed maxima at 430 as well as 530 mμ.

mixture was centrifuged at 1400*g* and the sediment was washed twice with 6-ml amounts of 0.85% NaCl to which 2.3 g of ammonium sulfate had been added. The precipitated SXII was then dissolved in water and dialyzed until free of ammonium sulfate.

B. PRECIPITATION WITH TYPE XII ANTISERUM. Type XII horse antiserum (4 ml), previously absorbed with pneumococcal C substance, was mixed with 1.2 mg of fraction A in 2 ml of 0.85% NaCl. After 3 days at 0°, the precipitate was collected by centrifugation at 0°, washed three times with 0.85% NaCl at 0°, and then extracted with 4% trichloracetic acid for 1 hr at 0° (Heidelberger *et al.*, 1955).

C. PURIFICATION WITH CONCANAVALIN A. A 0.2% solution of fraction A was mixed with 10 volumes of concanavalin A reagent, prepared as described earlier (Cifonelli and Smith, 1955). After 0.5 hr at room temperature, the mixture was centrifuged and the precipitate was washed once with 1% saline. Solution was effected with 0.1 N NaOH and protein was precipitated by the immediate addition of 40% trichloracetic acid to give a final concentration of 5%. After 10 min the precipitated protein was centrifuged and the supernatant solution was dialyzed against distilled H_2O at 4°.

Purification of Fraction C. Fraction C was refractionated by applying 150 mg on a 2 × 30 cm column of Dowex 1-2X, chloride. After washing the column with 200 ml of H_2O, fractions were eluted by means of salt gradients prepared by placing 250 ml of H_2O in the mixing flask and an equal volume of 2 M sodium chloride in the reservoir. Fractions (6 ml) were collected/0.5 hr. Samples were analyzed for uronic acid and hexosamine and those having equimolar ratios of these components were pooled and after dialyzing were concentrated and precipitated with 2 volumes of ethanol. The isolated material (70 mg) was analyzed for uronic acid, hexosamine, and sulfate contents and an infrared spectrum was obtained.

Periodate Oxidation of Fraction A. Fraction A (15 mg) was dissolved in 20 ml of 0.025 M $NaIO_4$ and the solution was kept in the dark at 24° for 4 days. After dialysis against four changes of distilled water at 2°, 10 mg of KBH_4 was added and the reaction was allowed to proceed for 4 hr at 2° and 2 hr at room temperature. The sample was heated with 1 N HCl at 100° for 2 hr and the hydrolysate was deacidified by use of Dowex 3, carbonate.

Isolation of Amino Sugars from SXII. Fraction A was heated as a 0.5% solution in 1 N HCl for 6 hr. The dried hydrolysate residue from 200 mg of fraction A was dissolved in 2 ml of H_2O and applied to a 2.2 × 40 cm column of Dowex 50-8X, H^+ (200–400 mesh). Hexosamines were eluted with 0.3 N HCl. Fractions of 20 ml were collected/hr and assayed for hexosamine content by the Elson–Morgan method. Two major peaks were obtained, one emerging from 350 to 550 ml (hexosamine peak 1) and a second at 700–800 ml (peak 11). The latter fraction was concentrated to a syrupy residue which was dissolved in 5 ml of methanol and acetone was added until the solution became opalescent. After several days at room temperature, small needles formed which were washed with a mixture of methanol–acetone (2:1, v:v). The weight was 16 mg.

Hydrolysates from SXII were also fractionated by a gas chromatographic procedure using the trimethylsilyl derivatives as described previously (Sweeley *et al.*, 1963). Galactosamine was isolated by this method as the trimethylsilyl-*N*-acetyl derivative.

Partial Acid Hydrolysis of SXII. A 0.1% solution of fraction A in 0.04 N HCl was heated in a boiling water bath for periods up to 6 hr. Aliquots were removed after heating for 0, 0.5, 1.0, 4.0, and 6.0 hr and cooled in an ice bath. After neutralizing by the addition of equal volumes of 0.04 N Na_2CO_3 solution, the hydrolysates were analyzed for reducing sugar and *N*-acetylhexosamine contents. Paper chromatography in solvent A was performed on the 4- and 6-hr hydrolysates. Gel filtration on a 200-cm long Sephadex G-25 column gave oligosaccharide fractions from 4-hr hydrolysates which were enriched in hexosamines and others predominating in neutral sugars. These are under current investigation.

