IMMUNOCHEMISTRY OF THE PNEUMOCOCCAL TYPES II, V, AND VI

III. Tests with Derivatives of the Specific Polysaccharides of Types II and VI

P. A. REBERS,[1] E. HURWITZ, M. HEIDELBERGER, AND S. ESTRADA-PARRA

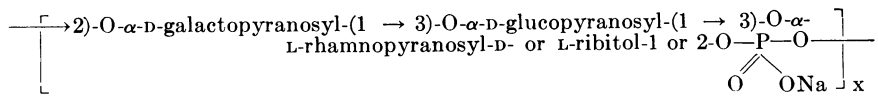
Institute of Microbiology, Rutgers, The State University, New Brunswick, New Jersey

Received for publication August 17, 1961

ABSTRACT

REBERS, P. A. (Rutgers, The State University, New Brunswick, N. J.), E. HURWITZ, M. HEIDELBERGER, AND S. ESTRADA-PARRA. Immunochemistry of the pneumococcal types II, V, and VI. III. Tests with derivatives of the specific polysaccharides of types II and VI. J. Bacteriol. **83**:335–342. 1962.—The type-specific pneumococcal polysaccharides, S II and S VI, were oxidized with periodate, and the capacity of the derivatives for precipitation by antibodies and adsorption by erythrocytes was studied. The derivatives were further modified by reduction, oxidation, and condensation with urea. Oxidation of S VI with periodate reduced its precipitation of homologous antibodies much less than did similar treatment of S II. About one-half of the components are attacked in both cases, but in S II, glucuronic acid end groups, the chief determinants of specificity, are destroyed by periodate, whereas in S VI, the structures responsible for specificity are either less affected or yield derivatives with almost as much affinity for antibody as the original polysaccharide. S II and S VI are not adsorbed by washed rabbit or human erythrocytes, but their periodate-oxidized derivatives are readily taken up, presumably owing to their newly formed reactive aldehyde groups, giving high titers in passive hemagglutination. The method should be applicable to other polysaccharides which do not adsorb readily on red cells.

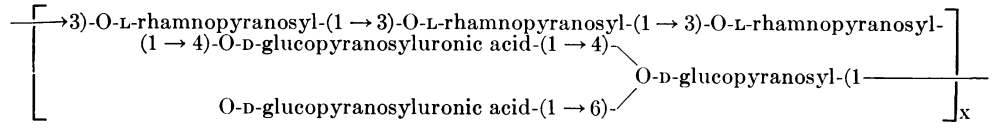
In the preceding paper of this series (Rebers, Hurwitz, and Heidelberger, 1961), it was shown that fragments of the type VI pneumococcal polysaccharide, S VI (structure given below), were potent inhibitors of the homologous reaction with rabbit antisera. Additional insight as to the antigenic importance of the various groupings of the polysaccharide has now been gained by oxidation of S VI with periodate, a reagent which attacks the 1,2-linked galactose and the —CHOH—CH_2OH grouping of the 1,3- or 2,3-linked ribitol, whereas the glucose, rhamnose, and phosphate-diester residues are resistant (Rebers and Heidelberger, 1959, 1961). The 1,2-linked galactose is converted to a dialdehyde which apparently exists as a cyclized hydrate (Hurd et al., 1953; Goldstein, Lewis, and Smith, 1958). The reactions of periodate on the galactose of S VI, one of the possible resulting ring structures, and the effects of subsequent oxidation with sodium chlorite or reduction with sodium borohydride are shown in Fig. 1. The ribitol, not shown in the figure, is oxidized by periodate to D- or L-erythrose, which yields erythritol on reduction or D- or L-erythronic acid on oxidation. Oxidized S VI was also condensed with urea (Sloan et al., 1956; Miles Chemical Co., 1959). The capacity of the modified derivatives to precipitate antibodies was determined, and supernatants were examined for their content of homologous and cross-reacting antibodies. For comparison, S II was oxidized with periodate. According to Butler and Stacey (1955), L-



rhamnose linked 1,3-, D-glucose linked 1,4,6-, D-glucuronic acid linked 1,4-, and D-glucuronic acid end groups are present. A possible structure, based on the results of methylation and of oxida-

