The Extracellular Glycosidases of *Diplococcus pneumoniae*. I. Purification and Properties of a Neuraminidase and a β-Galactosidase. Action on the α_1-Acid Glycoprotein of Human Plasma*

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Received May 13, 1964

Diplococcus pneumoniae Type I neuraminidase and β-galactosidase have been purified 400- to 500-fold in good yield from crude cultures of the organism. The neuraminidase releases quantitatively the bound *N*-acetylneuraminic acid of α_1-acid glycoprotein of human plasma, and of *N*-acetylneuraminyllactose. The rate of reaction is approximately three times greater with the second substrate than with the glycoprotein. The respective values for K_M are 3.5 and 18×10^{-4} M of *N*-acetylneuraminic acid residues per ml. The enzyme requires no metal ion for full activity and is not inhibited by ethylenediaminetetraacetate. The β-galactosidase releases galactose from several low-molecular-weight β-D-galactopyranosides and from sialic acid–free α_1-acid glycoprotein, and fucose from a β-D-fucoside The velocity of reaction with the last compound is about 0.1% of that with *o*-nitrophenyl β-D-galactopyranoside. The reaction of β-galactosidase with *o*-nitrophenyl β-D-galactoside is almost completely inhibited by the addition of 2 mM ethylenediaminetetraacetate to the reaction mixture. The inhibition is partially reversed on addition of the divalent cations, Ca^{2+}, Mg^{2+}, and Mn^{2+}. The reaction of the enzyme with α_1-acid glycoprotein, from which *N*-acetylneuraminic acid residues had been previously removed by treatment either with purified neuraminidase or by mild acid hydrolysis, has been studied. About 80% of the total galactose of the glycoprotein is removed, and this value was shown to be a truly limiting one for the reaction. The implications of the result for the structure of the carbohydrate chains of α_1-acid glycoprotein are discussed.

A study of the extracellular glycosidases elaborated by *Diplococcus pneumoniae* was undertaken since it was expected that these enzymes would prove to be useful tools for the structural analysis of various glycoproteins. The effect of *Diplococcus pneumoniae* cultures on serum proteins has been well studied and much qualitative data are available on the nature of the enzymes responsible for the degradation of the glycoproteins (Oncley *et al.*, 1958; Laurell, 1959; Laurell and Brönnestam, 1959; Li and Shetlar, 1961; Eylar and Jeanloz, 1962b). The monosaccharide units most commonly encountered in glycoproteins are D-galactose, D-mannose, L-fucose, 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine), 2-acetamido-2-deoxy-D-galactose (*N*-acetyl-D-galactosamine), and *N*-acetylneuraminic acid.

This paper describes the properties of a neuraminidase and of a β-galactosidase which have been isolated from the culture medium of *Diplococcus pneumoniae* free of other glycosidase or proteolytic activity. Action of the purified enzymes on a well-defined glycoprotein substrate, the α_1-acid glycoprotein of human plasma (Schmid, 1953), has also been studied. The following paper (Hughes and Jeanloz, 1964) describes the preparation from *Diplococcus pneumoniae* of a purified β-*N*-acetylglucosaminidase (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase) and some properties of the enzyme.

Materials and Methods

Bacteria.—A strain of *Diplococcus pneumoniae* type I was kindly made available by Miss L. Wetterlow of the State Biological Station, Jamaica Plain, Mass. The bacteria were grown on a medium containing beef heart infusion broth (24 g), neopeptone (10 g), $CaCl_2$ (0.02 g), $MgCl_2 \cdot 6\,H_2O$ (0.50 g), and K_2HPO_4 (2.2 g) per liter. Growth was continued at 37° for 30 hours without adjustment of *p*H, which remained almost constant at 6.8. The culture was centrifuged at room temperature at $800 \times g$ for 5–6 hours to remove the bulk of the cells. The clear brown supernatant could be stored at −5° with no loss of enzymic activities for at least 3 months.

Substrates.—*N*-Acetylneuraminyllactose was obtained from General Biochemicals, Chagrin Falls, Ohio. α_1-Acid glycoprotein was prepared from Cohn fraction VI of human plasma as described by Schmid (1953). The substance was shown to be homogenous by ultracentrifugation and by free-boundary electrophoresis in barbiturate-citrate buffer, *p*H 8.6. The composition of the purified α_1-acid glycoprotein was as follows: 11% *N*-acetylneuraminic acid, 13.5% 2-acetamido-2-deoxyglucose, 7.6% galactose, 5.5% mannose, and approximately 1% fucose. *p*-Nitrophenyl and *o*-nitrophenyl β-D-galactopyranosides were obtained from Mann Research Laboratories, Inc. *N*-Acetyllactosamine [2-acetamido-2-deoxy-(4-*O*-β-D-galactopyranosyl)-D-glucose] was kindly given by Dr. M. C. Glick, *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (Leaback and Walker, 1957) by Miss E. Walker, phenyl 2-acetamido-2-deoxy-α- and β-D-glucopyranosides (Leaback and Walker, 1957) by Dr. S. Roseman, and *p*-nitrophenyl α-D-, β-D-, α-L-, and β-L-fucopyranosides by Dr. P. G. Walker. Sephadex G-100 was purchased from Pharmacia, Uppsala, Swe-