Analytical Methods. Analysis for uronic acid was by the carbazole reaction (Dische, 1947) as well as by gas chromatography (Perry and Hulyalkar, 1965). Neutral sugars were estimated by the phenol–H_2SO_4

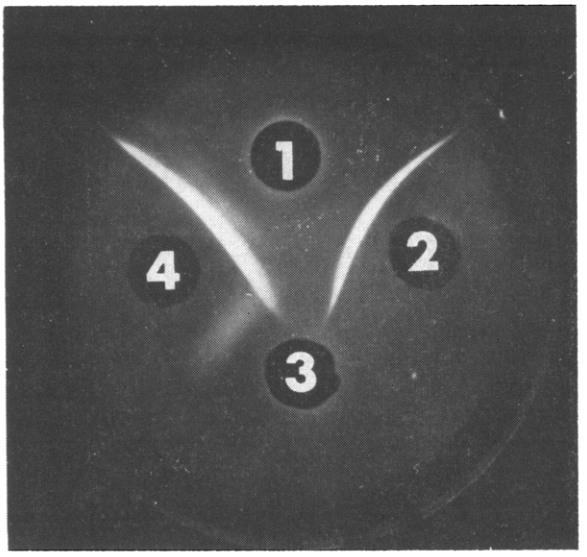

FIGURE 1: Immunodiffusion pattern of SXII fractions. Hole 1, type XII antiserum; hole 2, SXII, fraction A purified by precipitation with $(NH4)_2SO4$; hole 3, type VII antiserum; hole 4, SXII, fraction A. Volumes used in wells, 100 μl; concentration of SXII fractions, 200 μg/ml. Plate photographed 2 days after addition of reagents.

method (Dubois *et al.*, 1956). Hexosamine determinations were done by the Boas modification of the Elson–Morgan procedure (Boas, 1953) and *N*-acetylhexosamines were estimated as described by Levvy and McAllan (1959) or Reissig *et al.* (1955). Glucose and galactose estimations of paper chromatographic fractions were obtained by use of glucose oxidase (Hough and Jones, 1962) and galactose oxidase, respectively (Avigad *et al.*, 1962). Sulfate was estimated as described earlier (Cifonelli and Dorfman, 1960). Phosphate was analyzed by the Fiske–Subbarow method (1925). The Technicon amino acid autoanalyzer also was used for determining concentrations of amino sugars as well as amino acids present in the various preparations.

Paper chromatography was carried out by the descending technic using Whatman No. 1 paper for general purposes and Whatman No. 3 for preparative work. Solvents used included: A, 2 methyl-2-butanol–isopropyl alcohol–H_2O (8:2:3); B, 2,4-lutidine–H_2O (65:35); C, 1-butanol–acetic acid–H_2O (4:1:5); D, ethyl acetate–pyridine–H_2O (10:4:3).

Results

Fractionation of SXII on Dowex 1, Chloride. The presence of significant amounts of both uronic acid and sulfate in the type SXII polysaccharide preparation used indicated the possibility of contaminating mucopolysaccharides. Fractionation of a crude preparation of SXII on a column of Dowex 1, chloride gave the results shown in Table I. The largest fraction (A), representing approximately one-half of the total material, was obtained by elution with 0.2 M salt. This material contained little uronic acid as judged by the carbazole reaction which produced an anomalous yellow-pink color with absorption maxima at 430 and 530 mμ in contrast to a single maximum at 530 mμ shown by authentic glucuronic acid. The fraction contained no detectable sulfate but 0.1 molar equiv of phosphate was present indicating the possibility of contamination with C-polysaccharide (Lin and Gotschlich, 1963).

Fraction C, eluted with 2 M salt, comprised 8% of the total material. The finding of almost 1 molar equiv of uronic acid and 0.75 equiv of sulfate in conjunction with the optical rotation of $-26°$ suggested the presence of chondroitin sulfate. Refractionation of this material from Dowex 1, chloride yielded a main fraction containing molar ratios of uronic acid and sulfate to hexosamine of 1.09 and 1.20, respectively. Confirmation that this was chondroitin 4-sulfate was obtained from the similarity of its infrared spectrum to that of chondroitin 4-sulfate isolated from bovine nasal septum. Support was also provided by the finding that although incubation with testicular hyaluronidase eliminated its turbidity reaction with acid albumin, the digest products (Mathews and Inouye, 1961) showed little color in the Morgan–Elson reaction.