[1] Present address: National Animal Disease Laboratory, U. S. Department of Agriculture, Ames, Iowa.

tion with periodate, is given below:



Rhamnose is the only sugar combined in linkages resistant to periodate. The capacity of oxidized S II to precipitate antibodies in anti-Pn II and anti-Pn VI was studied quantitatively.

MATERIALS AND METHODS

These are fully described in the earlier papers of this series (Heidelberger and Rebers, 1960; Rebers et al., 1961).

RESULTS AND DISCUSSION

Oxidation of S VI. An aqueous solution of S VI, 9.5 mg/ml, was mixed with an equal volume of 0.1 M $NaIO_4$ at 0 C, kept at 0 C in the dark until the reaction was complete (Rebers and Heidelberger, 1959), and dialyzed against saline at 4 C until free from IO_4^- and IO_3^- as tested for by acidification and addition of KI and starch. A sample was analyzed for total phosphate, and since the oxidized derivative has a calculated P content of 4.6%, its concentration could be determined. Recoveries varied from 82 to 92%, suggesting that some degradation into dialyzable fragments had occurred. The solution was stored at 4 C, with toluene as a preservative (lyophilization led to insolubility in water). Freezing alone sometimes suffices to render the derivative produced by oxidation with periodate insoluble, as with glycogen (Abdel-Akher and Smith, 1959). The reactions of oxidized S VI (S VI-IO_4) with horse and rabbit anti-Pn VI sera are summarized in Table 1.

Since over 90% of the antibodies in the anti-Pn VI horse serum appeared to be precipitated by S VI-IO_4, it was conceivable that some non-specific combination between aldehyde groups of the antigen and serum proteins had occurred. However, this could have been only relatively minor, since the sum of the nitrogen precipitated first by S VI-IO_4 and then by S VI from the supernatants was only slightly greater than the amount precipitated by S VI alone (Table 1, footnote *b*). Similar results were obtained with rabbit serum (Table 1, footnote *c*). Also, simultaneous addition of S VI and S VI-IO_4 gave only slightly more precipitate than did S VI (Table 1). Addition of S VI-IO_4 to an unrelated anti-Pn horse serum, such as type III, gave no visible reaction. The high proportion of antibody N precipitated from the horse serum may therefore actually indicate that the cyclized hydrated dialdehyde postulated as a result of the attack

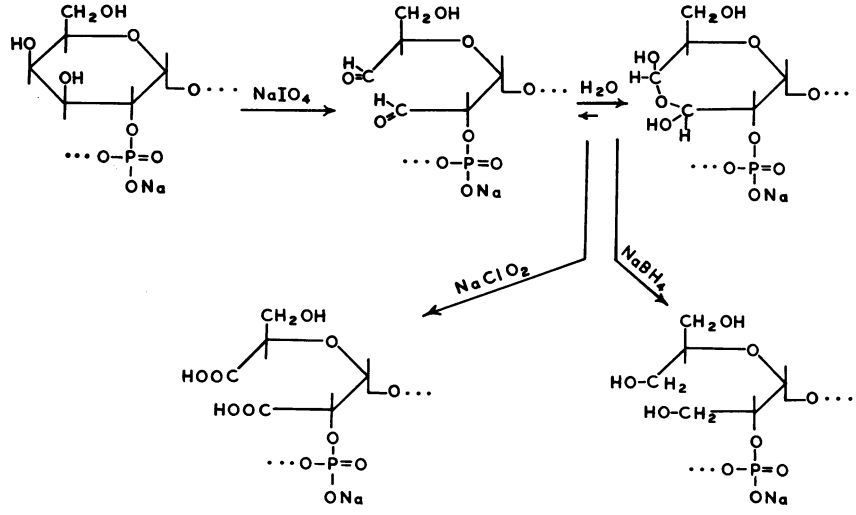


FIG. 1. *Reaction of 1,2-linked galactose in S VI with sodium periodate and subsequent oxidation with sodium chlorite or reduction with sodium borohydride.*

