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The Specific Polysaccharide of Type XVIII Pneumococcus. III*

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The galactose in the specific polysaccharide of type XVIII pneumococcus (S XVIII) has now been identified as the D enantiomorph by means of D-galactose oxidase. Glycerol was confirmed by paper electrophoresis and by the action of glycerol dehydrogenase. *O*-Acetyl was found as an additional constituent very important in the immunological specificity of S XVIII. Molar ratios of the components approximate: D-glucose 3, D-galactose 1, L-rhamnose 1, glycerol 1, *O*-acetyl 1, phosphate 1. The sugars appear to be in the pyranose form. Treatment with strong alkali splits off glycerophosphate and *O*-acetyl, yielding a product, Alk S XVIII, which retains the sugars with no change in ratios. Alk S VXIII, oxidized by periodate and reduced by borohydride, yields, in the proportions indicated: D-galactose 1, L-rhamnose 1, erythritol ≤ 1.5, glycerol 1. Mild acid hydrolysis of this product, followed by a second oxidation with periodate and reduction, results in destruction of the galactose and rhamnose with formation of glycerol and propylene glycol. These results, together with the cross reactions described and tests for their inhibition, point toward one or the other structure:

$$-\left[\rightarrow 3)\text{-D-gal-}(1\rightarrow 4)\text{-}\alpha\text{-D-glc-}(1\rightarrow 6)\text{-D-glc-}(1\rightarrow 3)\text{-L-rham-}(1\rightarrow 4)\text{-D-glc-}(1-\right]-$$
$$\left.\begin{array}{l}\text{Ac—}\\ \text{HOCH}_2.\text{CHOH.CH}_2\text{O.PO}_2-\end{array}\right]_x$$

or

$$-\left[\rightarrow 3)\text{-D-gal-}(1\rightarrow 4)\text{-D-glc-}(1\rightarrow 3)\text{-L-rham-}(1\rightarrow 4)\text{-}\alpha\text{-D-glc-}(1\rightarrow 6)\text{-D-glc-}(1-\right]-$$
$$\left.\begin{array}{l}\text{Ac—}\\ \text{HOCH}_2.\text{CHOH.CH}_2\text{O.PO}_2-\end{array}\right]_x$$

Initial studies on the capsular polysaccharide of type XVIII pneumococcus, S XVIII, showed the presence of D-glucose, L-rhamnose, and secondarily bound phosphate (Markowitz and Heidelberger, 1954). Because ribitol phosphate was found in the capsular polysaccharide of type VI pneumococcus, S VI (Rebers and Heidelberger, 1959, 1961), the study of S XVIII was resumed (Estrada-Parra *et al.*, 1962), resulting in the finding of two new components, galactose and glycerol, the latter present as glycerophosphate. The glycerophosphate could be removed by alkali without loss of the sugars (Alk S XVIII). Oxidation of S XVIII and Alk S XVIII by periodate indicated that rhamnose and galactose were probably 1,3-linked and that at least part of the glucose was bound 1,4- and 1,6-. The present paper is a report of further progress which has resulted in the proposal of partial structures for S XVIII.

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EXPERIMENTAL

Earlier preparations of S XVIII were used in part, e.g., 1104P (Estrada-Parra *et al.*, 1962). An additional batch was prepared from a strain of type XVIII pneumococci obtained from Dr. R. Austrian. This was passed through mice and grown in eleven 4-liter flasks, each containing 2 liters of Adams-Roe medium (1945) for 18 hours. S XVIII was obtained both from the cells (fractions A[1] and B) and culture fluid (fractions C and D) as in previous papers (Heidelberger *et al.*, 1950; Estrada-Parra *et al.*, 1962).

Properties of the fractions, as well as of another (1800H) obtained by combination and repurification of earlier preparations are given in Table I. *Aloe vera* polysaccharide was kindly given by Dr. F. Cordoba, S XVIII A by Dr. Rachel Brown, and the polysaccharide of *Klebsiella* E-26 by Dr. J. F. Wilkinson. Immunochemical methods are described in other papers (Rebers and Heidelberger, 1959, 1961; Rebers *et al.*, 1961).

Determination of O-Acetyl.—By the method of Hestrin (1949), with ethyl acetate as standard, duplicate 2.0-mg portions of S XVIII C gave 92 μg, or 4.6%. The molar ratio of *O*-acetyl to phosphate was therefore 0.81:1 (on ash-free basis).

[1] A small, acetic acid–precipitable fraction, apparently mainly protein.

