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OXIDATION OF THE CAPSULAR POLYSACCHARIDE OF PNEUMOCOCCAL TYPE IV BY PERIODATE*

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ABSTRACT

The immunologically specific capsular polysaccharide of pneumococcal type IV was oxidized with periodate before removal, and after partial and almost complete removal of pyruvic acid residues Destruction of the D-galactose residues was approximately proportional to the extent of removal of pyruvic acid residues, with relatively little effect on immunological specificity until more than one-third of the D-galactose residues had been affected The residues of the three amino sugars, 2-amino-2-deoxy-D-galactose, 2-amino-2-deoxymannose, and 2-amino-2,6-dideoxygalactose (fucosamine) were resistant to periodate oxidation and appeared to be separated from each other by one or more residues of (1→4)-linked *O*-pyruvyl-D-galactose There was also evidence for nonreducing end-groups of *O*-pyruvyl-D-galactose The pyruvyl residues are acetal-linked, possibly to positions 3 and 6 of D-galactose residues

INTRODUCTION

The type-specific capsular polysaccharide of pneumococcal type IV (S-IV) has become of renewed interest because of its content of pyruvic acid[1] and its acquisition of group-specificity when de-*O*-pyruvylated[2] The components of S-IV have been analyzed[3] and the specific inhibition of the reactions between the group-specific pneumococcal C-substance and anti-C and de-*O*-pyruvylated S-IV (dpS-IV) and anti-C has been described[4]. We now report on structural features which were elucidated by periodate oxidation of S-IV and dpS-IV

RESULTS

Treatment of S-IV with borohydride — This resulted in no loss of D-galactose or pyruvic acid residues and did not appreciably impair the immunological properties (Table I). D-Galactitol was not found by g l c of the products of hydrolysis The

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g l c peak corresponding to fucosamine was not appreciably diminished, but there was a 7% decrease in the absorbancy at 400 nm in the sugar determination by the cysteine–sulfuric acid method[5] Since this absorbancy corresponds, in part, to fucosamine[6], it is possible that a portion of the fucosamine residues in S-IV occurs as reducing end-groups

TABLE I

MAXIMAL PRECIPITATION OF ANTIBODY NITROGEN FROM ANTIPNEUMOCOCCAL TYPE IV SERA BY CAPSULAR POLYSACCHARIDE S-IV AND ITS DERIVATIVES[a]

Polysaccharide	*Antiserum*			
	Horse 608C[b]	*Horse 609*	*Horse 1026C (12/8/39)*	*Rabbit*[c]
S-IV	2340	2390	850	4300[d]
Reduced S-IV				4165[d]
Oxidized S-IV				4200
Oxidized-reduced S-IV				4110[d]
Alkali-treated S-IV				4250
39% DpS-IV				4025
Oxidized-reduced 39% dpS-IV				3115
DpS-IV	928	765	156	240
Oxidized-reduced dpS-IV	855[e]			36

[a]Precipitation from 1 0 ml of sera [b]Absorbed with group-specific C-polysaccharide [c]Purified, obtained from Squibb and Sons [d]Analyses performed at the same time with 0 15 ml of a 1 12 serum dilution, calc to 1 0 ml of undiluted serum [e]Supernatant + S-IV gave 1300 μg N

Oxidation of S-IV by periodate — A slight reduction of periodate could be traced to the extreme lability of some of the pyruvyl groups to acid and to the oxidation of the D-galactosyl residues exposed in this way

Oxidation of de-O-pyruvylated S-IV (dpS-IV) by periodate — DpS-IV containing 0 4–0 5% pyruvic acid residues reduced a maximum of 1 5 to 1 6 μmole of periodate per mg, with liberation of approximately 1 μmole of formic acid and no formaldehyde More than 90% of the D-galactose residues were oxidized and subsequently reduced and hydrolyzed[7] to threitol and traces of glycerol Oxidative destruction of the D-galactose residues almost abolished the already small precipitation of dpS-IV in antipneumococcal (anti-Pn) IV rabbit-antibody solution, but had little effect with antibodies raised in a horse (Table I) Reactivity of dpS-IV with the antibodies to the group-specific pneumococcal C-polysaccharide[2] was greatly reduced by the oxidation with anti-Pn I, from 269 μg N per ml to 11 μg, with anti-VII, from 579 μg to 125 μg, with anti-XII, from 334 μg to 56 μg (*cf* Table I, Ref 2) Since the residues of 2-acetamido-2-deoxy-D-galactose, the only sugar common to dpS-IV and C-polysaccharide[2], are unaffected by the oxidation, a large change in conformation must be involved on passage from dpS-IV to oxidized-reduced dpS-IV.