TABLE 1. *Precipitation of anti-Pn VI by S VI and derivatives*

Antigen	Quantity added	Antibody N pptd from 1.0 ml at 0 C	
		Rabbit serum N.Y. State No. 14	Horse serum N.Y. State No. 614
	μg	μg	μg
S VI	16		450
	37	485	
	75	890	
	89		1330
	150	1380	
	180		1200
	300	2300	1200
	600	3230	
	1410	3380	
	2820	3120	
	3360		1200
	4900	1750	
S VI-IO_4[a]	110		950[b]
	126	760	
	241	1070[c]	
	330		1050[b]
	515	1220	
	985		1300[b]
	1960		1200[b]
S VI[d]	160		1400
S VI-IO_4[d]	1010		1200
S VI + S VI-IO_4[d]	110 + 1010		1500
S VI-IO_4-BH_4-27 C	100		560[e]
	140	750	
	285	860	
	300		520[e]
	560	600	
	870		450[e]
S VI-IO_4-BH_4-0 C	60		545
	180		520
	210	740	
	268	772[f]	
	600		515
	850	370	
S VI-IO_4-ClO_2-29% oxd	118	490	745
	235	560	
	300		765
	341	535[g]	
	770		745
S VI-IO_4-ClO_2-69% oxd	320		380
S VI-IO_4-ClO_2-81% oxd	270		225
S VI-IO_4-urea[j]	60		670[h]
	185		815[h, i]
S VI-IO_4-urea[k]	183		543
	380		560
S VI-IO_4-urea[l]	190		650
	380		680

[a] Storage for 3 to 6 months did not affect the amount of rabbit antibody N precipitated.

[b] Addition of 110 μg S VI to each supernatant precipitated 365, 310, 260, and 210 μg N, respectively.

[c] Addition of 780 μg S VI to supernatant precipitated 2050 μg N.

[d] Data obtained on the same run.

[e] Addition of 110 μg S VI to each supernatant precipitated 700, 690, and 740 μg N, respectively.

[f] Addition of 780 μg S VI to supernatant precipitated 2700 μg N.

[g] Addition of 780 μg S VI to supernatant precipitated 2400 μg N.

[h] Corrected for amount N in S VI-IO_4-urea added.

[i] Single determination only.

[j] Modification 2, after 1 month.

[k] Modification 2, after 10 months; dilution twice that of preceding.

[l] Modification 1, after 10 months; dilution twice that in footnote *j*.

of periodate on the 1,2-linked galactose provides a configuration not too different from that of the original S VI (Fig. 1). Periodate, therefore, does not necessarily greatly alter the serological properties of a relatively resistant polysaccharide.

Reduction of S VI-IO_4. S VI-IO_4 (9 mg in 2 ml water) was treated with 40 mg $NaBH_4$. After 3 hr at 27 C the product was nonreducing in the Park-Johnson (1949) modification of the alkaline ferricyanide reaction, whereas S VI-IO_4 gave a reducing value equivalent to 291 μg as glucose per mg. The reaction mixture, at pH 9, was made slightly acid with acetic acid and dialyzed against daily changes of 0.9% NaCl solution for 3 days. Precipitation of rabbit and horse antisera with the product, designated S VI-IO_4-BH_4-27 C, is

