Inhibition by Aldobiouronates in the Precipitation of Pneumococcal Type II and III Systems*

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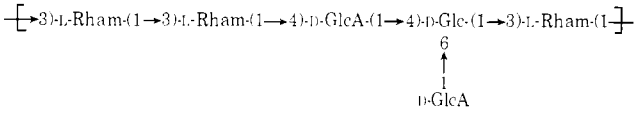
ABSTRACT: Inhibitory effects were studied of the sodium salts of D-glucuronic acid, maltouronic acid, cellobiouronic acid, isomaltouronic acid, and gentiobiouronic acid on precipitation of antipneumococcal type II and type III sera by the homologous capsular polysaccharides. Earlier conclusions of Goebel, based on uronic acid–protein conjugates, were confirmed and extended. The results exclude a recently proposed structure of the type II polysaccharide with side chains of cellobiouronic acid, and are in accord with the original data obtained by methylation.

The strong inhibition of the type II reaction by isomaltouronate indicates that the nonreducing end groups of D-glucuronic acid in the type II capsular polysaccharide are in the α anomeric form and are linked 1→6 to D-glucose. Isomaltose did not inhibit. Cellobiouronate was the best inhibitor of the type III reaction, in accord with the structure of the type III polysaccharide as a polycellobiouronic acid.

According to Butler and Stacey (1955) the capsular polysaccharide[1] of pneumococcal type II (Heidelberger and Avery, 1923; Heidelberger *et al.*, 1924) consists of nonreducing end groups of D-glucuronic acid, the same acid linked 1,4, L-rhamnose linked 1,3, and D-glucose in the form of 1,4,6-linked branch points. Of several possible structures based on this study, the following is in accord with the data on methylation (Butler and Stacey, 1955) and with subsequent studies on cross-reactivity (Heidelberger and Adams, 1956; Heidelberger, 1960; Heidelberger and McCarty, 1959; Goodman and Kabat, 1960), oxidation with periodate (Rebers *et al.*, 1962), and the inhibition of the SII anti-Pn II reaction by sugars (Goodman and Kabat, 1960).



In the meantime, Barker *et al.* (1967) have again subjected SII to oxidation with periodate and to degradation by induced enzymes and have proposed a structure with side chains of rhamnose and of cellobiouronic acid.

Several aldobiouronic acids were synthesized for the present study (Roy and Glaudemans, 1968) and were tested as inhibitors of the immune precipitation reactions, Pn SII anti-Pn II and Pn SIII anti-Pn III. The results are hardly compatible with the newer structure of Barker *et al.* (1967).

Experimental Section

Preparation of the Aldobiouronic Acids. Maltouronic acid was prepared by an unambiguous route as previously described (Roy and Glaudemans, 1968). Cellobiouronic acid was obtained from 1,6-anhydro-2,3,2′,3′,4′-penta-*O*-acetylcellobiose by oxidation with permanganate in acetic acid as described by Lindberg and Selleby (1960) The free acid, after deacetylation and hydrolysis of the 1,6-anhydro bridge, had $[\alpha]_D^{20}$ +7° (H_2O), lit. (Jayme and Demmig, 1960) $[\alpha]_D^{20}$ +7.6°. The acid was also prepared from the hydrolytic products of Pn S VIII, essentially according to Jones and Perry (1957); it had $[\alpha]_D^{24}$ +5° (H_2O).

Gentiobiouronic acid and isomaltouronic acid were obtained from the anomeric acetates of their methyl ester derivatives (Roy and Glaudemans, 1968) as follows. The fully acetylated methyl ester (200 mg) was dissolved in tetrahydrofuran (3 ml) and cooled in ice. To this was added ice-cold 1 N aqueous lithium hydroxide (10 ml) and the mixture was left for 0.5 hr in ice. The solution was passed through a column of Amberlite IR 120 and evaporated to a syrup. This was flashed off twice *in vacuo* with ethanol to remove H_2O and twice with toluene to remove ethanol. The resulting white powder was dried in a high vacuum. The yield of each free acid was approximately 100 mg. The products showed a single spot on paper chromatography with ethyl acetate–acetic acid–water (18:7:8). Isomaltouronic acid had R_{Glc} 0.59, and gentiobiouronic acid had R_{Glc} 0.50. Isomaltouronic acid, dissolved in water and neutralized with sodium hydroxide to phenol red, had $[\alpha]_D^{21.5}$ +85° (*c* 2.4).