* This is publication XXXIX of the series *Amino Sugars* and No. 375 of the Robert W. Lovett Memorial Group for the Study of Crippling Diseases, Harvard Medical School at the Massachusetts General Hospital, Boston, Mass. This investigation was supported by a research grant from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service (A-3564-2). It was presented at the 146th Meeting of the American Chemical Society, Denver, Colo., January, 1964.

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den; DEAE-cellulose (batch 1449) and CM-cellulose (batch 1468) ion-exchange agents from the Brown Co., Berlin, N. H.; and beef heart infusion broth and neopeptone from DIFCO Laboratories, Detroit.

Determination of Protein Content.—Protein content was routinely measured spectrophotometrically according to Warburg and Christian (1941) and checked by the quantitative method of Lowry *et al.* (1951). Specific activities are expressed as units/mg of enzyme protein, as measured by the method of Lowry.

Analyses of Sugar Components of Substrates.—The glycoprotein samples were hydrolyzed at 100° for 8 hours in 1 N sulfuric acid or 6 N hydrochloric acid prior to the determination of neutral hexoses or glucosamine, respectively. The sulfuric acid hydrolysates were neutralized by passage through Amberlite IR-45 anion exchanger in the acetate form and evaporated to dryness under reduced pressure below 40°. The hydrochloric acid hydrolysates were evaporated directly to dryness under reduced pressure below 40°. The neutral sugars, galactose and mannose, were separated by paper chromatography using ethyl acetate–pyridine–acetic acid–water (5:5:1:3) (Fischer and Nebel, 1955) or the upper phase of butanol-ethanol-water (4:1:5), and detected with aniline phthalate as described by Baar (1954). The developed spots were cut from the paper and eluted with 0.1 N hydrochloric acid in 80% ethanol for 1 hour at room temperature (Leloir, 1951). The optical density of the colored solutions was measured at 390 mμ. Standard sugar samples were included with each chromatographic separation. Glucosamine was determined after hydrolysis with 6 N hydrochloric acid as described by Blix (1948). *N*-Acetylneuraminic acid was determined in bound form by the resorcinol method (Svennerholm, 1957) and free *N*-acetylneuraminic acid by the method of Warren (1959). Fucose was estimated according to Dische and Shettles (1948).

Standard Assay of Neuraminidase with α_1-Acid Glycoprotein.—Suitable samples of enzyme in 0.10 ml of 0.1 M phosphate-citrate buffer, *p*H 6.5, were incubated at 37° with 0.10 ml of a solution containing 2.5 mg of α_1-acid glycoprotein. The volume was adjusted to 0.40 ml. After 30 minutes' incubation, 0.05-ml portions of the mixture were withdrawn and assayed for free *N*-acetylneuraminic acid (Warren, 1959), the enzymic reaction being terminated by the addition of the acidic sodium metaperiodate reagent. Color yields were measured at 550 mμ and corrected for substrate and enzyme blanks. The values were converted to total μg of free *N*-acetylneuraminic acid per 0.40 ml of enzyme reaction mixture. A unit of neuraminidase activity is defined as the release of 0.1 μg of *N*-acetylneuraminic acid from the α_1-acid glycoprotein per 0.40 ml reaction mixture under the standard conditions.

Assay of Neuraminidase with N-Acetylneuraminyllactose.—Samples (up to 0.4 ml) of the substrate solution (2.6 mg/ml) were incubated with the enzyme solution and 0.1 M phosphate-citrate buffer, *p*H 6.5 (0.10 ml). The final volume was adjusted to 0.8 ml. After incubation at 37° for 30 minutes, 0.10-ml portions were withdrawn and analyzed for free *N*-acetylneuraminic acid.

Standard Assay of β-Galactosidase with o-Nitrophenyl β-D-Galactopyranoside.—The enzyme in 0.5 ml of 0.1 M phosphate-citrate buffer, *p*H 6.3, was added to 1.0 ml of a solution of 10 mM substrate. The volume of the solution was adjusted to 2.0 ml. After incubation at 37° for 60 minutes, the reaction was stopped by addition of 2.0 ml of 0.2 M carbonate-bicarbonate buffer, *p*H 10.8, and the color was measured immediately at 430 mμ. The molar extinction coefficient of free *o*-nitrophenol under these conditions is 2.0×10^3. A unit of enzyme causes a change of optical density of 0.01 unit per 60 minutes under the conditions defined.