Degradation of oxidized-reduced dpS-IV with M hydrochloric acid for 24 h at room temperature, followed by gel filtration on Sephadex G-50, gave a small, excluded peak (6%) and a large one in the included region, with only traces of free

D-threitol before further hydrolysis On Sephadex G-25, the retarded peak gave a major fraction containing the three amino sugars and D-threitol, and two much smaller peaks

Oxidation of partially de-*O*-pyruvylated S-IV showed destruction of the D-galactose residues roughly proportional to the removal of the pyruvic acid residues The amino sugar residues remained intact

DISCUSSION

Intact S-IV is periodate-resistant and contains equimolar amounts of pyruvic acid and D-galactose residues[3] Partial de-*O*-pyruvylation (25–95%) renders a nearly equivalent portion of this sugar residue susceptible to oxidation Each residue of D-galactose is therefore substituted by one residue of pyruvic acid, and the resistance of D-galactose in S-IV to periodate is not due to (1→3)-linked D-galactose residues having no other substitution Hence, the three amino sugars are not pyruvylated and are linked internally in the 1,3 or 1,4 (or both) positions and not as non-reducing end-groups A few of the fucosamine residues may constitute reducing end-groups

Removal of 39% of the pyruvic acid residues exposed 35% of the D-galactosyl residues to oxidation and each of the latter residues consumed one equivalent of periodate Degradation of the oxidized-reduced product by mild hydrolysis yielded oligosaccharides with a terminal D-threitol residue The proportion of tetritol produced was approximately equivalent to that of the D-galactose oxidized, indicating that this portion of the sugar was bound at C-4 Since a small proportion of free D-threitol was also present, a few residues of D-galactose may be linked (1→4) consecutively and the presence of some residues linked at C-6 is not unequivocally ruled out

The proportion of periodate consumed by nearly fully de-*O*-pyruvylated S-IV (90–95%) was about 1 6 mole per mole of D-galactose, with liberation of about 1 equivalent of formic acid Mild hydrolysis of the oxidized-reduced product also yielded oligosaccharides with a terminal D-threitol residue as well as some free D-threitol and glycerol Therefore, dpS-IV contains some nonreducing end-groups of D-galactose which consume two equivalents of periodate Such end-groups are also indicated by the cross-reactivities of S-IV and dpS-IV in anti-Pn sera, which will be described elsewhere

The 39%-de-*O*-pyruvylated S-IV precipitated nearly as much antibody from rabbit anti-Pn IV as did intact S-IV, and a further reduction of only about 25% occurred after oxidation and reduction (Table I) This would indicate that the more stable pyruvylated end-groups of D-galactose are the principal antigenic determinants of S-IV, as has already been observed[2]

It is still uncertain which of the hydroxyl groups of D-galactose are substituted by pyruvic acid in S-IV The most probable are those at C-3 and C-6 A definitive answer must await experiments on methylation, as must also a decision as to whether or not only one type of linkage is involved

EXPERIMENTAL

General — The preparation of S-IV, the various methods of analysis of its components, and the estimation of precipitated-antibody nitrogen have been previously described[2–4] The reduction of periodate was measured titrimetrically[8].

Freeze-dried rabbit anti-Pn IV globulin (5 g, E R Squibb and Sons) were dissolved in chilled water (40 ml) A solution of 1% merthiolate (0 5 ml) was added and the pH was adjusted to 7 5 with M sodium hydroxide Sterile 0 9% sodium chloride was added to complete the volume to 60 ml As the solution contained about 4800 μg of antibody nitrogen per ml, most of the analyses were carried out at 1 6 or 1 12 dilutions at which the antibody content appeared somewhat less

Treatment of S-IV with borohydride — S-IV (10 mg) in water (27 ml) was treated with sodium borohydride (50 mg) added during 4 h The pH of the solution was maintained at 7–8 (pH meter) by a stream of carbon dioxide After 16 h at 4° and pH 7, excess borohydride was destroyed by addition of acetic acid to pH 4 and the solution was dialyzed against 0 1M sodium chloride and finally water The nondialyzable material was filtered off and the solution concentrated to 25 ml Aliquots (0 1 ml) were analyzed for D-galactose by the cysteine–sulfuric acid test together with aliquots of the original S-IV solution, for pyruvic acid, and for precipitation of rabbit anti-Pn IV Reduced S-IV (5 mg) was hydrolyzed (3M hydrochloric acid, 7 h, 100°) and analyzed by g l c Another sample (3 95 mg) was treated with M sodium hydroxide for 1 h at 37° The solution was neutralized with acetic acid, dialyzed, and the nondialyzable material analyzed by the cysteine–sulfuric acid test and for pyruvic acid

Oxidation of S-IV by periodate — S-IV (10 mg) in water (5 ml) was oxidized with 0 02M sodium metaperiodate solution (5 ml) at 4° in the dark Aliquots (0 2 ml) were analyzed at intervals for 30 h for formic acid content and for sodium periodate reduction Consumption of periodate was negligible The sodium periodate was reduced with 1,2-ethanediol and the solution was dialyzed against 0 1M sodium chloride and water The nondialyzable material was reduced with sodium borohydride as previously described and analyzed by the cysteine–sulfuric acid test and for activity against anti-Pn IV (Table I)