summarized in Table 1. Slow degradation of a control solution of S VI occurred at pH 9, indicated by reduction of its capacity to precipitate rabbit antibodies by 11% after 3 days at room temperature. Another sample of S VI-IO_4 was therefore reduced with $NaBH_4$ at 0 C in the presence of phenolphthalein, 5% HOAc being added as needed to discharge the pink color which appeared at pH 8.4. After 3 hr at 0 C, the sample was dialyzed against 0.9% NaCl solution as before. As shown in Table 1, the sample, S VI-IO_4-BH_4-0 C, precipitated almost the same amount of antibody as the 27 C sample. Therefore, the amount of degradation in 3 hr at both temperatures is either too small to be detectable, or possibly, but less likely, the same amount of degradation occurred under both sets of conditions. Less of the reduced derivative than of S VI-IO_4 is required for maximal precipitation of either horse or rabbit antibody and less antibody is precipitated. In the second paper of this series (Rebers et al., 1961) it was shown that S VI which was partly degraded with alkali precipitated as much antibody from horse anti-Pn VI as did the original S VI. Hence the decrease in the capacity of the SVI-IO_4-BH_4 to precipitate antibodies is probably due to the change in structure which occurs upon reduction (Fig. 1). The sum of the nitrogen precipitated from the sera first by S VI-IO_4-BH_4 and then by S VI from the supernatants was either equal to or slightly less than the amount precipitated by S VI alone (Table 1, footnotes *e*, *f*). The supernatant from the precipitation of horse anti-Pn VI with S VI-IO_4-BH_4 was tested with guar gum; 82 μg antibody nitrogen was precipitated per ml, calculated to the original serum volume, whereas guar precipitated 85 μg from the unabsorbed serum. Thus, 96% of the antibodies precipitable by guar as a result of its multiple end groups of galactose remained in the supernatant after absorption with S VI-IO_4-BH_4, in contrast to only 58% or less in the supernatant from S VI-IO_4. This furnishes added evidence that some of the antibodies in anti-Pn VI which precipitate with polysaccharides containing multiple end groups of galactose also precipitate with S VI-IO_4, and accords with the cyclized structure for the oxidized galactose in S VI-IO_4, a structure eliminated by reduction.

Oxidation of S VI-IO_4. Since weakly acidic sodium chlorite is a relatively specific oxidant for sugar aldehyde groups (Hofreiter, Wolff, and Mehltretter, 1957), 3.6 mg S VI-IO_4 in 1 ml of water was treated with 90 mg $NaClO_2$ at pH 3.2 for 3 hr at room temperature and dialyzed against 0.9% NaCl solution at 4 C until free of ClO_2^- as shown with acidified starch-KI. Of the aldehyde groups, 29% were converted to COOH as measured by the loss in reducing power with alkaline ferricyanide (Park and Johnson, 1949). Extension of the time of reaction resulted in products in which 69 and 81% of the aldehyde groups were oxidized. These materials, S VI-IO_4-ClO_2 (29%), S VI-IO_4-ClO_2 (69%), and S VI-IO_4-ClO_2 (81%) also precipitated horse and rabbit anti-Pn sera (Table 1) in amounts which decreased with increasing extent of oxidation. Although some degradation into small nonprecipitating fragments might have occurred, the fractions tested had been retained by a cellophane dialysis bag. It had been shown in the second paper of this series that partially alkali-degraded S VI corresponding roughly to a trimer of the repeating unit and small enough to pass through a dialysis bag was nevertheless large enough to precipitate most of the antibody in horse anti-Pn VI. Thus the additional ionized carboxyl groups evidently influence precipitation adversely.