TABLE I
PROPERTIES OF FRACTIONS OF S XVIII

Fraction	Yield (mg)	$[\alpha]_D^{23}$ [a] (degrees)	N[a] (%)	P[a] (%)	Reducing Value[b] as Glucose (μg/mg)	Ash as Na^+ (%)
S XVIII B	162	+85	0.8	3.3	1.3	3.4
S XVIII C	454	+79	0.3	3.0	0.8	1.8
S XVIII D	201	+78	0.3	3.0	1.8	3.0
S XVIII 1800H	80	+85	0.8	3.1	7.2	1.9

[a] Not calculated to the ash-free basis. [b] By method of Park and Johnson (1949).

TABLE II
TREATMENT OF S XVIII WITH ALKALI

Alk S XVIII Preparation	Length of Alkali Treatment (days)	Temperature (degrees)	Final *p*H	Experimental Values of P Calculated as % of Total Found		Reducing Value[b] as Glucose (μg/mg)
				Split Off (%)	Residual (%)	
0% Alk S XVIII[a]	1	22	10	0	100	
0% Alk S XVIII	3	22	10	0	100	
5% Alk S XVIII	3	22	12	5	95	
6% Alk S XVIII	10	28	12	6	94	
50% Alk S XVIII	21	37	12	50	50	
92% Alk S XVIII	26	37	14	92	8	3.1
94% Alk S XVIII	25	37	14	94	6	9.3
97% Alk S XVIII	40	37	14	97	3	
98% Alk S XVIII	25	37	14	98	2	6.0

[a] The % indicates the amount of P split off. [b] By method of Park and Johnson (1949).

Attempt to Hydrolyze S XVIII at pH 2.4.—S XVIII (1800H) was treated with wet Dowex-50 and after removal of the resin the filtrate, at *p*H 2.4, was heated in a sealed tube in boiling ethanol for 4 hours. Paper chromatograms gave no evidence of the splitting off of sugars or glycerol.

Identification of Galactose as the D-*Enantiomorph.*—Eight mg of S XVIII (1800H) was hydrolyzed with 2.5 N H_2SO_4, neutralized with Duolite A, and concentrated, and the galactose was separated by paper chromatography with solvent A (1-butanol-pyridine-water-benzene, 5:3:3:1; Jeanes, *et al.*, 1951), as described by Whistler and BeMiller (1962). The area corresponding to galactose was eluted with 5.0 ml of water, yielding 80 μg/ml by the phenol method (Dubois *et al.*, 1956). Determination of galactose with D-galactose oxidase (Avigad *et al.*, 1962) in 1.0 and 2.0 ml of the solution yielded 75 and 148 μg.

Isolation and Further Identification of Glycerol.—Fifty-seven mg (i.e., 57.5 μmoles of repeating unit) of S XVIII (1104P) was degraded at 28° for 8 days with 0.1 N NaOH. The dialyzable material, after treatment with Duolite A (to remove glycerophosphate) and Dowex-50, was dried *in vacuo* and extracted with 5 ml of absolute ethanol. Paper chromatograms run in solvents A and B (1-butanol-ethanol-water-ammonia, 40:10:49:1; Block, 1952) then showed only a spot corresponding to glycerol and its mobility when subjected to paper electrophoresis in 0.05 M sodium borate buffer at *p*H 9.9 at 500 v and 7 ma, was the same as that of an authentic sample. Determination of glycerol in the alcoholic solution by the chromotropic acid method (Lambert and Neish, 1950) showed 1.34 μmoles/ml. An aliquot portion was evaporated and redissolved in the same volume of water. With glycerol dehydrogenase (Hagen, 1962) the amount found was 1.36 μmoles/ml.

Degradation by Alkali.—When S XVIII is treated with 1 N NaOH at 37° under N_2 for 3 weeks, 90% or more of its phosphate is eliminated, mostly as glycerophosphate (Estrada-Parra *et al.*, 1962). The amount of P split off varies with the *p*H, time of reaction, and temperature. A summary of data on the products is given in Table II and their reactivities toward antisera are given in Table III.

Determination of O-Acetyl in Alkali-degraded Preparations.—By Hestrin's method (1949) S XVIII treated 1 day and 3 days at *p*H 10 gave 35 and 41 μg of *O*-acetyl, respectively, per mg of polysaccharide, but no *O*-acetyl remained after 3 days at *p*H 12 and 22° (5% Alk S XVIII).

Titration Curves of S XVIII and Alkali-cleaved S XVIII.—S XVIII (1104P) was converted into the free acid with Dowex-50. Two 10-ml portions of a solution containing 2.5 mg S XVIII/ml (i.e. 80.5 μg P/ml) were titrated under N_2 with 0.0137 N $Ba(OH)_2$. With a *p*H meter, a curve resulted, almost identical with that of a monobasic acid (Figure 1). Also, S XVIII which had been treated with 1 N NaOH for 5 weeks at 37° was passed through Dowex-50 to remove Na^+, concentrated, lyophilized, and dried to constant weight. Ten ml, containing 2 mg/ml alkali-cleaved S XVIII and 67.7 μg P/ml, were titrated as above with 0.0116 N $Ba(OH)_2$, reacting as a dibasic acid (Fig. 1).

