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The Extracellular Glycosidases of *Diplococcus pneumoniae*. II. Purification and Properties of a β-*N*-Acetylglucosaminidase. Action on a Derivative of the α_1-Acid Glycoprotein of Human Plasma*

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The purification of β-*N*-acetylglucosaminidase from the culture fluids of *Diplococcus pneumoniae* is described. The product is a highly active and stable preparation. The enzyme releases 2-acetamido-2-deoxy-D-glucose from *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside. The kinetics and nature of this reaction are reported. Values of V_{max} and K_M were 0.26 μmoles of substrate hydrolyzed per minute per mg of enzyme and 0.22×10^{-3} M, respectively. The enzyme also releases 26% of the 2-acetamido-2-deoxy-D-glucose residues from a derivative of α_1-acid glycoprotein of human plasma from which 100% of the neuraminic acid and 80% of the galactose residues had been previously removed by enzymic treatment. This result suggests that only seven to eight chains of the glycoprotein are terminated by the sequence *N*-acetylneuraminyl-β-D-galactosyl-2-acetamido-2-deoxyglucose.

The preparation of a highly purified neuraminidase and of a β-galactosidase from culture fluids of *Diplococcus pneumoniae* has been described in the preceding paper (Hughes and Jeanloz, 1964). The pure enzymes have appreciable activity on glycoprotein substrates of high molecular weight, and have proved to be of value in the structural study of these substances. The presence of a highly active β-*N*-acetylglucosaminidase in the culture medium of *Diplococcus pneumoniae* (Hughes and Jeanloz, 1964; Li and Shetlar, 1963) and the occurrence of 2-acetamido-2-deoxy-D-glucose (*N*-acetylglucosamine) in many glycoproteins prompted the work to be described in the present paper. The β-*N*-acetylglucosaminidase has been purified extensively in a form free of other glycosidase or proteolytic activity, and the kinetics of the reaction of the enzyme with *p*-nitrophenyl 2-acetamido-2-deoxy-D-glucopyranoside (Leaback and Walker, 1957) has been studied. In addition, the action of the enzyme on a macromolecular substrate derived by enzymic modification of α_1-acid glycoprotein of human plasma (Hughes and Jeanloz, 1964) is reported.

MATERIALS AND METHODS

Bacteria.—The strain of *Diplococcus pneumoniae* used and the conditions of growth of the organism are described in the preceding paper (Hughes and Jeanloz, 1964). Preliminary experiments indicated that the culture fluid contained a highly active β-*N*-acetylglucos-

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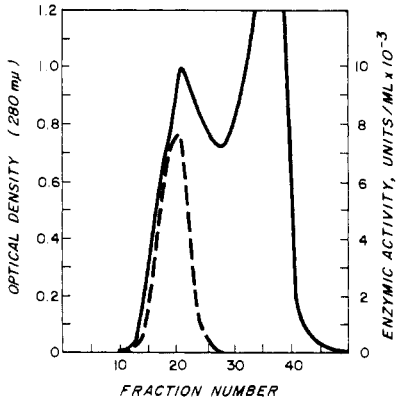

FIG. 1.—Gel-filtration on Sephadex G-100 of partially purified enzyme. The enzyme fraction (30 ml) from stage 1 of the purification scheme was applied to the column. Fractions (5 ml) were collected and measured for ultraviolet absorption (solid line) and enzymic activity (broken line).

aminidase, when assayed with PNPGNAc;[1] it was used as the source of the enzyme.

Substrates.—PNPGNAc was prepared according to Leaback and Walker (1957) and had mp 211–213°, decomp (literature mp 210–212°). 2-Acetamido-2-deoxy-D-glucose was obtained from Charles Pfizer and Co. All other substances were as described in the preceding paper (Hughes and Jeanloz, 1964). Buffers were 0.1 M phosphate-citrate, *p*H 2.5–8.4, and 0.1 M sodium acetate–acetic acid, *p*H 5.3.