Condensation of S VI-IO_4 with urea. Starch oxidized with periodate adds one mole of urea per repeating unit when heated at 40 to 60 C for 2 to 4 hr at pH 5 to 7 (Sloan et al., 1956). Under similar conditions, S VI-IO_4 failed to react with urea, but condensation was effected by either of two modifications: (i) S VI-IO_4 (6 mg in 11 ml H_2O and 50 mg urea) was evaporated to dryness in vacuo, heated at 50 to 60 C for 15 min, dissolved in water, and dialyzed against daily changes of distilled water for 3 days at 4 C; the nondialyzable portion contained 3.7 moles N (1.9 moles urea) per mole of P. (ii) S VI-IO_4 (5.4 mg in 6 ml H_2O and 50 mg urea) was evaporated to dryness in vacuo at 40 C, about 5 ml methanol was added, the mixture was warmed for 30 min as a slurry (Miles Chemical Co., 1959), evaporated to dryness in vacuo, heated for 1 hr at 57 C, and dialysed as before. Analysis showed 8 moles N per mole P. The reactions of both products with anti-Pn VI horse serum are shown in Table 1.

Methylation of S VI. Diazoalkanes have been used to convert phosphate diesters to triesters

(Atherton, Howard, and Todd, 1948). After exposure to diazomethane for 30 min was shown to be insufficient, S VI in the H^+ form was lyophilized and treated with diazomethane in ether for 10 hr at 4 C and 7 hr at room temperature. Titration with alkali showed 94% conversion to phosphate triester, but the methoxyl content (Steyermark, 1956) was 14%. Hence, two alcoholic groups were methylated in addition to the phosphate diester. Addition of 18, 36, and 54 μg of derivative precipitated 17, 27, and 34 μg antibody nitrogen per 0.20 ml rabbit serum which had been diluted 0.7 → 5.0, whereas the same amounts of S VI precipitated 81, 62, and 44 μg N. The triester was sensitive to acid; after 1 hr at 100 C and pH 3.5 it lost 90% of its activity, while S VI was scarcely affected. The methylated derivative was not studied further because of its possible inhomogeneity and the excessive extent of methylation.

Oxidation of S II by periodate. Inasmuch as end groups of glucuronic acid are present in S II (Butler and Stacey, 1955), the oxidation was carried out with HIO_4 so as to minimize side reactions (Smith and Montgomery, 1959); 18.8 mg of anhydrous S II, fraction B I, purified by E. A. Kabat (Beiser, Kabat, and Schor, 1952), was dissolved in 25 ml of 0.02 M aqueous HIO_4 and oxidized at 4 C. The periodate consumed was determined at 3- to 7-day intervals by the arsenite method (Fleury and Lange, 1933*a*,*b*). After 19 days, consumption was 6.9 moles per mg S II and 7.0 after another week. Since terminal glucuronic acid groups would probably be completely oxidized and consume 5 moles periodate, while the 1,4-linked glucuronic acid and 1,4,6-linked glucose also present (Butler and Stacey, 1955) would each consume one mole (Smith and Montgomery, 1959), the formula of the repeating unit (formula weight ca. 1000) previously given is proposed as a possibility best in accord with the present somewhat fragmentary knowledge of S II. The rhamnose content, 44%, is not far from the 49 to 51% previously reported (Beiser et al., 1952).

Periodate and iodate were removed from another sample of the product, S II-IO_4, with $Ba(OH)_2$ to pH 6. The precipitate was centrifuged off. The supernatant was dialyzed against saline until free of iodate, and analyzed for sugars by the phenol method (Dubois et al., 1956). It contained 239 μg/ml, calculated as rhamnose.

TABLE 2. *Precipitation of antipneumococcal type VI and type II horse sera by S II and S II-IO_4*

Antigen	Quantity added	Nitrogen pptd per ml at 0 C	
		Type VI 771 C	Type II 513
	μg	μg	μg
S II[a]	80	152	
	200	155	
	1250		3600
S II-IO_4	60		410
	75	175	
	126	190[b]	470
	200	171	
	240		470
S II-IO_4-BH_4	52		170
	105		215
	210		200

[a] Data from Heidelberger and Rebers (1960).
[b] Addition of 66 μg S II to 0.50 ml of supernatant gave no additional precipitate.