The material eluted with 1.0 M salt (fraction B) comprised 40% of the total preparation applied to the resin column. This fraction appeared to represent a mixture as judged by the phosphate and uronic acid contents. The results suggested that both chondroitin 4-sulfate and C-polysaccharide were present but further efforts to identify these in fraction B were not made.

Purification and Composition of Fraction A. Gel diffusion studies indicated that fraction A was contaminated with C-polysaccharide. Further purification of this preparation by precipitation with ammonium sulfate, type-specific antisera, or concanavalin A yielded products which no longer gave an immune reaction for C-polysaccharide. This is illustrated in Figure 1 where it is noted that fraction A purified by precipitation with ammonium sulfate forms a single line with type XII antiserum and does not react with type VII antiserum. However, unpurified fraction A produces a weak second line with homologous antiserum and a line also with type VII. The amount of antibody precipitated by this fraction with homologous C-absorbed horse antiserum was as follows: 1.92, 3.84, and 9.60 μg of fraction A precipitated 25.8, 51.8, and 99.2 μg of antibody N, respectively, from 0.1-ml portions of serum. The purified substances contained no uronic acid, phosphate, or other titratable acidic functions. Hydrolysis of purified SXII followed by paper chromatography of the hydrolysate in solvent A showed spots that migrated similar to glucosamine, galactose, and glucose. In solvent B, two major ninhydrin-positive spots were seen, one corresponding in mobility to galactosamine and one moving slightly faster than glucosamine (R_{gN} 1.12). Both spots were also observed after spraying the chromatogram with an alkaline–silver reagent, indicating that these sub-

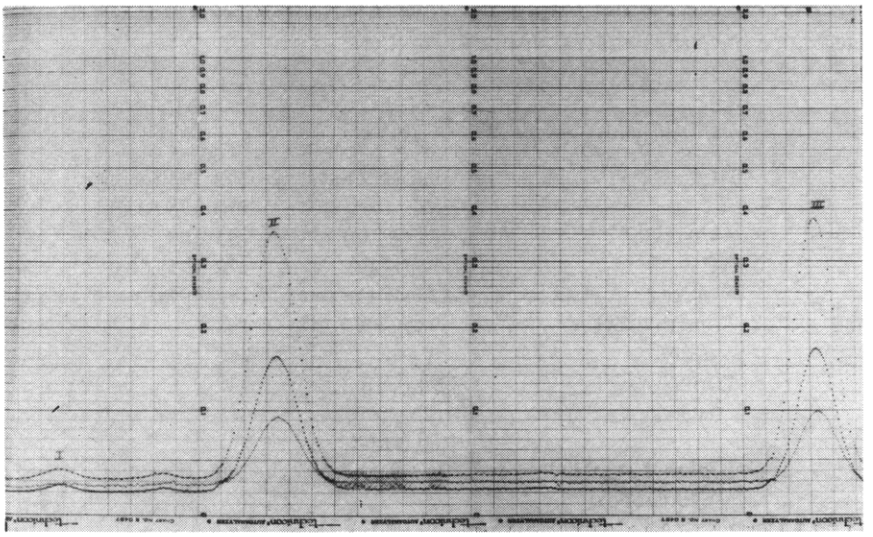

FIGURE 2: Identification and estimation of hexosamines present in SXII fraction A by use of the Technicon amino acid autoanalyzer. Hydrolysis was performed by heating in 6 N HCl for 20 hr. Identification of peaks is described in the text.

stances were hexosamines rather than amino acids.

Application of hydrolysates to the Technicon amino acid autoanalyzer revealed two major peaks of similar size (Figure 2), one at the galactosamine location (peak II) and the other in the region for norleucine (peak III) and identified as L-fucosamine, as described later. Glucosamine (peak I) and amino acids were negligible components in the precipitated substances. However, as indicated in Table II, glucosamine was appreciable in fraction A and amounted to about one-eighth of the galactosamine present. That the glucosamine appears to be due to contaminating C-polysaccharide, as demonstrated in Figure 1, is supported by the presence of muramic acid in fraction A but not in the repurified products (Table II). Fraction B, as expected from the relatively high phosphate content, contained the largest proportions of glucosamine and muramic acid, components of C-polysaccharide (Lin and Gotschlich, 1963; Hornung and Berenson, 1963).

