PREDICTED AND UNPREDICTED CROSS-REACTIONS OF AN ACETYLPHOSPHOGALACTAN OF SPOROBOLOMYCES YEAST

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The teichoic acid of streptococcal Group N, which has nonreducing end groups of D-galactose phosphate (1), cross-reacts with antipneumococcal (anti–Pn) horse sera of types VI, XIV, XVI, and XXVII (2). In anticipation of the actual experiments, precipitation in antisera to the first two types was considered highly probable because (*a*) the capsular polysaccharidic determinant of the specificity of type VI, S VI, contains residues of D-galactose-2-phosphate in its repeating unit (3); (*b*) that of type XIV, S XIV, has nonreducing end groups of D-galactose (4, 5) which should not be very different immunologically from those of galactose phosphate; and (*c*) the quantitative precipitin theory of Heidelberger and Kendall (6) permits the prediction that, when two antigens contain multiples of the same sugar or sugars in the same or similar linkage, cross-reactivity may occur (7). Little is known of the capsular polysaccharides of types XVI and XXVII, S XVI and S XXVII, except that the former contains, in part, galactose and glycerophosphate, the latter galactose and phosphate (8), so that these portions of the determinants undoubtedly account for the reactions in anti–Pn XVI and anti–Pn XXVII.

In the meantime, much of the structure of an acetylphosphogalactan isolated from culture fluids of *Sporobolomyces* yeast has been clarified (9) and shown to be characterized by the sequences:

$$\alpha\text{-D-gal-1-PO}_3\text{-6-D-gal-(1} \rightarrow 6 \text{ and/or } 1 \rightarrow 3)\text{-D-gal...}$$
$$\Big|$$
$$\text{OK}$$

Since nonreducing end groups of D-galactose-1-phosphate were present, one could predict that the galactan would not only precipitate the same anti-Pn sera as did the teichoic acid of streptococcal Group N, but that it would also precipitate anti–Group N. These predictions were readily verified. Several

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additional unexpected and highly instructive reactions occurred as well, and all of these are quantitatively described in Tables I and II and discussed below.

Materials and Methods

Pneumococcal polysaccharides were obtained from E. R. Squibb and Sons,[1] kindness of Mr. T. D. Gerlough, and from Dr. Rachel Brown (10). Teichoic acid of streptococcal Group N was supplied by Dr. Stuart Elliott. Antipneumococcal horse sera were obtained from the New York City and New York State Departments of Health, anti-group N rabbit sera from Dr. Rebecca Lancefield, and the "O" polysaccharide of *Salmonella paratyphi A* and an equine antiserum to the microorganisms from Dr. Anne-Marie Staub. All analyses were carried out in duplicate at 0°C as in previous papers (11, 12) with the use of a cold box during drainage of the tubes preparatory to the washings (13).

Degradation Products of the Galactan.—Deacetylation of the intact material has been described (9). The product is designated DeAcPO$_4$ galactan (Table II).

Phosphomonoester galactan: 1 g of intact phosphogalactan from strain NRRL Y6502 in 100 ml H_2O was decationized and heated for 10 min in boiling water, neutralized to pH 6.7 with solid KOAc, and precipitated with 1 volume of MeOH. The precipitate was dissolved in H_2O, dialyzed, and lyophilized. Yield, 639 mg; $[\alpha]_D^{25}$- + 120° (c 0.45, 0.1 M KCl); gal:P, 5.9; acetyl: P, 0.6. Titration showed quantitative liberation of secondary phosphoryl groups.

Deacetylated dephosphorylated galactan (*DeAcdePO$_4$galactan*): 203 mg phosphomonoester, 200 μmoles $MgCl_2$, 1200 μmoles 2-amino-2-methyl-1,3-propanediol buffer, pH 9.8, and 10 mg commercial calf intestinal alkaline phosphatase were incubated at 37°C in the presence of toluene. Dephosphorylation was nearly complete in 24 hr. After removal of protein according to Sevag (14), the galactan was precipitated with 1 volume MeOH, redissolved in H_2O, reprecipitated as before, redissolved in H_2O, dialyzed, clarified at 20,000 *g*, and lyophilized. Yield, 101 mg; $[\alpha]_D^{25}$- + 129° (c 0.66, H_2O); gal: P, 42; no acetyl.