Protein Determination.—Protein was estimated spectrophotometrically (Warburg and Christian, 1941) or by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Enzyme Assay.—Appropriate amounts of enzyme solution were incubated with 0.5 ml of 0.008 M PNPGNAc and 0.25 ml of 0.1 M phosphate-citrate buffer, *p*H 5.3. The volume was diluted to 1.0 ml. After 30 minutes at 37°, the reaction was stopped by addition of 1.0 ml of 0.25 M sodium carbonate and the intensity of the yellow chromophore was measured at once at 400 mμ (Borooah *et al.*, 1961).

Definition of Unit.—A unit of activity is defined as a change of 0.01 optical density units at 400 mμ after incubation for 30 minutes under the standard conditions. Specific activity is expressed as units/mg of enzyme protein as measured by the Lowry reaction.

Estimation of Liberated 2-Acetamido-2-deoxy-D-glucose.—The release of 2-acetamido-2-deoxy-D-glucose during the hydrolysis of PNPGNAc in the standard assay was measured by the Morgan-Elson reaction (Gardell, 1958). Portions of 0.1 ml of the standard assay mixture were withdrawn and pipetted into tubes containing 0.9 ml of water. The tubes were heated at 100° for 3 minutes to stop the reaction and stored at −5° until required. To each tube was added 0.1 ml of 0.5 M sodium carbonate, and the tubes were heated in a boiling-water bath exactly 4 minutes, after which time the tubes were cooled in ice for 10 minutes. Glacial acetic acid (8.0 ml) and Ehrlich's reagent (1.0 ml) were added consecutively, and the color intensity which developed after 1 hour at room temperature was read at 550 mμ. The amounts of sugar released were estimated from a standard curve made up using 2-acetamido-2-deoxy-D-glucose.

[1] The following abbreviation is used: PNPGNAc, *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside.

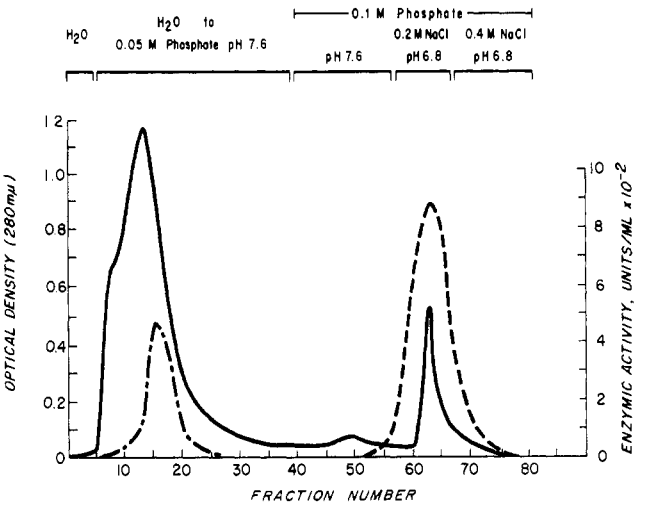

FIG. 2.—Chromatography of partially purified enzyme on DEAE-cellulose. The enzyme solution (5 ml) from stage 3 was applied to the column and the protein mixture was eluted with buffers containing increasing concentrations of salt at decreasing *p*H. Fractions (5 ml) were examined for ultraviolet absorption at 280 mμ (——) and for β-*N*-acetylglucosaminidase activity (- - -). For comparison the position at which the β-D-galactosidase activity was eluted is indicated (— · —).

Purification Procedure

The general procedures and the starting source of enzyme were identical with those described in the preceding paper (Hughes and Jeanloz, 1964).