TABLE II: Amino Sugar Composition of SXII Fractions.[a]

Preparation	GalN	FucN	GlcN	MA
Fraction A, pptd with antiserum	1.06	1.00	0.05	0
Fraction A, pptd with concanavalin A	1.09	1.00	0.02	0
Fraction A, pptd with ammonium sulfate	1.02	1.00	0.03	0
Fraction A	1.13	1.00	0.13	0.03
Fraction B	1.14	1.00	0.40	0.07
SXII, crude	1.44	1.00	0.31	0.06

[a] Abbreviations used: GalN, galactosamine; FucN, fucosamine; GlcN, glucosamine; MA, muramic acid.

Data from the amino acid autoanalyzer, not shown in the table, indicated that the amino acid contents of the various preparations ranged from 5% of the total hexosamine concentrations for the refractionated preparations to approximately 10 and 15%, respectively, for fractions A and B.

Hydrolysis of fraction A by 0.04 N HCl proceeded readily as shown in Figure 3. The reducing sugar values (denoted by solid line) increase much more rapidly than those for *N*-acetylhexosamine (dotted line) suggesting that the neutral sugar linkages of SXII are more rapidly cleaved than those of the hexosamines, or alternatively, that oligosaccharides having hexosamine reducing ends are partially 1→4 linked and thus are unreactive in the Morgan–Elson method. Paper chromatography of the acid hydrolysates produced by 4 and 6 hr of heating show the presence of galactose with little free hexosamine or *N*-acetylhexosamine supporting the suggestion that only the neutral sugars are rapidly released by acid hydrolysis.

Isolation and Characterization of SXII Components. Isolation of the constituents of SXII polysaccharide was accomplished by use of paper, gas, and resin chromatographic techniques. Separation of amino sugars from Dowex 50 as described by Gardell (1953) resulted in the isolation of two major fractions. Paper chromatography in solvent B indicated the first fraction (Heidelberger *et al.*, 1954) consisted mainly of galactosamine with a small amount of glucosamine. Fraction II showed a single substance with R_{gN} 1.12. The properties of this substance agreed with those

TABLE III: Characterization of Carbohydrate Constituents of SXII.

Component	Derivative	$[\alpha]_D$ (deg)	Mp (°C)	Enzyme Reactions
L-Fucosamine	HCl	−92[a] (H_2O)	192–193 (dec)[a] 170–175 (dec)[b]	
	N-Acetyl	−79[c] (H_2O)	195–198[c]	
Peak II	HCl	−85 (H_2O)	178–195 (dec)	
	N-Acetyl	−75 (H_2O)	192–197	
D-Galactosamine	Trimethylsilyl-*N*-acetyl	+93 ($CHCl_3$)	144	
Peak I	Trimethylsilyl-*N*-acetyl	+94 ($CHCl_3$)	142	
D-Galactose		+80 (H_2O)		
	1-Methyl-1-phenyl-hydrazone		186	
Paper fraction I		+79 (H_2O)		Oxidized by galactose oxidase
	1-Methyl-1-phenyl-hydrazone		186	
D-Glucose		+52.5 (H_2O)	146	
Paper fraction II		+50 (H_2O)	145	Oxidized by glucose oxidase

[a] Reported by Kuhn *et al.* (1959). [b] Crumpton and Davies (1958). Given for D-fucosamine hydrochloride. [c] Properties reported by Barker *et al.* (1961).