Dephosphorylated acetylgalactan (*AcdePO$_4$*): Phosphomonoester galactan (101 mg) in 50 ml 0.05 M acetate buffer at pH 5 was treated with 20 mg wheat-germ acid phosphatase in 2 ml buffer. 2 ml 0.1 M $MgCl_2$ and toluene were added. After 2 wk at 25°C an additional 20 mg enzyme was added and the mixture was left 2 wk longer. Spontaneously denatured enzyme was centrifuged off and acetylgalactan precipitated with 3 volumes of 95% EtOH, redissolved, and further deproteinized by eight Sevag treatments. The product was precipitated with 1.5 vols EtOH plus 1 drop saturated KCl, dialyzed, and lyophilized. Yield, 51 mg; $[\alpha]_D^{25}$- + 109° (c 0.33,0.1 M KCl); gal: P, 23; acetyl: P, 2.1.

Oxidized Acetyl Phosphogalactan (*OxAcPgal*).—To 205 mg AcPgal Y6502 in 50 ml H_2O were added 4 ml of 0.6 M $NaIO_4$ solution at 4°C. After 4 days, 2 ml $(CH_2OH)_2$ were added and the solution was dialyzed against H_2O. OAc, 0.7 μM/mg original substance; with 0.8 μM/mg.

Reduction of OxAcPgal.—

1. Oxidized-reduced acetyl phosphogalactan (OxredAcPgal): To 23 ml of a solution of OxAcPgal calculated to contain 3.4 mg/ml, 0.25 g $NaBH_4$ was added in small portions during 1 hr while N_2 was passed over to reduce foaming and CO_2 passed in intermittently to keep the pH near 7 (it went to 8.2 during 10 min and was brought back to 7 with 2 drops HOAc). The reaction mixture was dialyzed. Analysis showed that no deacetylation had occurred and that, after hydrolysis in 0.02 N HCl for 30 min at 100°C, glycerol and α-glycerophosphate were the principal mobile components on chromatograms.

2. Oxidized-reduced phosphogalactan (OxredPgal): A dialyzed solution of OxAcPgal,

[1] S VI, S XIV, and S XVIII were further purified.

prepared as above, was brought to room temperature, treated with 0.5 g $NaBH_4$ in small portions during 1 hr, and let stand overnight, after which it was dialyzed vs. H_2O in the cold. The product contained no acetyl.

Before use in serological tests, NaCl was added to bring the above oxidized substances to approximately 0.15 M NaCl concentration.

TABLE I

*Predicted Cross-Reactions of Sporobolomyces Phosphogalactan**

Substance (μg)	Antibody nitrogen precipitated at 0°C calculated to 1.0 ml antiserum				
	Antipneumococcal sera type				Anti-streptococcal group N
	VI H681C‡	XIV H635C	XVI H594C	XXVII H668C	R2013
	μg	μg	μg	μg	μg
Homologous (max.)	690	910	900	260	840
Intact PO_4 galactan					
50	126	81§		22‖	135¶
100	166	89§	100**	16‖	151¶
200	173		91**		124¶
400	155‡‡				
$DeAcPO_4$ galactan					
100			104		
200			99		

* Phosphogalactans from strains Y6493 and Y6502 precipitated identical amounts of antibody N from antisera tested with both.

‡ C indicates sera absorbed with pneumococcal C-substance if appreciable amounts of anti-C were present; H, horse; R, rabbit.

§ Supernatants + guar gave 113 μg N; intact serum gave 204 μg.

‖ Supernatants + streptococcal N teichoic acid gave 64 μg N; intact serum gave 90 μg (extrapolated value). Supernatants + *Rhizobium radicicola* at the 50 μg level gave 55 μg N as in intact serum (2).

¶ Supernatants + S XVI gave 7 μg N; intact serum gave 21 μg.

** Supernatants + strep. N teichoic acid gave 140 μg N; intact serum gave 250 μg (extrapolated value).

‡‡ Supernatants from all analyses mixed: + guar, gave 12 μg N; intact antiserum gave 106 μg.

RESULTS AND DISCUSSION

From the structure of the phosphogalactan (9) and the quantitative theory of the precipitin reaction (6), it could be predicted that the galactan would not only cross-react with antipneumococcal sera of types VI, XIV, XVI, and XXVII, those precipitated by the teichoic acid of streptococcal Group N, but would also react with antiserum to Group N. The quantitative data in Table I show that the predicted reactions occurred and were extensive in all but

anti–Pn XXVII. In qualitative tests, removal of the acetyl group slightly reduced reactivity in anti–Pn XIV but did not appreciably increase or diminish precipitation in anti–Pn VI. This would tend to confirm other evidence (see reference 9, and below) that the acetyl groups in the phosphogalactan are not on the terminal residues of D-galactose-1-phosphate. Deacetylation of the phosphogalactan also failed to influence precipitation visibly in anti-streptococcal Group N serum, but removal of the end group with formation of the phosphomonoester greatly reduced reactivity in anti–Pn VI, anti–Pn XIV, and anti-N, as anticipated from the known structures of S VI, S XIV, and the Group N teichoic acid.