Ratios of the Components of S XVIII and 92% Alk S XVIII.—To 5.0 ml each of S XVIII C (1 mg/ml) and Alk S XVIII (1.26 mg/ml), 0.5 ml of 5 N H_2SO_4 was added and the solutions were heated for 6 hours at 100° in sealed tubes. Quantitative determinations of the components are given in Table IV. The molar ratios in S XVIII come close to glucose-galactose-rhamnose-phosphorus, 3:1:1:1. The ratios of the sugars remain unchanged in Alk S XVIII. The same ratios of sugars (3.1:1.0:1.1) were obtained upon hydrolysis of preparation 1800H after separation by paper chromatography and elution with water.

The ratio of glycerol to sugars in S XVIII was obtained on an aliquot portion of 1.5 ml of the acid hydrolysate of S XVIII C after 15 hours' further heating. One ml was neutralized with Duolite A, after which

TABLE III
MAXIMAL PRECIPITATION OF HORSE (H), RABBIT (R), ANTI-PN[a] XVIII SERA BY S XVIII AND DERIVATIVES AT 0°

Antigen		μg Antibody N pptd, Calcd to 1.0 ml			
Substance	Taken (μg)	H 495C[b]	H 632C	R 43C "Diluted"[c]	R 43C
S XVIII	100		308		
	300	2200			
	1200			3460	4590
0% Alk S XVIII	100		282		
	600	1860			
	1200			3400	
5% Alk S XVIII	30		25		
	100	445			
	400			1150	
50% Alk S XVIII	33		20		
	300	349			
90% Alk S XVIII	33		2		
	100	285[d]			233
98% Alk S XVIII	30		2		
	600	275			
S XVIII-IO_4	111		143[d]		
	300			164	
	540	1750[d]			
S XVIII-IO_4-BH_4	60		62		
	100				50
	1200	800			
Alk S XVIII-IO_4	50	0			
	150	0			

[a] Pn, pneumococcal. [b] Absorbed with Pn C-substance. [c] 43C "dil": The amounts given are calculated to 1.0 ml undiluted serum, but as the tests were carried out in serum diluted 1 → 4 comparison with antibody N precipitable by intact S XVIII at the same dilution was necessary. [d] From Estrada-Parra *et al.* (1962).

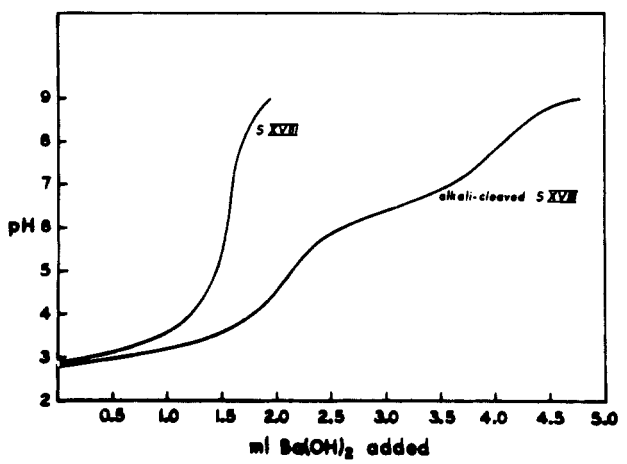

FIG. 1.—Titration curves of S XVIII and alkali-cleaved S XVIII.

TABLE IV
RATIOS OF THE COMPONENTS OF S XVIII AND 92% ALK S XVIII

Components	S XVIII (μg/ml)	S XVIII (μmoles/ml)	92% Alk S XVIII (μg/ml)	92% Alk S XVIII (μmoles/ml)
Total sugars by phenol	680		1102	
Rhamnose by Dische (1948)	126	0.77	203	1.24
Total hexose by Dische	567	3.15	945	5.25
Glucose by "glucostat"[a]	417	2.32	706	3.92
Galactose by difference[b]	150	0.83	239	1.32
Total P	26	0.85		
Ratio of hexose/rhamnose	4.1		4.2	
Ratio of glucose/galactose	2.8		2.9	

[a] Solution hydrolyzed for 21 hours and neutralized before determination with "glucostat" (Saloman and Johnson, 1959). [b] Glucose subtracted from total hexose.

glycerol dehydrogenase showed 0.652 μmole of glycerol/ml. Total sugars by the phenol method were 572 μg/ml, or 3.3 μmoles. The ratio, sugars to glycerol, was 3.3/0.652, 5.1:1.