Stage 1: Ammonium Sulfate Fractionation.—Addition of solid ammonium sulfate to the clarified growth medium up to 0.75 saturation was made as described previously. The precipitate was dissolved in cold water and dialyzed exhaustively against cold distilled water. Solid ammonium sulfate (210 g/liter) was added to the dialyzed solution with rapid stirring to give 0.35 saturation. After 1 hour the mixture was centrifuged and the precipitate was discarded. The supernatant solution was adjusted to 0.45 saturation by addition of solid ammonium sulfate (62 g/liter), and the precipitate was allowed to settle overnight. The precipitate was isolated by centrifugation, dissolved in the minimum volume of water, and dialyzed against 0.01 M phosphate buffer, *p*H 6.8, overnight.

Stage 2: Gel-Filtration through Sephadex G-100.—A portion (30–50 ml) of the fraction from stage 1 was applied to a column of Sephadex G-100 (2.2 × 70 cm) which had been equilibrated with 0.01 M phosphate buffer, *p*H 6.8, containing 0.4 M sodium chloride. The elution was made with the same buffer and fractions of 5 ml were collected, and assayed for enzymic activity and ultraviolet absorption at 280 mμ. The β-*N*-acetylglucosaminidase activity was eluted in the breakthrough peak (Fig. 1), together with about 60% of the neuraminidase and β-galactosidase activities. A large heavily pigmented fraction was eluted later in the filtration, and was enzymically inactive. The yield of β-*N*-acetylglucosaminidase activity was quantitative and a considerable purification from inactive protein was achieved in this step.

Stage 3: Concentration of Active Fractions by Precipitation with Ammonium Sulfate.—It was not possible at this stage to lyophilize the effluent fractions from the gel filtration, because excessive foaming tended to occur. Therefore the fractions were concentrated by precipitation with ammonium sulfate. Solid ammonium sulfate (51.5 g/100 ml) was added with stirring, and the pre-

TABLE I
PURIFICATION OF β-D-N-ACETYLGLUCOSAMINIDASE

Stage	Fraction[a]	Volume (ml)	Protein (g)	Total Activity (units × 10^{-6})	Specific Activity (units/mg protein)	Overall Yield (% units)
1	Ammonium sulfate precipitate 0–0.75 satn	930	8.80	2.5	290	100
2	Ammonium sulfate precipitate 0.35–0.45 satn	130	2.03	1.2	590	48
3	Sephadex G-100 filtration	13	0.30	1.0	3300	40
4	DEAE-cellulose chromatography	6	0.04	0.3	7400	12

[a] Starting volume of medium was 6 liters.

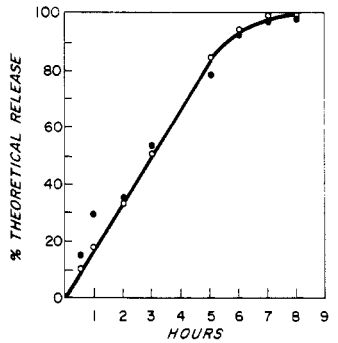

FIG. 3.—Stoichiometry of the hydrolysis of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside by the enzyme. The enzyme was incubated with the substrate under the standard assay conditions and the rate of release of phenol (O) and 2-acetamido-2-deoxyglucose (●) was followed as described in the text.

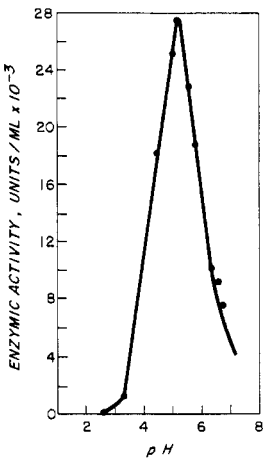

FIG. 4.—*p*H Optimum of the purified enzyme. The enzyme was assayed in phosphate-citrate buffers.

cipitate which formed was isolated after 1 hour by centrifugation; the precipitate was dissolved in cold water and dialyzed overnight against 0.005 M phosphate buffer, *p*H 6.8.