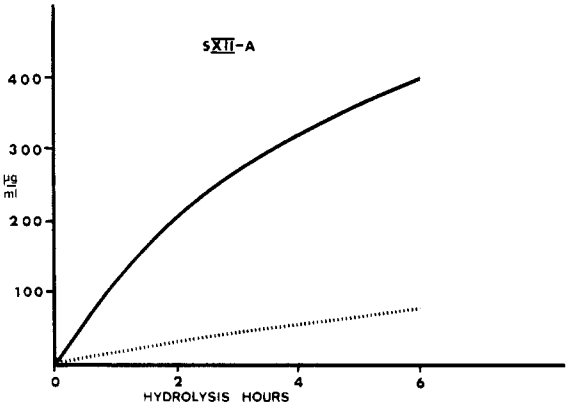

FIGURE 3: Hydrolysis of SXII fraction A with 0.04 N HCl for up to 6 hr. The broken line refers to *N*-acetylhexosamine values and the solid line is based on reducing sugar estimations. Maximum values for reducing sugars were obtained by 10 hr of hydrolysis.

reported for L-fucosamine while the properties for peak 1 demonstrate the galactosamine in SXII to be of the D form. Both of the isolated hexosamine compounds, after acetylation as described by Levvy and McAllan (1959), gave color yields in the Morgan–Elson reaction which corresponded to 31–36% of that produced by *N*-acetylglucosamine. In the Elson–Morgan reaction the two substances gave color yields similar to that given by glucosamine.

Gas chromatographic data further established the identity of the hexosamine components. The retention times of the *N*-acetyl-*O*-trimethylsilyl derivatives for both hexosamine constituents were similar to those from authentic galactosamine and fucosamine. Results from the amino acid autoanalyzer demonstrated the presence of two amino sugars in major amounts, one with the mobility of galactosamine and the other moving similarly to fucosamine obtained from SV pneumococcal polysaccharide (Barker *et al.*, 1961). Further characterization of this component as L-fucosamine was obtained in the following way. Oxidative degradation with ninhydrin (Stoffyn and Jeanloz, 1954) gave a product with R_F in solvents C and D identical with 5-deoxy-L-lyxose. Similarly, electrophoretic mobility in 0.1 M borate, pH 10 of the *N*-acetyl derivative was the same as that shown by authentic *N*-acetyl-L-fucosamine. In addition, periodate oxidation of the *N*-acetyl derivative produced 0.98 molar equiv of acetaldehyde.

The neutral sugar components of SXII were isolated from hydrolysates by preparative paper chromatography using solvent D. Two substances which moved similarly to glucose and galactose in solvents A, C, and D were observed and quantitative measurements by the phenol–sulfuric acid method indicated that these were present in equimolar amounts. Identification of fractions I and II as D-galactose and D-glucose was made by melting points, optical rotations, and enzymatic reactions as shown in Table III. In addition, the 1-methyl-1-phenylhydrazone derivative of fraction

I corresponded to that from authentic D-galactose.

Oxidation of fraction A (containing 3.5 mg of hexosamine and 5.6 mg of neutral sugars) with periodate followed by reduction with sodium borohydride gave a product in which hexosamine recovery was 3.4 mg and that of neutral sugars was 2.7 mg as estimated by the Elson–Morgan and phenol–sulfuric acid methods, respectively. Separate fractions containing amino and neutral sugars were isolated from hydrolysates of the oxidized and reduced polysaccharide by use of Dowex 50 columns. As illustrated by paper chromatography, the effluent fraction obtained from Dowex 50 contained mainly glycerol with relatively little glucose or galactose. This suggests the lack of C-3 or C-4 substitution in the neutral sugars of SXII. The absence of arabinose indicates that internal galactofuranose units of a type found in SXXXIV (Roberts *et al.*, 1963) are not present. The relatively high carbohydrate value obtained in the phenol–sulfuric method is thought to be due at least partially to breakdown products from the periodate oxidation.

Paper chromatography of the amino sugar fraction, obtained by elution from the cation resin with 1 N hydrochloric acid revealed substances moving similarly to galactosamine and fucosamine. These were shown by the amino acid autoanalyzer to be present in a molar ratio of 1.11:1.00, respectively.

Discussion

Type XII pneumococcal polysaccharide has been reported to contain galactose, glucose, and *N*-acetylhexosamine (Heidelberger *et al.*, 1954) and possibly phosphate and uronic acid (Brown, 1939). Examination of crude SXII showed the presence of all these components in addition to a significant content of sulfate (Table I). Purification of the pneumococcal polysaccharide by means of chromatography on Dowex 1 yielded three fractions after elution with 0.2, 1.0, and 2 M saline solutions. The first of these (A) possessed only small amounts of uronic acid, phosphate, or sulfate, suggesting that these constituents were present as contaminants. Fraction B had most of the phosphate-containing material in addition to an appreciable content of sulfate. The 2 M salt-eluted fraction C appeared from its high sulfate to hexosamine ratio to be mucopolysaccharide in nature. Refractionation of this preparation on Dowex 1 gave material shown by infrared spectrum and analytical data to be chondroitin 4-sulfate. This presumably was a contaminant introduced by the culture medium used in growing the pneumococci.