Studies with Periodate.—(a) Consumption of Periodate by S XVIII and Alk S XVIII and Determination of Formic Acid and Formaldehyde.—At 4°, S XVIII consumes 4.36 μmoles of periodate per μmole of P in 14 days and 4.4 μmoles in 21 days. Under similar conditions duplicate samples of 2.72 mg of 97% Alk S XVIII consumed 13.3 μmoles of periodate in 13 days. The molecular weight of the presumed "repeating unit" of Alk S XVIII, as 3 glucose, 1 galactose, and 1 rhamnose, minus 5 H_2O, is 794. Therefore 2720/794 = 3.4 units of Alk S XVIII and the μmoles of periodate consumed per unit are 13.3/3.4, or 3.9, a value which remained the same after 4 more days. Determination of formic acid in the oxidation of 97% Alk S XVIII, as described in Kabat and Mayer (1961), showed that after 13 days 10.8-mg portions of Alk S XVIII had each liberated 1.21 μmoles of HCOOH, or 0.9 μmole per "repeating unit." Determination of formaldehyde upon oxidation of S XVIII gave 1.26 μmoles per μmole of P (Estrada-Parra *et al.*, 1962). Five per cent Alk S XVIII liberates a similar amount, while 92% Alk S XVIII yields a negligible quantity.

(b) Oxidation of S XVIII and Alk S XVIII with Periodate, Reduction by Borohydride, and Acid Hydrolysis.—Parallel oxidations, with a 4-fold excess of reagent, were carried out with 10-ml portions of S XVIII, 1 mg/ml, and 92% Alk S XVIII, 1.26 mg/ml, during 4 days at 4°. Periodate and iodate were precipitated with 27 mg each of $BaCl_2$ and the solutions were dialyzed. To aliquot portions of each, 14 mg of sodium borohydride was added, and after 24 hours the solutions were dialyzed, concentrated, and hydrolyzed with 0.5 N H_2SO_4. The solutions were neutralized with Duolite A, concentrated, and chromatographed on paper with the solvent systems A and B. In A the S XVIII-IO_4-BH_4 hydrolysate, sprayed with $AgNO_3$-NH_3 (Block, 1952), showed spots corresponding to galactose, glucose,

TABLE V
MAXIMAL CROSS PRECIPITATION OF DEXTRANS FROM *Leuconostoc* IN HORSE ANTI-PN XVIII 495C SERUM, 1.0 ML AT 0°

Dextran	Types of Linkages (%)					Dextran Added (μg)	Antibody N Pptd (μg)
	1 → 6	1 → 4 (like)	1 → 3	1 → 2[a]	1 → 4		
B-1146	97	3				150	55
N-236	*ca.* 95[f]		4			50	47[e]
B-512	95	5				50	44
B-1254	92	4	4			150	44
B-1351	85	4	11			150	28
B-742	66	25	9			150	21
B-1299 (L)[b]	58	36	6	34	2	150	50
B-1355 (S)[c]	57	8	35	−5		150	18
B-1299 (S3)[d]	50	50		38	12	50	16

[a] Given by rotation in cuproammonium solution (Scott *et al.*, 1957). [b] Fraction L. [c] Fraction S has 1 → 6 linkages in branches. [d] Fraction S. [e] Supernatant + glycogen pptd 4 μg N; supernatant from this + *A. vera* gave 149 μg N (see Table VI). [f] Kabat and Berg (1953).

erythritol, and rhamnose, while Alk S XVIII-IO_4-BH_4 gave the same spots except for glucose. A chromatogram in solvent B gave spots corresponding to galactose, glucose, erythritol, rhamnose, and glycerol, the last being weaker with S XVIII. Another chromatogram in solvent B, sprayed with KIO_4-benzidine (Cifonelli and Smith, 1954), gave an additional spot corresponding to ethylene glycol with the solution from S XVIII, but not with that from Alk S XVIII. In similar experiments with 0%, 5%, and 6% Alk S XVIII, paper chromatograms showed the components found on oxidation of S XVIII. The reactions of S XVIII-IO_4, Alk S XVIII-IO_4, and their products of reduction toward anti-Pn XVIII sera are given in Table III.

(c) Quantitative Determination of Sugars, Glycerol, and Erythritol in Oxidized-reduced Alk S XVIII.—Alk S XVIII preparations, 94% and 98% degraded, were mixed, oxidized for 10 days, and reduced, hydrolyzed, and neutralized as in the above experiment. Determination of sugars by the phenol method gave 465 μg/ml, corresponding to 465/172 or 2.9 μmoles/ml of galactose-rhamnose (172 = mean mw of galactose, 180, and rhamnose, 164), the only sugars remaining. Determination of glycerol-erythritol by the chromotropic acid method gave 4.5 μmoles/ml. Estimation of glycerol by glycerol dehydrogenase gave 1.5 μmoles/ml; therefore μmoles of erythritol by difference are 3.0 and the molar ratios of the products are close to galactose 1, rhamnose 1, glycerol 1, erythritol <1.5.