Stage 4: Chromatography on DEAE-Cellulose.—DEAE-cellulose was treated as previously described (Hughes and Jeanloz, 1964), and packed under pressure into a column (1 × 45 cm). The column was washed with a large excess of cold water. The enzyme solution from stage 3 was passed through the column at a flow rate of 60 ml/hour. The column was eluted with increasing concentrations of salt, as indicated in Figure 2. The β-*N*-acetylglucosaminidase activity was tightly held to the column, and was eluted with 0.1 M phosphate buffer, *p*H 6.8, containing 0.2 M NaCl. The enzyme was thereby well separated from β-galactosidase and neuraminidase activities, which were eluted with low salt concentrations. The fractions containing β-*N*-acetylglucosaminidase were pooled and lyophilized. The white powder was dissolved in water and dialyzed against 0.1 M phosphate, *p*H 6.8, overnight.

The solution was water-clear and showed no neuraminidase, β-galactosidase, nor proteolytic activity, when assayed at optimum *p*H with appropriate substrates. The yield of enzyme was rather low in this last step, but the purification from other glycosidase activities was complete. The preparation remained fully active on storage at 4° for at least 3 months. The enzyme was clearly heterogeneous, since the ratio of ultraviolet extinction at 280 mμ and 260 mμ was 1.03, indicating the presence of about 3% nucleic acid. Use of streptomycin sulfate at various stages of the fractionation procedure to improve this ratio was not successful. A similar finding was experienced during the purification of the neuraminidase and β-galactosidase of *Diplococcus pneumoniae* (Hughes and Jeanloz, 1964). The yield and purification at each stage are given in Table I.

RESULTS

Stoichiometry of the Reaction.—The nature of the reaction of the enzyme with PNPGNAc was studied by following the release of the phenol and of 2-acetamido-2-deoxy-D-glucose in the standard assay mixture at various times. Free *p*-nitrophenol was estimated by measurement of the optical density of suitably diluted aliquots of the reaction mixture in alkaline solution, using a value of 18 × 10^3 for the molar extinction coefficient. The release of free 2-acetamido-2-deoxy-D-glucose was followed by the Morgan-Elson reaction (Gardell, 1958). As shown in Figure 3, the molar ratio of phenol to sugar release was essentially unity up to the end of the reaction. The enzymic reaction therefore is a purely hydrolytic one under the standard conditions.

Effect of pH.—The effect of *p*H upon the activity of the enzyme is shown in Figure 4. The *p*H optimum was found to be 5.3. It is of interest that at *p*H 5.3 the purified β-galactosidase of *D. pneumoniae* exhibits less than 25% of full activity (Hughes and Jeanloz, 1964). Similarly, at the *p*H optimum of the latter enzyme (6.3), the β-*N*-acetylglucosaminidase is less than 30% active. It would be expected therefore that small amounts of one enzyme in purified preparations of the other would not be greatly detrimental to the use of these enzymes in the structural study of a glycoprotein.

Velocity and Substrate Concentration.—The velocity of the hydrolysis of PNPGNAc by the purified enzyme was determined for a range of final substrate concentrations from 0.4 to 5.6 × 10^{-3} M. A limiting velocity

was attained, and a Lineweaver-Burk plot of the results gave values for V_{max} and K_M of 0.26 μmole substrate hydrolyzed per minute per mg protein, and 0.22 $\times 10^{-3}$ M, respectively. The standard assay was carried out at a limiting substrate concentration. The value of the Michaelis constant is one order of magnitude smaller than was found for the crude enzymes of rat testis (Borooah *et al.*, 1961), rat kidney (Pugh *et al.*, 1957), and ox liver (Watanabe, 1936).