The reactions of S II and S II-IO_4 with types II and VI anti-Pn horse sera are summarized in Table 2. In contrast to S VI-IO_4, S II-IO_4 precipitated only a small part of the antibodies in anti-Pn II horse serum 513, in which an unusually large proportion of the antibodies had been shown to be reactive with gums containing multiple end groups of glucuronic acid (Heidelberger, 1960; Goodman and Kabat, 1960). The large decrease in the amount of antibody nitrogen precipitated by S II-IO_4 from anti-Pn II may thus be explained by the loss of the glucuronic acid end groups of S II. The same reason might be invoked for the slight increase in reactivity with anti-Pn VI 771 C, since the ionized glucuronic acid end groups of intact S II could interfere sterically with the approach, in anti-Pn VI, of the reaction sites complementary to multiple groupings of 1,3-linked rhamnose in the main chain of S II and S II-IO_4. As in the case of S VI-IO_4, reduction of S II-IO_4 with sodium borohydride reduces the amount of antibody which can be precipitated, and an explanation based upon the loss of cyclized structures may also be advanced in this instance.

Tests by passive hemagglutination. Adsorption

TABLE 3. *Passive hemagglutination of erythrocytes sensitized with pneumococcal polysaccharides and derivatives*

Species of erythrocyte used	Polysaccharide	Hemagglutination[a]	
		Extent	Titer
Human[b]	S III	−	10, 100, 1000
	S VIII	−	10, 100, 1000
	S II	−	10, 100, 1000
	S II-IO_4	++++	10,000
	S VI	−	10, 100, 1000
	S VI-IO_4	++++	1000
Human[c]	S III	−	10, 100, 1000
	S VIII	−	10, 100, 1000
	S II	−	10, 100, 1000
	S II-IO_4	++++	1000
	S VI	−	10, 100, 1000
	S VI-IO_4	+++	1000
Human[d]	S III	−	10, 100, 1000
	S VIII	−	10, 100, 1000
	S II	−	10, 100, 1000
	S II-IO_4	++++	10,000
	S VI	−	10, 100, 1000
	S VI-IO_4	−	10, 100, 1000
Rabbit	S II	−	5, 125, 1250
	S II-IO_4	++++	2000
	S III	++	10,000
	S VI	−	1000, 10,000
	S VI-IO_4[e, f]	++++	3200
	S VI-IO_4-BH_4	−	10, 250, 1250
	S VI-IO_4-ClO_2-81%	−	10, 250, 1250
	S XVIII	−	10, 250, 1250
	S XVIII-IO_4	++++	1250
Mouse	S VI	++++	10,000

[a] Agglutination: ++++ = complete, perfect mat; +++, ++, + = decreasing amounts of mat, increasing amounts of button; − = negative, disc or button. The titers given are the reciprocals of the highest dilutions of the antisera for which the extent of agglutination is reported. Negative results are usually reported for three titers. The following sera were used: Anti Pn II, N. Y. State No. 513; anti Pn III, N. Y. City No. 792; Anti Pn VI, N. Y. State No. 614; Anti Pn VIII, N. Y. City 1008; Anti Pn XVIII, N. Y. State No. 495, which contained 3600, 717, 1300, 1288, and 2218 μg antibody nitrogen, respectively, precipitable by the homologous polysaccharide.

[b] Cells from P.A.R., 1 and 13 days after bleeding, type A DCe/dce (R_1r).