(d) Mild Acid Hydrolysis of Periodate-oxidized, Borohydride-reduced 97% Alk S XVIII.—After the titration for HCOOH (see section a) 207 mg of $NaBH_4$ was added to each sample. The samples were left 24 hours at 4° and dialyzed against water. The undialyzable material was concentrated to 0.5 ml and treated with 0.5 ml of 2 N H_2SO_4. After 20 hours at 28° the solution was neutralized with Duolite A and concentrated, and the products were separated on paper in solvent A. Three strong spots appeared on a strip sprayed with $AgNO_3$-NH_3: a fast one, F, with R_F = glycerol; another, M, with R_{Glu} = 1.11; and a slow one, S, R_{Glu} = 0.67. The areas of the unsprayed paper corresponding to each spot were eluted with water, concentrated, hydrolyzed with 0.5 N H_2SO_4 at 100° overnight, and neutralized as before. Paper chromatograms run in A and B gave for S two spots corresponding to galactose and erythritol; that of M showed strong spots corresponding to erythritol and rhamnose and a weak one corresponding to galactose, while F gave a strong spot corresponding to glycerol and a weak one corresponding to galactose.

(e) Second Oxidation with Periodate of 93% Alk S XVIII-IO_4-BH_4 after Mild Acid Hydrolysis.—Twenty ml of a solution of 93% Alk S XVIII, 2 mg/ml, was oxidized, reduced, and subjected to mild hydrolysis as above, except that the oxidation was carried out during 1 month. The neutralized solution was concentrated to 1.0 ml and again oxidized for 10 days at 4° with 1 ml of 0.25 M $NaIO_4$. Four mg $NaBH_4$ was added and after 24 hours the mixture was passed through Duolite A and Dowex-50 and evaporated *in vacuo*. Methanol was added twice and evaporated to eliminate boric acid. The residue was hydrolyzed at 100° for 18 hours, neutralized, and concentrated. Paper chromatograms in solvent B, sprayed with KIO_4-benzidine, gave only two spots, a strong one corresponding to glycerol and the other to 1,2 propanediol, neither of which showed when sprayed with aniline oxalate (Partridge, 1951).

Cross Reactions of the Pn XVIII System and Their Inhibition.—Cross Precipitation of S XVIII and Derivatives in Antipneumococcal Horse Sera.—S XVIII and Alk S XVIII precipitate in anti-Pn VI sera in an interpretable fashion, and also in anti-Pn VII, and VIII horse sera (Estrada-Parra *et al.*, 1962). Oxidation of S XVIII by periodate reduces precipitation in anti-Pn VIII from 40 μg N to 26, while oxidation of Alk S XVIII abolishes precipitation in both anti-Pn VI and VIII.

Cross Reactions of Various Carbohydrates and Polysaccharides with Horse Anti-Pn XVIII Sera.—In Table V are given data on cross reactions with dextrans of different linkages (Jeanes *et al.*, 1953; 1954; Scott *et al.*, 1957), while in Table VI the values for several other carbohydrates and polysaccharides are given.

Inhibition of the Cross Reactions.—As no appreciable inhibition occurred in the homologous reaction

TABLE VI
MAXIMAL PRECIPITATION OF HORSE ANTI-PN XVIII SERA BY VARIOUS CARBOHYDRATES AT 0°

Substance	Amount Taken (μg)	μg Antibody N Pptd, Calcd to 1.0 ml	
		495C	632C
S XVIII A	600	357	21
Aloe vera	100		5
	600	175[a]	
S VII	75		21
	150	72	
Limit dextrin	500	27	
S VIII	75	14	
S VIII reduced	50	21	
Klebsiella E-26	150	12	

[a] Supernatant + dextran N 236 pptd 18 μg N (Table V).

TABLE VII

INHIBITION OF THE CROSS REACTION BETWEEN 90% ALK S XVIII AND HORSE ANTI-PN XVIII 495C BY DIVERSE SUBSTANCES[a]

Inhibitor		Antibody N Pptd	Inhibition
Substance	Amount (μmoles)	(μg)	(%)
None	—	16	
D-Glucose	55	10	38
D-Mannose	55	12	25
D-Galactose	55	12	25
L-Rhamnose	55	13	19
Ribitol	66	15	6
Mannitol	55	14	13
Glycerol	109	14	13
α-Methyl mannoside	53	13	19
α-Methyl glucoside	53	9	44
Lactose	27.7	12	25
Melibiose	26.4	14	13
Maltose	27.7	9	44
Cellobiose	28.4	10	38
None	—	14	
Gentiobiose	2.9	12	14
Isomaltose	2.9	9	36
Maltose	2.8	11	21
Cellobiose	2.8	12	14

[a] Final volume of the reaction mixture, 0.4 ml.