Activators and Inhibitors.—No difference in enzymic activity was found if the buffer in the standard assay was replaced by 0.1 M sodium acetate–acetic acid buffer, *p*H 5.3. Therefore, the enzyme was not inhibited by acetate ion in contrast to the results obtained for the crude enzyme of rat kidney by Pugh *et al.* (1957). The effects of various cations upon the activity of the purified bacterial enzyme is shown in Table II. No activation was found with any of the metals

TABLE II
EFFECT OF ADDED SUBSTANCES ON THE ACTIVITY OF THE PURIFIED ENZYME[a]

Substance	Final Concn (mM)	Activity (% of control)
Hg^{2+}	2.0	21
Cd^{2+}	2.0	34
Mg^{2+}	2.0	84
Ca^{2+}	2.0	82
Mn^{2+}	2.0	90
EDTA	2.0	100
EDTA	20.0	80

[a] Reagents were added to the concentrations indicated, to the enzyme in 0.10 M sodium acetate–acetic acid buffer (0.25 ml), *p*H 5.3. After the enzyme solution had stood at 37° for 15 minutes, 8 mM *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (0.50 ml) was added. The volume was adjusted to 1.0 ml with water and the enzymic activity was assayed as described in the text.

tested, and at 2 mM final concentration Mg^{2+}, Ca^{2+}, and Mn^{2+} were somewhat inhibitory. Mercuric ion inhibited the enzyme markedly, and cadmium ion slightly less so, indicating the presence of an essential, sensitive sulfhydryl group in the enzyme. Table II also shows that EDTA has little or no effect, even at very high concentration (20 mM), on the activity of the enzyme, indicating that the enzyme has no divalent-cation requirement. It may be noted that the β-galactosidase of *D. pneumoniae* is inhibited almost completely by 2 mM EDTA (Hughes and Jeanloz, 1964).

Action on Enzymically Modified α_1-Acid Glycoprotein.—In the preceding paper (Hughes and Jeanloz, 1964) was described the preparation of a derivative of the α_1-acid glycoprotein, by successive degradation of the glycoprotein with the purified neuraminidase and β-galactosidase of *Diplococcus pneumoniae*. It was shown that 100% of the neuraminic acid, and about 80% of the galactose of the intact glycoprotein, were removed enzymically. There was little or no other degradation of the macromolecule. It was of interest to test the activity of the purified β-*N*-acetylglucosaminidase on the modified glycoprotein, on the assumption that the previous degradations had exposed nonreducing terminals of β-*N*-acetylglucosamine residues, which would be susceptible to hydrolysis by the enzyme. A sample of 0.5 ml containing 8.15 mg of the modified glycoprotein was incubated at 37° with phosphate-citrate buffer, *p*H 5.3 (0.25 ml), and with the enzyme preparation (0.25 ml). The release of 2-acetamido-2-deoxyglucose was followed, as described under Materials and

TABLE III
RELEASE OF NONREDUCING TERMINAL 2-ACETAMIDO-2-DEOXYGLUCOSE FROM A DERIVATIVE OF α_1-ACID GLYCOPROTEIN[a]

Time (hours)	2-Acetamido-2-deoxyglucose Released (%)
0	0
6	20
16	26
46	26

[a] This derivative was prepared by prior successive degradation of the glycoprotein with purified neuraminidase and β-D-galactosidase of *Diplococcus pneumoniae*. The experiment was performed as described in the text. The enzyme used was eluted from the DEAE-cellulose column (stage 4) and had a specific activity of 7400 units/mg. The enzyme contained no neuraminidase, nor β-galactosidase activity.

Methods, on suitable aliquots of the reaction mixture. The amount of sugar released rapidly approached, after 16 hours, a limiting value of 26% of the total 2-acetamido-2-deoxyglucose residues of the glycoprotein derivative (Table III). Further prolonged incubation of the substrate with the enzyme failed to release more 2-acetamido-2-deoxyglucose residues from the glycoproteins. A sample of the enzymically modified glycoprotein obtained as described was examined for sedimentation behavior. Figure 5 shows the sedimentation pattern obtained for the derivative of the α_1-acid glycoprotein and includes for comparison the results given by intact α_1-acid glycoprotein. The results indicate that no gross alteration of the macromolecule had taken place during enzymic treatment. The approximate sedimentation constants for intact and enzymically treated α_1-acid glycoprotein were 2.7 and 2.4, respectively.