So much for the expected reactions. Entirely unexpected was the massive reactivity of the intact galactan in anti–Pn XVIII, for in the one horse serum used, 73% of the type-specific antibody was precipitated. The structure of the antigenic determinant of Pn XVIII, S XVIII, was shown to be:

$$\left[\begin{array}{c}\rightarrow 3)\text{-D-gal-}(1\rightarrow 4)\text{-}\alpha\text{-D-glc-}(1\rightarrow 6)\text{-D-glc-}(1\rightarrow 3)\text{-L-rham-}(1\rightarrow 4)\text{-D-glc-}(1- \\ \text{Ac-} \\ HOCH_2CHOHCH_2OPO_2O\text{-}\end{array}\right]_x$$

or the alternative structure in which the places of the galactose and rhamnose are exchanged. The positions of the acetyl and α-glycerophosphate groups were not known (15), but an acetylated sugar proved to be the principal antigenic determinant of the repeating unit, as only 20% of the type-specific antibody was precipitated upon removal of the O-acetyl. Had the single residue of 1,3-linked D-galactose in the repeating unit of S XVIII been responsible for the cross-reactivity of the *Sporobolomyces* phosphogalactan in anti–Pn XVIII, only weak precipitation could have occurred. A closer reading of the Slodki paper (9) was evidently necessary and this supplied the clue: the phosphogalactan had been shown to have roughly one O-acetyl group for each phosphorus! Its relevance was quickly established, for the deacetylated phosphogalactan gave no precipitate whatsoever in the anti–Pn XVIII horse serum and only 7–17% as much in rabbit anti–Pn XVIII sera as did the intact galactan (Table II). This behavior was reminiscent of S XVIII itself, for, strangely enough, its deacetylation reduced precipitation less in rabbit antisera than in that raised in a horse (15).

The magnitude of this unpredicted cross-reaction leads to two predictions, which should, of course, be tested for their validity by direct chemical study: (*a*) In the phosphogalactan the O-acetyl group is necessarily on one of the residues of galactose, therefore the acetyl group of S XVIII must also occur on the single galactose in the repeating unit; and (*b*) since this galactose is linked 1,3-, the acetyl group in the yeast phosphogalactan will probably also be found on a 1,3-linked galactose.

The terminal residue of D-galactose-1-phosphate in the yeast phosphogalactan is of negligible importance in this cross-reaction, since removal either of

TABLE II

Unpredicted Cross-Reactions of Sporobolomyces Phosphogalactan and its Derivatives

Substance (μg)	Antibody nitrogen precipitated at 0°C, calculated to 1.0 ml antiserum.					
	Antipneumococcal sera type					Anti-Salmonella paraty A
	XVIII H495C	XVIII R42C	XVIII R500	XXIII H912	XXV H513C	H152
	μg	μg	μg	μg	μg	μg
Homologous polysaccharide (max.)	2200	5360	785	275	186	730
$AcPO_4$ galactan						
25					26	
50				42	32	
100			210*	44		305
200	1185	345	265*			445‡
400	1495	385				450
800	1610					
1600	1515§					
$DeAcPO_4$ galactan						
50	0	25	46	35		
100		13	23	32		
200	0					400
400						415‖
$AcPO_4$ monoester						
50				39		
100				38		
200	1260					390
400	1410					395
800	1290					
$AcDePO_4$ galactan						
400	1260					
800	1400					
$DeAcDePO_4$ galactan						
50				22		
100				16		
200						425
400						420
OxAcPgal						
100						363
200						481
400	1620					545¶
800	1885					
OxredAcPgal						
400	730					
800	1200					
2000	1160					
OxredPgal						
200						154
400						200
800						215

* Combined supernatants + S XVIII gave 550 μg N; mean total, 790 μg.

‡ In another run at this level, supernatant + paraty A polysaccharide gave 316 μg N; total, 761 μg.

‖ Single determination only.

§ All supernatants combined; + S XVIII gave 675 μg N; mean total, 2125 μg.