TABLE VIII

INHIBITION OF THE CROSS REACTION BETWEEN DEXTRAN N236 AND HORSE ANTI-PN XVIII 495C BY DISACCHARIDES[a]

Inhibitor		Antibody N Pptd	Inhibition
Substance	Amount (μmoles)	(μg)	(%)
None	—	20	
Isomaltose	2.9	0	100
Cellobiose	2.8	15	25
Maltose	2.7	8	60
Gentiobiose	2.9	15	25

[a] Final volume of the reaction mixture, 0.6 ml.

of S XVIII with anti-Pn XVIII horse or rabbit sera with simple sugars and disaccharides, the sugars were tested in several of the cross reactions. The results are given in Tables VII and VIII.

DISCUSSION

Quantitative determinations of the components show that S XVIII is a polymer of D-glucose, D-galactose, L-rhamnose, glycerol, *O*-acetyl, and phosphate in the approximate ratios 3:1:1:1:1:1 (*cf.* Table IX). The configuration of the galactose had not been determined, and it has now been identified as D-galactose through its alteration by galactose oxidase. The sugars appear to be in the pyranose form, since none is liberated when S XVIII is subjected to mild hydrolysis. Degradation by alkali shows S XVIII to be composed of a main chain of sugars carrying one acetyl group and a side chain of glycerophosphate for each five sugars. The phosphate is in diester linkage, as indicated by the titration curves of S XVIII before and after treatment with alkali and by the splitting off of glycerophosphate. This seems to be α-glycerophosphate, because, upon oxidation of S XVIII with periodate, formaldehyde and ethylene glycol are formed, products which are absent in a similar oxidation of Alk S XVIII, from which practically all of the glycerophosphate had been eliminated.

The action of alkali on S XVIII varies with the *p*H, temperature, and length of treatment (Table II). Removal of *O*-acetyl, with only 5% splitting off of P, results in a product (5% Alk S XVIII) which behaves toward periodate as does intact S XVIII. Hence the *O*-acetyl does not esterify any hydroxyl otherwise susceptible to periodate, so that the incomplete destruction of glucose probably depends upon the glycerophosphate. However, 5% Alk S XVIII behaves differently from intact S XVIII toward antisera, precipitating only one-fifth, one-twelfth, and one-third as much antibody as S XVIII in anti-Pn XVIII horse sera 495C and 632C and rabbit serum 43C (Table III). The importance of *O*-acetyl groups in immunological specificity has been shown with other polysaccharides. Pappenheimer and Enders (1933) and Avery and Goebel (1933) found that S I was labile to alkali, which eliminated *O*-acetyl. Alkali-degraded S I precipitated less antibody than intact S I from anti-Pn I rabbit sera (Heidelberger *et al.*, 1936). Kotelko *et al* (1961) found that *O*-acetyl plays a very important role in the specificity of the antigenic group *O*:5 of *Salmonella*, since removal of *O*-acetyl abolishes its precipitating power in anti-*O*:5. Further removal of glycerophosphate with formation of 50% Alk S XVIII and 90% Alk S XVIII caused another small reduction in the precipitation of anti-Pn XVIII 495C horse serum, but in anti-Pn XVIII horse 632C and rabbit 43C sera the fully alkali-degraded material precipitated much less antibody than did 5% Alk S XVIII (Table III). In this instance glycerophosphate as well as *O*-acetyl probably played a part. Determination of "reducing sugars" in unhydrolyzed S XVIII and Alk S XVIII

TABLE IX

RATIOS OF COMPONENTS IN S XVIII AND ITS DERIVATIVES; DATA ON PERIODATE OXIDATION

Component	S XVIII[a]			Alk S XVIII 5%[a]	Alk S XVIII 92%[b]	Alk S XVIII 97%[b]	96% Alk S XVIII-IO_4-BH_4[c]
Glucose	2.7[d]		3.1[f]		3.0[d]		
Galactose	1.0[d]	5.1[f]	1.0[f]		1.0[d]		2.0[j]
Rhamnose	0.9[d]		1.1[f]		0.9[d]		
Glycerol		1.0[e]					1.0
P	1.0[d]						
Acetyl	0.8			0			
Erythritol							≤1.5
$NaIO_4$ uptake	4.4[g]					3.9[h]	
HCOOH prodn.						0.9[h]	
HCHO prodn.	1.3[g,i]			1.2[h]	Neglig.		