of polysaccharides on erythrocytes may be detected by agglutination in antisera (Keogh, North, and Warburton, 1948; Middlebrook and Dubos, 1948; Landy, 1954). In such instances, periodate has been reported either not to affect the hemagglutination titers or to diminish them (Hayes, 1951). In our study of the factors controlling the adsorption, erythrocytes from human beings, mice, and rabbits were treated with pneumococcal polysaccharides and their derivatives. Red cells, not less than 1 day nor more than 3 weeks old in Alsever's solution, were washed three times with Locke's solution at pH 6.8 (NaCl, 8.5 g; KCl, 0.42 g; $CaCl_2 \cdot 2H_2O$, 0.24 g; $NaHCO_3$, 0.20 g; H_2O, 1000 ml). Packed cells (0.1 to 0.2 ml) were added to 0.20 ml of 0.85% NaCl solution containing 50 to 200 μg of polysaccharide, incubated 2 hr at 37 C, and either washed directly three times with Locke's solution or stored overnight at 4 C and subsequently washed; 0.1 ml of a 1 to 2% suspension of the treated cells was added to 1 ml of the homologous horse antiserum diluted with Locke's solution in 10 by 74 mm test tubes. As controls, a 1 to 2% suspension of washed untreated cells was added to each serum dilution, and both untreated and treated cells were added to saline. Since untreated rabbit cells were occasionally agglutinated by antipneumococcal horse sera diluted 1 to 200, such sera were given a preliminary absorption with washed red cells. After addition of the cell suspension to the test solution, the tubes were shaken and allowed to stand overnight at 4 C. Before reading, the tubes were warmed for 10 min in a water bath at 37 C to eliminate possible cold agglutinins. The results are summarized in Table 3. S II, S VI, and S XVIII gave negative results with rabbit cells, although their derivatives produced by oxidation with periodate reacted positively. Human cells from three individuals behaved likewise and gave consistently negative results with S II and

[c] Cells from A.M.R., 15 days after bleeding, type A_2 dce/dce(rr).

[d] Cells from J.M.T., 8 and 12 days after bleeding; 4 days after bleeding S III and S VI-IO_4 gave ++ at a titer of 1000.

[e] Rabbit cells as old as 7 weeks still fix S VI-IO_4 as well as fresh cells.

[f] Cells treated with S VI-IO_4 retained their capacity for agglutination by antiserum after storage in Locke's solution at 4 C for at least 5 days.

positive with S II-IO_4. The cells from two of these individuals also showed excellent fixation of S VI-IO_4, but those from the other showed weak fixation or none. No explanation of this variation is available at present. The fixation of the oxidized polysaccharides is probably mediated by combination of aldehyde groups in the derivative with amino groups of the proteins of the red cell. These derivatives still possess structures with serological specificities sufficiently like those of the original polysaccharides to react with high dilutions of antisera to the homologous pneumococcal types. Reduction of the aldehyde groups of the oxidized polysaccharides to alcohols or oxidation to carboxylic acids eliminated the fixation to red cells as determined by hemagglutination.

ACKNOWLEDGMENTS

Carried out under a grant from the National Science Foundation.

We wish to thank N. C. Palczuk for his advice on the hemagglutination experiments.

LITERATURE CITED

ABDEL-AKHER, M., AND F. SMITH. 1959. Oxidation of glycogen with periodic acid. J. Am. Chem. Soc. **81**:1718–1721.

ATHERTON, F. R., H. T. HOWARD, AND A. R. TODD. 1948. Studies on phosphorylation. Part IV. Further studies on the use of dibenzylchlorophosphonate and examination of certain alternative phosphorylation methods. J. Chem. Soc., p. 1106–1111.

BEISER, S. M., E. A. KABAT, AND J. M. SCHOR. 1952. Immunochemical studies on the specific polysaccharide of type II pneumococcus. J. Immunol. **69**:297–309.

BUTLER, K., AND M. STACEY. 1955. Immunopolysaccharides. Part IV. Structural studies on the type II pneumococcus specific polysaccharide. J. Chem. Soc., p. 1537–1541.

DUBOIS, M., K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH. 1956. A colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.

FLEURY, P., AND J. LANGE. 1933*a*. Determination of periodic acid in the presence of iodic acid. J. pharm. chim. **17**:107–13.