Examination of fraction A by an agar immunodiffusion method indicated that C-polysaccharide was present, explaining the occurrence in this fraction of small amounts of phosphate, glucosamine, and muramic acid, as shown in Table II. This contaminant was easily removed by precipitation of SXII with specific antiserum, concanavalin A, or ammonium sulfate. The purified products (Table II) contained no muramic acid or phosphate and showed no immunodiffusion bands for C-polysaccharide as indicated in Figure 1.

Paper chromatography of acid hydrolysates from fraction A showed the presence of two hexosamines, one with the mobility of galactosamine and the other moving slightly faster than glucosamine in solvents A and B. Analysis by the Technicon amino acid autoanalyzer indicated that the two amino sugars were present in equimolar amounts (Figure 2). The amino sugar components isolated by means of chromatography on Dowex 50 or by gas–liquid partition chromatography, were characterized as D-galactosamine and L-fucosamine (Table III).

The neutral sugar components of SXII were isolated from hydrolysates by paper chromatography in solvent D. Equimolar amounts of glucose and galactose were found which were characterized as the D sugars (Table III). Acid hydrolysis under mild conditions has been shown to release free neutral sugars prior to liberation of hexosamines. Preliminary results from partial acid hydrolysis suggest that at least a portion of the neutral sugars is linked in succession since oligosaccharides have been isolated from such hydrolysates which are composed mainly of galactose and glucose. Analogous fractions enriched in hexosamines have been found also. These data suggest that SXII may contain sections with hexosamine repeating units and other sections with neutral sugar components, possibly as branch components.

Results from periodate oxidation of SXII indicate that neutral sugars are almost entirely destroyed although hexosamines are unaffected. Glycerol was the only polyalcohol observed from hydrolysates of the oxidized and reduced material. This would indicate that C-3 and C-4 of the neutral sugars must be free for the most part. The possibility that 1→2 linkages may be present appears attractive from the suggestions by Suzuki and Hehre (1964) and Goodman and Kabat (1960) that this type of linkage may be involved in reactivity with type-specific antiserum. Further efforts to determine whether this linkage type occurs in SXII are in progress. The failure of periodate to cleave amino sugar units indicates that these are likely substituted at C-3 or C-4.

References

Avigad, G., Amaval, D., Asensio, C., and Horecker, B. L. (1962), *J. Biol. Chem. 237*, 2736.

Barker, S. A., Brimacombe, J. S., How, M. J., Stacey, M., and Williams, J. M. (1961), *Nature 189*, 303.

Boas, N. F. (1953), *J. Biol. Chem. 204*, 553.

Brown, R. (1939), *Immunology 37*, 445.

Cifonelli, J. A., and Dorfman, A. (1960), *J. Biol. Chem. 235*, 3283.

Cifonelli, J. A., and Smith, F. (1955), *Anal. Chem. 27*, 1639.

Crumpton, M. J., and Davies, D. A. L. (1958), *Biochem. J. 20*, 729.

Dische, Z. (1947), *J. Biol. Chem. 167*, 189.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28*, 350.