[a] Mol wt of repeating unit taken as 991. [b] Mol wt of residual repeating unit, 794. [c] Mixture of 94% and 98% Alk S XVIII. [d] See Table IV. [e] Determined on whole hydrolysate (21 hours). [f] Sugars from 21-hour hydrolysate separated on paper. [g] μMoles/μmole P. [h] μMoles/repeating unit. [i] See Estrada-Parra *et al.* (1962). [j] Galactose + rhamnose.

gave no evidence of a breakdown with alkali into small units as in the case of S VI (Rebers and Heidelberger, 1961). This was confirmed by the failure of more than 1% of the sugars to dialyze out even after the most drastic treatments with alkali.

Oxidation of S XVIII with periodate, which destroys glycerol and most of the glucose, yields a product which reacts strongly with the two anti-Pn XVIII horse sera, but precipitation in rabbit sera is greatly decreased (Table III). Reduction of S XVIII-IO_4 with sodium borohydride further reduces reactivity toward antisera, as in the case of S VI (Rebers *et al.*, 1962). Oxidation by periodate of S XVIII and Alk S XVIII greatly decreases or abolishes their capacity to precipitate in other antipneumococcal sera. This is probably due, in the case of anti-Pn VIII, to the destruction of glucose.

The chemical studies of oxidation of S XVIII and Alk S XVIII by periodate, as well as the ratios galactose 1, rhamnose 1, glycerol 1, erythritol $\lessdot$1.5, found after the hydrolysis of Alk S XVIII-IO_4-BH_4, are accounted for if D-galactose and L-rhamnose are 1,3-linked and two of the glucose residues are bound 1,4- and one 1,6-. A second oxidation of Alk S XVIII-IO_4-BH_4, after mild acid hydrolysis, resulted in destruction of galactose and rhamnose, yielding glycerol and propylene glycol. Since mild hydrolytic conditions result in cleavage of the hemiacetals formed upon oxidation, while glycosidic linkages are resistant (Smith and Montgomery, 1959), failure of galactose and rhamnose to survive the second oxidation shows that they must be separated by one or more of the glucose residues. Two of the glucose residues must therefore be linked. Moreover, mild hydrolysis of Alk S XVIII-IO_4-BH_4 resulted in three main products: glycerol, derived from 1,6-linked glucose, and two "disaccharides" which appear to be galactosyl-erythritol and rhamnosyl-erythritol and which account for the products obtained after the second oxidation of Alk S XVIII-IO_4-BH_4. Since formic acid is obtained in the first oxidation and glycerol after mild hydrolysis of Alk S XVIII-IO_4-BH_4, the two consecutive glucose residues are linked 1,6-. Consideration of these findings, together with the cross reactions and tests for their inhibition, discussed below, suggest that S XVIII may have either of the two formulas:

Pn S XVIII

$$-\left[\rightarrow 3)\text{-D-gal-}(1\rightarrow 4)\text{-}\alpha\text{-D-glc-}(1\rightarrow 6)\text{-D-glc-}(1\rightarrow 3)\text{-L-rham-}(1\rightarrow 4)\text{-D-glc-}(1-\right]-$$

$$\left.\begin{array}{l}\text{Ac—}\\ \text{HOCH}_2\text{.CHOH.CH}_2\text{O.PO}_2\text{—}\end{array}\right]_x$$

or

$$-\left[\rightarrow 3)\text{-D-gal-}(1\rightarrow 4)\text{-D-glc-}(1\rightarrow 3)\text{-L-rham-}(1\rightarrow 4)\text{-}\alpha\text{-D-glc-}(1\rightarrow 6)\text{-D-glc-}(1-\right]-$$

$$\left.\begin{array}{l}\text{Ac—}\\ \text{HOCH}_2\text{.CHOH.CH}_2\text{O.PO}_2\text{—}\end{array}\right]_x$$

As to the cross reactions in anti-Pn XVIII sera, all polysaccharides tested containing α-1,6-linked glucose, such as dextrans, glycogen, and β-amylase-limit dextrin, precipitate anti-Pn XVIII 495C horse serum (Tables V and VI). The dextrans, especially, contain α-1,6-linked glucose, and those with the highest proportions of these linkages precipitate the most antibody (Table V), with the exception of dextran 1299 L. These cross reactions, together with the inhibition studies in which isomaltose is the best inhibitor (Tables VII, VIII), give support for the presence of antibodies in anti-Pn XVIII 495C with specificity against multiples of α-1,6-linked glucose and this would be expected if there were isomaltose residues in S XVIII.

As to the other cross reactions, those between S XVIII and anti-Pn VIII and between S VIII and anti-Pn XVIII seem to depend upon the presence in both S VIII and S XVIII of multiple residues of 1,4-linked glucose (for the structure of S VIII see Jones and Perry, 1957).