Antisera were supplied by the New York City and New York State Department of Health Laboratories and by Dr. Bertil Björklund of the State Bacteriological Laboratory, Stockholm. Cellobiouronic acid was isolated from SVIII by Dr. Mervyn J. How. SII was supplied by Dr. E. A. Kabat (Beiser *et al.*, 1952).

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† Aided by Grant GB-5747 from the National Science Foundation.

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[1] Hereinafter designated S, with the appropriate type numeral. Other abbreviations used are: Pn, pneumococcal; CB, 4-*O*-β-D-glucopyranosyluronate-D-glucose (cellobiouronate); MU, 4-*O*-α-D-glucopyranosyluronate-D-glucose (maltouronate); GB, 6-*O*-β-D-glucopyranosyluronate-D-glucose (gentiobiouronate); IMU, 6-*O*-α-D-glucopyranosyluronate-D-glucose (isomaltouronate); IM, 6-*O*-α-D-glucopyranosyl-D-glucose (isomaltose); Glc, glucose; GlcA, glucuronate; Rham, rhamnose.

TABLE I: Inhibition of Pn SII Anti-Pn II H513[a] by Na Salts of Uronic Acids and by Isomaltose.

Uronate μM Added	Total Vol (ml)	At 0° (days)	Antibody N Pptd (μg)	Inhibn (%)
None	0.5	6	32	
CB,[b] 15	0.5	6	26	19
None	0.5	4	31	
GlcA, 15	0.5	4	25.5	18
None	0.6	7	30	
GlcA, 30	0.6	7	23	23
CB,[b] 29	0.6	7	27	10
None	0.5	11	31.5	
GlcA, 30	0.5	11	22	30
CB,[c] 30	0.5	11	30.5	3
MU,[c] 30	0.5	11	26.5	16
IMU,[c] 30	0.5	11	16.5	48
GB,[c] 30	0.5	11	33	0
None	0.5	6	35.5	
IMU, 15	0.5	6	16.5	54
None	0.5	7	35.5	
IMU, 7.5	0.5	7	19.5	45
None	0.5	7	32	
IM,[d] 35.5	0.5	7	30	6
IM,[d] 71	0.5	7	30.5	5

[a] Anti-Pn II horse 1054, C absorbed, and a reconstituted rabbit anti-Pn II globulin showed 6% or less inhibition with GlcA or CB at the 30 μM (1054) or 22 μM (rabbit anti-II) level. [b] Isolated from Pn S VIII by M. J. How. [c] Synthetic. [d] Provided by E. A. Kabat.

TABLE II: Inhibition of Pn SII Anti-Pn II Horse Swedish by Na Salts of Uronic Acids.

Uronate μM Added	Total Vol (ml)	At 0° (days)	Antibody N Pptd (μg)	Inhibn (%)
None	0.45	10	38	
GlcA, 30	0.45	10	16.5	57
CB,[a] 30	0.45	10	28.5	25
MU,[a] 30	0.45	10	21	45
None	0.5	6	38.5	
GlcA, 20	0.5	6	15	61
IMU,[a] 15	0.5	6	10.5	73
None	0.5	7	35.5	
IMU,[a] 7.5	0.5	7	10.5	70
None	0.5	8	29	
GlcA, 10	0.5	8	14	52
IMU,[a] 4	0.5	8	7.5	74
IMU,[a] 2	0.5	8	7.5	74
None	0.5	7	(30)[b]	
IMU,[a] 0.75	0.5	7	(12.5)[b]	(58)

[a] Synthetic. [b] Not run in duplicate.