Oxidation of de-O-pyruvylated S-IV (dpS-IV) by periodate — S-IV (10 mg) was de-*O*-pyruvylated (0 01M hydrochloric acid, 30 min, 100°) and analyzed for residual pyruvic acid The dpS-IV was oxidized for 43 h, reduced, and analyzed as just described and also analyzed for production of formaldehyde as well An additional amount (50 mg) of dpS-IV was oxidized for 17 h, the experiment just described having shown no further oxidation after this period After reduction, the immunological activity of the substance was recorded (Table I) Aliquots of oxidized-reduced dpS-IV were analyzed with the cysteine–sulfuric acid test and, after acid hydrolysis (4M hydrochloric acid, 7 5 h, 100°), by g l c

Mild-acid hydrolysis and fractionation of oxidized-reduced dpS-IV — Oxidized-reduced dpS-IV (30 mg) was dissolved in M hydrochloric acid (10 ml) and kept for

24 h at room temperature before fractionation on a column (84 × 2 6 cm) of Sephadex G-50 and elution with water at 4° Aliquots (0 5 ml) from fractions (5 ml) were analyzed after hydrolysis (4M hydrochloric acid, 3 h, 100°), for amino sugars and by g l c both before and after hydrolysis, as just described The two fractions obtained from the column were isolated and the second fraction was adsorbed on Sephadex G-25 (80 × 2 6 cm) and eluted and analyzed as just described

Oxidation of partially de-O-pyruvylated S-IV (PdpS-IV) by periodate — S-IV (purified on DEAE Sephadex[2], 50 mg) was partially de-*O*-pyruvylated by treatment with 0 01M hydrochloric acid for 1 5 min at 100°, and the solution was poured into ice–water and dialyzed to give $Pdp_{1\ 5}$S-IV An aliquot was analyzed for residual pyruvic acid (5 4%) and the remainder oxidized with 0 01M sodium periodate for 28 h at 4°, followed by dialysis and reduction with borohydride The oxidized-reduced $Pdp_{1\ 5}$S-IV was analyzed by the cysteine–sulfuric acid test and by g l c after hydrolysis (2M hydrochloric acid, 6 h, 100° and 3M hydrochloric acid, 7 h, 100)° The pyruvate content was the same as before oxidation, but the D-galactose content had decreased to 35% Samples of $Pdp_{1\ 5}$S-IV and oxidized-reduced $Pdp_{1\ 5}$S-IV were also analyzed for residual immunological activity against rabbit anti-Pn IV Similarly, S-IV (purified on DEAE-Sephadex, 10 mg) was de-*O*-pyruvylated (0 01M hydrochloric acid, 1 min, 100°) to give $Pdp_{1\ 0}$S-IV (6 7% pyruvic acid) This was oxidized for 40 5 h, reduced as just described and aliquots were analyzed for pyruvic acid and by the cysteine–sulfuric acid test Losses of pyruvate and residues of D-galactose were 26% and 36%, respectively

Mild-acid hydrolysis and fractionation of oxidized-reduced PdpS-IV — A control study was made of the recovery of possible degradation products from oxidized-reduced $Pdp_{1\ 0}$ S-IV after treatment with 0 25M sulfuric acid and neutralization with barium hydroxide Samples (12 to 20 mg) of glycerol, erythritol, and D-galactose were dissolved in water (25 ml) To aliquots (1 0 mg) sulfuric acid was added to 0 25M concentration, and the solutions were kept 24 h at room temperature After neutralization, barium sulfate was centrifuged off and the combined supernatants and three washings were analyzed by g l c , as well as aliquots of the original solutions Recoveries for the 3 compounds were 46, 79, and 83%, respectively The low recovery of glycerol appears due to losses on concentration *in vacuo* Similar treatment of 200-μg samples of S-IV resulted in a loss of 50% of acetal-linked pyruvic acid

A solution of oxidized-reduced $Pdp_{1\ 0}$S-IV (8 mg) in 0 05M sulfuric acid (10 ml) was kept for 24 h at room temperature and a 1-ml aliquot (A) was removed Sulfuric acid was added to the remainder of the solution to concentration 0 25M and the mixture was kept for a further 24 h at room temperature, when a second 1-ml aliquot (B) was taken The residual solution was neutralized as just described and concentrated to 1 ml (C) Samples A and B were neutralized, aliquots of one-half of each were dried and silylated directly and hydrolyzed (2M hydrochloric acid, 5 h, 100°) and silylated, the four samples were examined by g l c Sample C was fractionated on a column (104 × 1 cm) of Sephadex G-25 and eluted with water

Aliquots (0 1 ml) of the fractions were analyzed by the cysteine–sulfuric acid test and by g l c before and after hydrolysis (2M hydrochloric acid, 5 h, 100°)

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