DISCUSSION

As in the purification of the neuraminidase and β-galactosidase activities of *Diplococcus pneumoniae*, fractionation of the enzyme was considered complete when no contaminating glycosidase nor proteolytic activities could be detected in the final preparation of the enzyme. The main qualifications required for an enzyme to be useful in a study of a glycoprotein are that the enzymic reaction should be rapid and purely hydrolytic in nature and that, in the cases of β-galactosidase and β-*N*-acetylglucosaminidase, only nonreducing terminal residues should be susceptible to hydrolysis. These properties appear to be met by the enzymes described in the present and preceding papers. Thus no transfer of monosaccharide units was detected when the enzymes were incubated with various substrates and, similarly, paper chromatographic examination of enzyme-incubation mixtures with suitable derivatives of the α_1-acid glycoprotein revealed only the presence of the expected, simple monosaccharide. Hydrolysis of interchain linkages would by contrast be expected to release oligosaccharides containing reducing-terminal galactose or 2-acetamido-2-deoxyglucose residues. The release of 100 and 80%, respectively, of the *N*-acetylneuraminic acid and galactose units of the α_1-acid glycoprotein by the successive actions of neuraminidase and β-galactosidase of *Diplococcus pneumoniae* has been reported in the preceding paper (Hughes and Jeanloz, 1964). Further treatment of the enzymically modified glycoprotein with β-*N*-acetylglucosaminidase released 26% of the total 2-acetamido-2-deoxyglucose of the α_1-

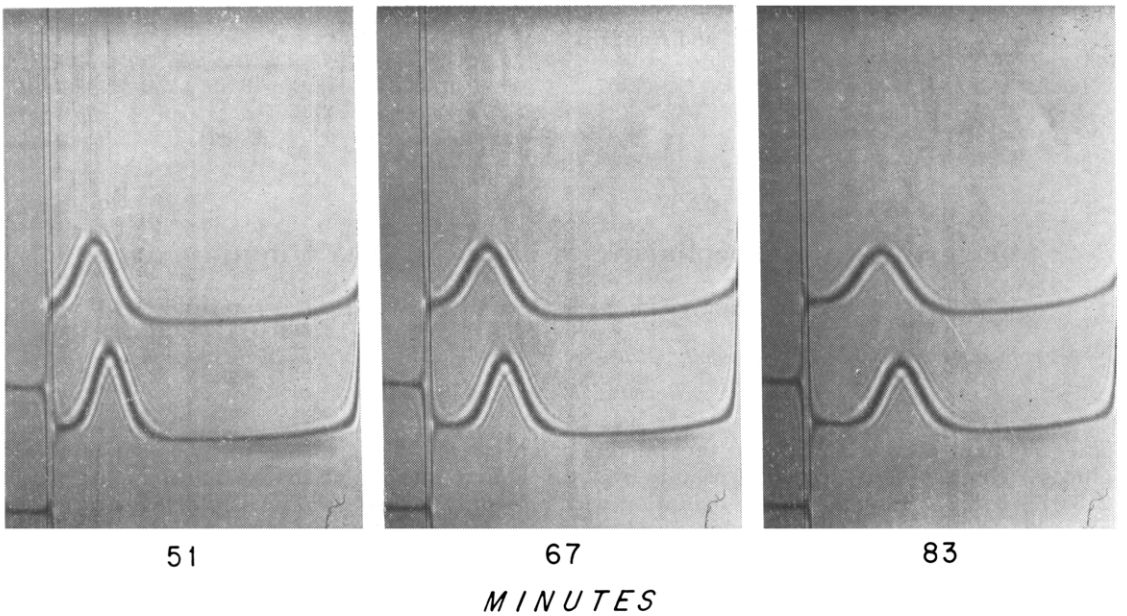

FIG. 5.—Sedimentation patterns of α_1-acid glycoprotein (lower) and of α_1-acid glycoprotein after successive treatments with purified neuraminidase, β-galactosidase, and β-N-acetylglucosaminidase (upper). Concentration, 5 mg/ml; speed of rotation, 59780 rpm; buffer, 0.1 M barbiturate-citrate, *p*H 8.6; temperature, 20°.