¶ Supernatants plus paratyphoid A "O" polysaccharide gave 440 μg N; total, 985 μg showing that some nonspecific protein had been precipitated by OxAcPgal.

the galactose alone to form the phosphomonoester, or of both the sugar and its attached phosphate fails to influence appreciably the amount of antibody precipitated provided the O-acetyl groups are retained (Table II). These reactions were tested only in the horse serum.

The other major cross-reaction which turned up was in equine antiserum to *S. paratyphi A*. Little was known of the linkages of the sugars in the "O" polysaccharide of this bacillus except that paratose occupied some of the terminal positions and glucose and rhamnose others (16). The data in Table II, which indicate involvement of up to 60% of the anti-"O" in the serum,[2] establish clearly that the acetyl, terminal galactose, and phosphate residues of the phosphogalactan are not implicated. This leaves only multiples of the internal residues of D-galactose and, since the cross-reaction is so massive, probably at least two consecutive units form the corresponding determinant in the paratyphoid A polysaccharide. They are thus shown for the first time to belong in the D- series and to be linked either 1,6-, 1,6-; 1,6-, 1,3-; 1,3-, 1,6-; or 1,3-, 1,3-. There is, then, a major determinant of *S. paratyphi A* in addition to the generally recognized "antigens" 1, 2, and 12, or else D-galactose is an essential part of one or another of these.

A smaller cross-reaction in anti-Pn XXIII must also be due to multiple occurrences of 1,3- or 1,6-linked D-galactose, since it, too, is unaffected by removal of acetyl or terminal galactose from the phosphogalactan. S XXIII contains D-galactose, D-glucose, L-rhamnose, and phosphate,[3] but details have not yet appeared. Principal determinants of S XXIII are multiple nonreducing end groups of L-rhamnose, and D-galactose is a minor determinant (17). Little can be said about the reaction in anti-Pn XXV except that galactose[4] and phosphate are both said to occur in S XXV (8).

The effects of oxidation of the acetyl phosphogalactan by periodate and reduction of the oxidized product were also studied. Qualitatively, OxredPgal showed only weak reactions in anti-Pn VI, XIV, and XXIII and doubtful to negative ones in anti-Pn XVI and XXVII. Quantitative analyses of the precipitation of anti-Pn XVIII and anti-paraty A by OxAcPgal indicated that nonspecific protein as well as antibody was covalently precipitated, as had occasionally been observed before (18), and this was further indicated by the opalescence of the supernatants and washings and by difficulty in dissolving the washed precipitates in alkali. Reduction of the oxidized galactan with borohydride was accompanied by removal of -OAc and near abolition of precipitation in anti-Pn XVIII, as expected, but when precautions were taken

[2] *Note added in proof:* Rabbit anti-*S. paratyphi A* serum (Sylvana Chemical Co., Millburn' N. J., lot No. 062067-21) with 87 μg N/ml precipitable by the homologous O-polysaccharide, gave 69 μg N, or 79%, with $AcPO_4$-galactan.

[3] Jones, J. K. N., and M. B. Perry. Personal communication.

[4] Confirmed by Dr. Amalendu Das.

to maintain the reduction as close to neutrality as possible, -OAc was retained and precipitation in anti-Pn XVIII was heavy, although somewhat less than with the intact galactan (Table II). In most instances reduction of periodate-oxidized polysaccharides has reduced their serological activity (15, 19, 20). This is shown also by the data on OxredPgal in anti-paraty A.

SUMMARY

The teichoic acid of streptococcal Group N, with end groups of galactose phosphate, had been shown to cross-react with antipneumococcal sera of types VI, XIV, XVI, and XXVII. End groups of D-galactose-1-phosphate in the phosphogalactans of *Sporobolomyces* yeasts made it predictable that these galactans would precipitate the same antipneumococcal sera and also antisera to streptococcal Group N. The predictions were verified, and other unpredicted reactions were found. Precipitation of much of the antibody in an antipneumoccal type XVIII horse serum was shown to be due to O-acetyl-D-galactose residues in the phosphogalactan, in accord with earlier information that an O-acetyl sugar was a principal determinant of S XVIII. The new results identify this sugar as D-galactose. Since it is linked 1,3- in S XVIII, the O-acetyl group in the *Sporobolomyces* galactan is probably also on a 1,3-linked residue. Another major cross-reaction in anti-*S. paratyphi A* serum characterizes the galactose residues in the "O" polysaccharide of the bacillus as members of the D-series probably linked in tandem 1,6-, 1,6-; 1,6-, 1,3-; 1,3-, 1,6-; or 1,3-, 1,3-. Reactions of periodate-oxidized–reduced products confirm the conclusions stated above. Quantitative data are given.

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