Estrada-Parra, S., Rebers, P. A., and Heidelberger, M. (1962), *Biochemistry 1*, 1175.
Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.
Gardell, S. (1953), *Acta Chem. Scand. 7*, 207.
Goldstein, I. J., Hollerman, C. E., and Marrick, J. M. (1965), *Biochem. Biophys. Acta 97*, 68.
Goodman, J. W., and Kabat, E. A. (1960), *J. Immunol. 84*, 347.
Heidelberger, M., Barker, S. A., and Stacey, M. (1954), *Science 120*, 781.
Heidelberger, M., Dische, Z., Neely, W. B., and Wolfrom, M. L. (1955), *J. Am. Chem. Soc. 77*, 3511.
Heidelberger, M., and Kendall, J. E. (1935), *J. Exptl. Med. 61*, 559.
Hornung, M. O., and Berenson, G. S. (1963), *Proc. Soc. Exptl. Biol. Med. 114*, 31.
Hough, L., and Jones, J. K. N. (1962), *Methods Carbohydrate Chem. 1*, 400.
Kuhn, R., Bister, W., and Dafeldecker, W. (1959), *Ann. Chem. 628*, 186.
Levvy, G. A., and McAllan, A. (1959), *Biochem. J. 73*, 127.
Lin, T.-Y., and Gotschlich, E. C. (1963), *J. Biol. Chem. 238*, 1928.
Mathews, M. B., and Inouye, M. (1961), *Biochim. Biophys. Acta 53*, 509.
Perry, M. B. (1964), *Can. J. Biochem. 42*, 451.
Perry, M. B., and Hulyalkar, R. K. (1965), *Can. J. Biochem. 43*, 573.
Reissig, J. L., Strominger, J. L., and Leloir, L. F. (1955), *J. Biol. Chem. 217*, 959.
Roberts, W. K., Buchanan, J. G., and Baddiley, J. (1963), *Biochem. J. 88*, 1.
Stoffyn, P. J., and Jeanloz, R. W. (1954), *Arch. Biochem. Biophys. 52*, 373.
Suzuki, H., and Hehre, E. L. (1964), *Arch. Biochem. Biophys. 104*, 305.
Sweeley, C. C., Bentley, R., Makita, M., and Wells, W. W. (1963), *J. Am. Chem. Soc. 85*, 2497.

Fructose 1,6-Diphosphatase from Rabbit Liver. VI. Functional Tyrosyl Residues in the Active Center*

S. Pontremoli,† E. Grazi, and A. Accorsi

ABSTRACT: When fructose 1,6-diphosphatase from rabbit liver is acetylated with acetylimidazole, the catalytic activity toward fructose 1,6-diphosphate is decreased almost 90%. The changes in catalytic activity are prevented by the presence of the substrate and can be reversed by deacetylation with hydroxylamine. On the basis of difference spectra and the conditions for hydroxamate formation, it may be concluded that the alterations in enzymatic activity are correlated with acetylation of four tyrosyls. Other groups may be acetylated but only the *O*-acetylation of tyrosine residues can be related to the changes in catalytic activity.

In the course of efforts to identify the functional amino acid residues in fructose 1,6-diphosphatase (FDPase),[1] the authors have previously studied the effects of 2,4-dinitrofluorobenzene on the catalytic activity of the enzyme from rabbit liver (Pontremoli *et al.*, 1965a,b). Marked changes in catalytic activity resulted from the specific dinitrophenylation of a single sulfhydryl group in the protein molecule; the most significant of these was the large increase in catalytic activity at neutral pH when the enzyme was tested with Mn^{2+} as the metal activator.

In the search for other amino acid residues which may participate in the catalytic activity of FDPase, the authors have now found that treatment with acetylimidazole abolishes the hydrolytic activity almost completely. These alterations in catalytic activity correlate closely with the acetylation of four tyrosine residues. The participation of other functional groups, such as ϵ amino, aliphatic hydroxyl, imidazole, and sulfhydryl, in inactivation due to acetylation seems to be excluded.

Materials

Acetylimidazole was prepared by the method of Boyer (1952) and stored *in vacuo* over phosphorus pentoxide. *N,O*-Diacetyltyrosine and *N*-acetyltyrosine

* From the Istituto di Chimica Biologica, University of Ferrara, Ferrara, Italy. *Received May 24, 1966.* Supported by a grant from the Italian Consiglio Nazionale delle Richerche, Impresa Enzimologia, by the Grant GM 12291-02 from the National Institutes of Health, and by the Nato Grant No. 218.

† Istituto di Chimica Biologica, Università di Ferrara, Ferrara, Italy.

[1] The following abbreviations are used: FDPase, native fructose diphosphatase; Ac-FDPase, acetylated fructose diphosphatase; Ac-P-FDPase, fructose diphosphatase acetylated in the presence of the substrate fructose 1,6-diphosphate; TPN^+, triphosphopyridine nucleotide; TPNH, reduced TPN^+.