acid glycoprotein. This indicates the existence in the α_1-acid glycoprotein of carbohydrate chains having as the nonreducing terminals the sequence: *N*-acetylneuraminyl-β-D-galactosyl-2-acetamido-2-deoxy-D-glucose. The existence of part of this sequence has been inferred also (Eylar and Jeanloz, 1962) by the isolation from partial acid hydrolysates of α_1-acid glycoprotein of *N*-acetyllactosamine [2-acetamido-2-deoxy-4-*O*-(β-D-galactopyranosyl)-D-glucose]. Clearly not all of the galactose of α_1-acid glycoprotein is present in this simple sequence since, after removal of approximately sixteen residues of galactose from the α_1-acid glycoprotein by β-galactosidase, only about seven to eight residues of 2-acetamido-2-deoxyglucose are exposed for hydrolysis by β-*N*-acetylglucosaminidase. Therefore the maximum number of carbohydrate chains per molecule of α_1-acid glycoprotein having the nonreducing terminal sequence indicated above is seven to eight. It is possible that in other chains the 2-acetamido-2-deoxyglucose residues of the suggested sequence form branch points, and are substituted by structures unaffected by treatment with the enzymes. These substitutions would protect the residues from enzymic release, even after prior removal of galactose units. It is interesting that, after methylation and complete acid hydrolysis of α_1-acid glycoprotein, the methylated hexosamine derivatives isolated were shown to have the structures 2-amino-2-deoxy-6-*O*-methyl-D-glucose (N. R. Williams and R. W. Jeanloz, unpublished data) and 2-amino-2-deoxy-3,6-di-*O*-methyl-D-glucose (Jeanloz and Eylar, 1959). The isolation of a monomethyl derivative indicates that some of the 2-acetamido-2-deoxyglucose units of α_1-acid glycoprotein are doubly substituted and form branch links, probably through position 3. Further structural study of the product remaining after degradation of α_1-acid glycoprotein with the enzymes would be of interest.

The *p*H optimum of 5.3 for the β-*N*-acetylglucosaminidase of *Diplococcus pneumoniae* is slightly higher than the optimum (4.2–4.7) found for the enzymes obtained from mammalian tissues (Borooah *et al.*, 1961; Pugh *et al.*, 1957; Watanabe, 1936). The Michaelis constant of 0.22×10^{-3} M is about 10-fold smaller than the corresponding constants found for the mammalian enzymes. The inhibition of β-*N*-acetylglucosaminidase activity by acetate ion observed for the enzyme of rat kidney (Pugh *et al.*, 1957) was not found in the present case, but in other respects the effect of various cations and anions was identical with the results published by the other workers. It is interesting that the β-*N*-acetylglucosaminidase activities isolated from rat testis (Woollen *et al.*, 1961) and from pig epididymis (Findlay and Levvy, 1960) catalyze the hydrolysis of 2-acetamido-2-deoxy-β-D-galactosides, and purification did not eliminate this dual enzymic function. The enzyme from *Diplococcus pneumoniae* has not been tested for reactivity with 2-acetamido-2-deoxy-β-D-galactosides but, in view of the occurrence of 2-acetamido-2-deoxygalactose as well as 2-acetamido-2-deoxyglucose residues in many glycoproteins, it would be desirable that this possibility be investigated.

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The Specific Polysaccharide of Type XVIIIA Pneumococcus*

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The capsular polysaccharide of the pneumococcal type XVIIIA consists of D-galactose, D-glucose, rhamnose, *N*-acetylglucosamine, glycerol, and phosphate in the approximate ratios 2:3.5:1:1:1:1. Its behavior on oxidation with periodate and some of its serological reactivities resemble those of the *O*-deacetylated polysaccharide of pneumococcal type XVIII. The residues of galactose and rhamnose are probably linked 1,3-, and the *N*-acetylglucosamine is also stable to periodate.