The polysaccharide of *Aloe vera*, which gave very strong precipitation in anti-Pn XVIII 495C, is composed of mannose and glucose, with small amounts of galactose and pentoses. However, if it is recovered from the precipitate with anti-Pn XVIII 495C the galactose is lost (Felix Cordoba, personal communication). Moreover, oxidation of *A. vera* results in almost complete destruction of glucose, yielding erythritol (Estrada-Parra, unpublished results). Therefore multiples of 1,4-linked glucose would seem to be responsible for the cross reaction. S VII contains glucose, galactose, rhamnose, glucosamine, and galactosamine (Tyler and Heidelberger, 1962) with the glucose probably linked 1,4-, 1,6-, or 1,4,6-, thus accounting for the cross reaction. S XVIII A is presently under study in this laboratory.

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Radiolysis of Reduced Diphosphopyridine Nucleotide in Aqueous Solution

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The radiolysis of DPNH solutions by 250-kvp X rays has been followed utilizing the absorption at 340 mμ, the corresponding fluorescence at 470 mμ, and the coenzymatic activity with alcohol dehydrogenase. All three parameters are decreased simultaneously and this reduction in DPNH concentration is neither linear nor logarithmic with dose. The coenzyme is protected in the presence of protein; the protection afforded by glutamic dehydrogenase, a DPNH-binding enzyme, is equal to that of serum albumin at the same weight concentration. The radiolysis of DPNH is independent of *p*H over the range 4.8–7.5 and is accompanied by an initial increase in *p*H, 260-mμ absorption, and cyanide reactivity. These observations suggest that DPN is the first product formed which is, in turn, degraded logarithmically. A kinetic model for the radiolysis has been formulated which gives a *G*(-DPNH) of 2.3 in air and 1.2 in oxygen-free nitrogen.

A number of investigations have indicated that ionizing radiation may have a more profound effect on the tissue levels of DPNH[1] than on the oxidized coenzyme. For example, irradiation of grasshopper eggs leads to a decrease in the DPNH-DPN ratio during the period of irradiation, with the ratio returning toward normal following the X-ray exposure (Tahmisian and Wright, 1956). In addition, rat liver concentrations of DPNH are reduced by whole-body doses of X rays that do not change the concentration of DPN appreciably (Eichel and Spirtes, 1955). These findings suggest that irradiation either alters cellular metabolism immediately in favor of a decreased DPNH-DPN ratio or that DPNH is sufficiently sensitive within cells to be attacked directly by the aqueous radicals. As a preliminary to the further elaboration of these *in vivo* observations the comparative radiolysis of reduced and oxidized DPN solutions is described in this report.

Experimental

Solutions of purified DPN and DPNH, obtained from the Sigma Chemical Co. (St. Louis, Mo.), were irradiated with 250-kvp unfiltered X rays at a dose rate of approximately 2000 rad per minute. Ferrous ammonium sulfate dosimeters of the identical geometry were employed and a *G* value of 15 was assumed in the dosage calculations (Swallow, 1960). The solutions were made with deionized, glass-distilled water except in those instances where specific buffer solutions are indicated. The samples and ferrous ammonium sulfate solutions were irradiated in Parafilm-covered Pyrex beakers kept at ice temperature. For the anerobic irradiations, oxygen-free nitrogen was bubbled through the solutions in a sealable manifold prior to X-ray exposure (Laser, 1962).

The optical absorbancy of the solutions was measured in a Beckman ultraviolet spectrophotometer (DU) and the fluorescence in a Bowman-Aminco spectrofluorometer. The coenzymatic activity of DPNH was measured by the reduction in 340 mμ absorbancy following the addition of 30 μg of yeast alcohol dehydrogenase (C. F. Boehringer and Son) at *p*H 7.5 in the presence of 8 mM acetaldehyde. The coenzymatic activity of DPN was measured by the increase in 340 mμ absorbancy following the addition of 30 μg of yeast alcohol dehydrogenase at *p*H 10.1 in the presence of 0.7 M ethanol. The cyanide addition product was determined by adding 1 ml of the original and irradiated solutions to 2 ml of 1.0 M potassium cyanide, and measuring the absorption at 325 mμ.

Purified human serum albumin was a gift from the Protein Foundation and glutamic acid dehydrogenase was obtained from C. F. Boehringer and Son.

Results

The radiolysis of DPNH was followed by its optical absorbancy at 340 mμ, its fluorescence at 470 mμ when excited at 340 mμ, and its coenzymatic capability in the presence of acetaldehyde and alcohol dehydrogenase. From inspection of Figure 1 it is apparent that the radiolysis proceeds to the same extent under each measurement. The degradation of DPNH is neither

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[1] The following abbreviations are used: DPN, diphosphopyridine nucleotide, nicotinamide adenine dinucleotide; DPNH, reduced diphosphopyridine nucleotide, reduced nicotinamide adenine dinucleotide; *G*, molecules converted per 100 ev of energy absorbed.