FLEURY, P., AND J. LANGE. 1933*b*. Action of periodic acid on polyhydroxylated compounds. I. Technic used. J. pharm. Chim. **17**:196–208.

GOLDSTEIN, I. J., B. A. LEWIS, AND F. SMITH. 1958. The structure of the dialdehyde formed by periodate oxidation of methyl α-L-rhamnopyranoside. J. Am. Chem. Soc. **80**:939–941.

GOODMAN, J. W., AND E. A. KABAT. 1960. Immunochemical studies on cross-reactions of antipneumococcal sera. I. Cross-reactions of types II and XX antipneumococcal sera with dextrans and of type II antipneumococcal sera with glycogen and Friedländer Type B polysaccharide. J. Immunol. **84**:333–346.

HAYES, L. 1951. Specific serum agglutination of sheep erythrocytes sensitized with bacterial polysaccharides. Australian J. Exptl. Biol. Med. Sci. **29**:50–62.

HEIDELBERGER, M. 1960. The immunological specificity of type II pneumococcus and its separation into partial specificities. II. J. Exptl. Med. **111**:33–43.

HEIDELBERGER, M., AND P. A. REBERS. 1960. Immunochemistry of the pneumococcal types II, V and VI. I. The relation of type VI to type II and other correlations between chemical constitution and precipitation in antisera to type VI. J. Bacteriol. **80**:145–153.

HOFREITER, B. T., I. A. WOLFF, AND C. L. MEHLTRETTER. 1957. Chlorous acid oxidation of periodate oxidized cornstarch. J. Am. Chem. Soc. **79**:6457–6460.

HURD, C. D., P. J. BAKER, R. P. HOLYZ, AND W. H. SAUNDERS. 1953. Cyclic modification of the dialdehyde from periodate oxidation of methyl α-D-glucopyranoside. J. Org. Chem. **18**:186–191.

KEOGH, E. V., E. A. NORTH, AND M. F. WARBURTON. 1948. Adsorption of bacterial polysaccharides to erythrocytes. Nature **161**:687–688.

LANDY, M. 1954. On hemagglutination procedures utilizing isolated polysaccharide and protein antigens. Am. J. Public Health **44**:1059–1064.

MIDDLEBROOK, G., AND R. J. DUBOS. 1948. Specific serum agglutination of erythrocytes sensitized with extracts of tubercle bacilli. J. Exptl. Med. **88**:521–528.

MILES CHEMICAL CO. 1959. Sumstar dialdehyde starch. Tech. Bull. No. 6–129.

PARK, J. T., AND M. J. JOHNSON. 1949. A submicrodetermination of glucose. J. Biol. Chem. **181**:149–151.

REBERS, P. A., AND M. HEIDELBERGER. 1959. The specific polysaccharide of type VI pneumococcus. I. Preparation, properties and reactions. J. Am. Chem. Soc. **81**:2415–2419.

REBERS, P. A., AND M. HEIDELBERGER. 1961.

The specific polysaccharide of type VI pneumococcus. II. The repeating unit. J. Am. Chem. Soc. **83**:3056–3059.

REBERS, P. A., E. HURWITZ, AND M. HEIDELBERGER. 1961. Immunochemistry of pneumococcal types II, V, and VI. II. Inhihition tests in the type VI precipitating system. J. Bacteriol. **82**:920–926.

SLOAN, J., B. T. HOFREITER, R. L. MELLIES, AND I. A. WOLFF. 1956. Properties of periodate oxidized starch. Ind. Eng. Chem. **48**:1165–1172.

SMITH, F., AND R. MONTGOMERY. 1959. Periodate oxidation in structural studies of gums and mucilages, p. 194–222. *In* F. Smith and R. Montgomery, Chemistry of plant gums and mucilages. Reinhold Pub. Co., New York.

STEYERMARK, A. 1956. Report on microanalytical determination of alkoxyl groups. Part III. J. Assoc. Off. Agr. Chem. **39**:401–407.