Quantitative estimations of antibody nitrogen were carried out as in earlier papers (Heidelberger *et al.*, 1957; Heidelberger and Tyler, 1964). Inhibition reactions were set up in duplicate at 0°, usually with 0.10 ml of a dilution of the antiserum. To one pair of tubes was added a definite volume of 0.85% saline, and to the others a known quantity of (usually) 0.15 M sodium glucuronate or aldobiouronate, as indicated in Tables I, II, and III. After 0.5–1 hr, an amount of the homologous type-specific polysaccharide, calculated to reach equivalence, was added and the tubes were allowed to stand in a 0° bath for a number of days. They were then washed with saline in the cold, once or twice depending upon the concentration of serum, and the precipitates were dissolved with a drop of 1 N NaOH and rinsed into Markham flasks. Antibody nitrogen precipitated was estimated according to Markham (1942).

Results and Discussion

Goebel (1939, 1940) showed that a synthetic antigen of protein-coupled cellobiouronic acid (CB) evoked antibodies in rabbits which agglutinated type III pneumococci (but not those of types II and VIII[2]) and conferred passive immunity in mice against infection with pneumococcal types III, VIII, and II. Goebel concluded that this passive immunity was due to the terminal GlcA residues of the cellobiouronic acid antigen, since protein-coupled gentiobiouronic acid also elicited antibodies which conferred passive immunity in mice against pneumococcal type II, but not against types III or VIII. Inhibition of the precipitin reactions of the cellobiouronic antigen in anti-Pn II, III, and VIII also indicated that it was only the GlcA and not the whole CB that mediated the activity in anti-Pn II and the inference was made that neither CB nor GB was a constituent of S II.

The present studies reinforce Goebel's conclusions, extend them to two of the homologous immune systems instead of to a cross-reaction, and furnish new information as to the structure of S II.

Tables I and II show clearly that except in one dubious instance D-glucuronate is a better inhibitor of precipitation in the S II anti-Pn II reaction than are cellobiouronate, maltouronate, and gentiobiouronate. The last one did not inhibit at all at the concentrations used and even appeared to enhance precipitation somewhat. The outstanding inhibitor in this reaction in two different anti-Pn II horse sera was isomaltouronate and it was effective at relatively low concentrations. This provides evidence that, in S II, at least one of the residues of D-glucuronic acid in the presumed repeating unit is linked α,1→6 to D-glucose. A 1→6-linkage is compatible with structures based on the data of Butler and Stacey,

[2] This corrects a quotation from the literature in Roy and Glaudemans (1968).

TABLE III: Inhibition of Pn SIII Anti-Pn III 792C by Na Salts of Uronic Acids.

Uronate μM Added	Total Vol (ml)	At 0° (days)	Antibody N Pptd (μg)	Inhibn (%)
None	0.5	6	23	
CB,[a] 15	0.5	6	15	35
None	0.5	4	24	
GlcA, 15	0.5	4	23.5	2
None	0.6	7	24	
GlcA, 30	0.6	7	23.5	2
CB,[a] 29	0.6	7	13	46
None	0.5	9	30.5	
GlcA, 45	0.5	9	29	5
MU[b]	0.5	9	32	0
GB[b]	0.5	9	36	0
None	0.5	8	31	
CB,[b] 23	0.5	8	20.5	34
IMU[b] 19	0.5	8	31.5	0

[a] From Pn S VIII. [b] Synthetic.

as shown at the beginning of the present paper, and is actually postulated as occurring in the main chain in the newer structure of Barker *et al.* However, it has been shown that a large proportion of the antibodies in serum II 513, for example, is directed toward the nonreducing end groups of D-glucuronic acid (Goodman and Kabat, 1960; Heidelberger, 1960). The far greater inhibitory effect of isomaltouronate than of glucuronate therefore indicates that there is at least one residue of isomaltouronic acid with D-glucuronic acid as a nonreducing end group in the presumed repeating unit of S II.

With regard to the S III anti-Pn III reaction (Table III), the results are as expected from the known constitution of S III (Hotchkiss and Goebel, 1937; Reeves and Goebel, 1941). Cellobiouronate is a better inhibitor than glucuronate, as was anticipated in a system in which the antigenic determinant is a polycellobiouronic acid.

The data given in this paper again demonstrate the utility of quantitative immunochemical methods as checks and guides in the choice between alternative fine structures of polysaccharides.

Acknowledgment

The able technical assistance of W. P. Grosvenor is acknowledged.

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