The capsular polysaccharides of pneumococcus are the antigenic determinants of type specificity in this heterogeneous group of microorganisms. The type-specific antibody found in animals as a result of infection, or injection of the killed cells or the polysaccharides (in man and the mouse) is both curative for and protective against a given pneumococcal type. Hence the study of the chemistry of these capsular substances advances both the practical and theoretical knowledge of the relation between chemical constitution and immunological specificity (for a review see Heidelberger, 1960). As the fine structures of relatively few pneumococcal substances are known, this field is being actively developed in several laboratories.

Clarification of the major structural features of the capsular polysaccharide of type XVIII pneumococcus (Estrada-Parra and Heidelberger, 1963) led to an interest in the closely related type XVIIIA. An early preparation of its polysaccharide, S XVIIIA, was available (Brown and Robinson, 1943) and was further purified. The substance not only showed many similarities to the type-specific determinant of type XVIII, but exhibited several major differences as well.

Experimental

Fractionation of S XVIIIA.—Preparation 1 (Brown and Robinson, 1943), 2.2 g, was dissolved in 50 ml of water, chilled, treated briefly in a Waring Blendor with chloroform-butanol (5:1), and centrifuged in the cold. The aqueous supernatant and the washings of the not very large middle layer were combined, diluted to a concentration of 3% NaOAc with neutralized saturated sodium acetate solution, and precipitated with an equal volume of chilled 2-propanol. A small fraction, deposited upon addition of another volume of ethanol, was not further characterized. The principal fraction was taken up in 70 ml of water and precipitated with 125 ml of saturated ammonium sulfate solution at *p*H 5.4. This step had been found to remove the group-specific C-substance from S XVIII (Estrada-Parra, *et al.*, 1962). After thorough dialysis of the precipitate against neutralized 5% aqueous sodium acetate in the cold, the S XVIIIA was precipitated with chilled ethanol, redissolved in water, dialyzed against water, and poured into 500 ml of cold ethanol containing 7 g of sodium acetate neutralized with a few drops of glacial acetic acid. The fibrous product was squeezed out, broken up into redistilled ethanol, treated with redistilled acetone, and filtered off. The yield was 1.45 g. A comparison of the material with the original substance and with S XVIII is given in Table I.

TABLE I
PROPERTIES OF THE SPECIFIC POLYSACCHARIDES OF TYPE XVIII AND TYPE XVIIIA PNEUMOCOCCUS

Preparation	$[\alpha]_D$ (°C)	N (%)	P (%)	Acetyl (%)	Ash (%)
S XVIIIA					
Original No. 1[a]	+82	1.7	2.9	6.1	10.6
Purified	+97[b]	1.5	2.9	0[c]	1.7[d]
S XVIII					
Fraction C[b]	+79	0.3	3.0	4.6[c]	1.8[d]

[a] Brown and Robinson, 1943. [b] Ash-free; Estrada-Parra and Heidelberger, 1963. [c] *O*-Acetyl. [d] As Na^+.

Immunochemical methods used were the same as described in other papers (Rebers and Heidelberger, 1959, 1961; Rebers *et al.*, 1962). Rabbit antipneumococcal (anti-Pn) type XVIIIA sera were kindly supplied by Miss Jessie L. Hendry of the Division of Laboratories and Research, New York State Dept. of Health, Albany, N.Y. Anti-Pn XVIIIA horse serum was not available. The present initial chemical study was carried out as far as possible along the same lines as that of S XVIII.

Results and Discussion

Hydrolysis of S XVIIIA by Acid.—A solution of S XVIIIA was heated in 1.5 N H_2SO_4 at 100° for 17 hours, passed through Duolite A in the OH^- form, and concentrated *in vacuo* to small volume. A chromatogram in solvent A, 1-butanol-pyridine-H_2O-benzene, 5:3:3:1 (Jeanes *et al.*, 1953), showed components corresponding to glucose, galactose, rhamnose, and glycerol when

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