Assay of β-Galactosidase with p-Nitrophenyl β-D-Galactopyranoside and p-Nitrophenyl β-D-Fucoside.—Samples of 0.05 to 1.0 ml of 10 or 9.5 mM solution, respectively, of substrate in water were incubated with 0.5 ml of enzyme solution in 0.1 M phosphate-citrate buffer, *p*H 6.3. After adjustment of the volume to 2.0 ml, the solution was kept at 37° for 60 or 120 minutes, when the reaction was terminated by addition of 2.0 ml of 0.25 M sodium carbonate (Borooah *et al.*, 1961). The chromophore was measured at 400 mμ and the amount of the *p*-nitrophenol liberated by the enzyme was estimated from the molar extinction coefficient (18×10^3) of the chromophore at this wavelength.

Assay of β-Galactosidase with N-Acetyllactosamine.—Samples of up to 0.25 ml of a solution of 26 mM *N*-acetyllactosamine in water were incubated for 120 minutes at 37° with 0.25 ml of enzyme solution in 0.1 M phosphate-citrate buffer, *p*H 6.3. The volume was adjusted to 1.0 ml. Samples (0.2 ml) were withdrawn and the amount of released 2-acetamido-2-deoxyglucose was measured by the Morgan-Elson reaction (Gardell, 1958). *N*-Acetyllactosamine did not give a color when assayed under the given conditions.

Purification Procedure

All operations were carried out at 4° unless otherwise specified. Centrifugations were usually carried out in a refrigerated Servall centrifuge at $8000 \times g$ for 40 minutes.

Stage 1: Concentration of the Enzymes.—For each liter of clarified medium, 510 g of solid ammonium sulfate was added with stirring to give a 0.75 saturation. The mixture was left for 10 hours at 4°, then centrifuged. The sticky brown precipitate was dissolved in a minimum volume of water (about 0.1 of starting volume) and dialyzed exhaustively against cold distilled water for 2 days.

Stage 2: Ammonium Sulfate Fractionation.—To the dialyzed fraction (*p*H 5.8) was added solid ammonium sulfate (175 g/liter) to give 0.30 saturation. After 5 hours in the cold the precipitate was removed by centrifugation and discarded. The saturation of the supernatant solution (*p*H 5.8) was increased to 0.75 by addition of solid ammonium sulfate (310 g/liter). After 5 hours in the cold the precipitate was isolated by centrifugation, dissolved in a minimum amount of water, and dialyzed exhaustively against cold distilled water until free of sulfate ions. It is essential that dialysis be efficient in order to obtain reproducibility at stage 3. The dialyzed fraction was adjusted to approximately 15 mg/ml of protein for the next stage.

Stage 3: Batch-by-Batch Absorption to DEAE-Cellulose.—The DEAE-cellulose ion-exchange resin was washed thoroughly with water, and then successively with dilute sodium hydroxide, dilute hydrochloric acid, water, dilute sodium hydroxide, and finally water until the effluent was neutral. The resin was dried by suction on the filter and stored in a brown tightly stoppered bottle at 4°. DEAE-cellulose (10 g) was added at 4° with vigorous stirring to each 100 ml of the solution from stage 2. The suspension (*p*H 7.6) was centrifuged after 30 minutes and the pellet was washed several times with small portions of cold water. The supernatants, which were almost colorless, were pooled and contained quantitatively the neuraminidase activity and 10–20% of the galactosidase activity of the starting fraction.

The pellet was kept for the preparation of the β-galactosidase. This stage has proved to be the most variable one of the fractionation scheme, and it is advisable

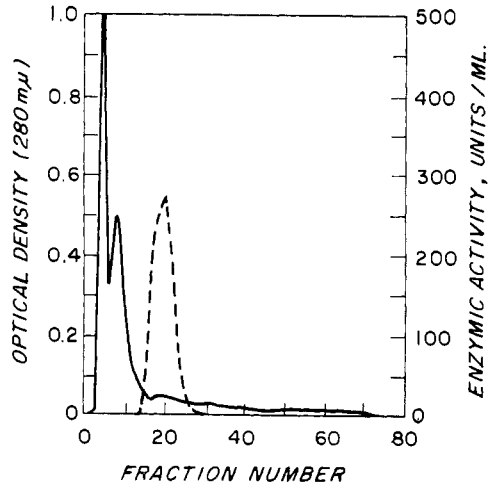

FIG. 1.—Chromatography of partially purified neuraminidase from stage 4a on CM-cellulose. Enzyme fraction (8.5 ml) was placed on the column which was developed with 50 ml of 0.01 M phosphate buffer, pH 6.8, and then with a gradient made using 225 ml of 0.01 M phosphate buffer, pH 6.8, in the mixing chamber and 225 ml of 0.10 M phosphate buffer, pH 6.8, in the reservoir. Fractions (10 ml) were examined for ultraviolet absorption at 280 mμ (solid line) and for enzymic activity using the standard assay (broken line).

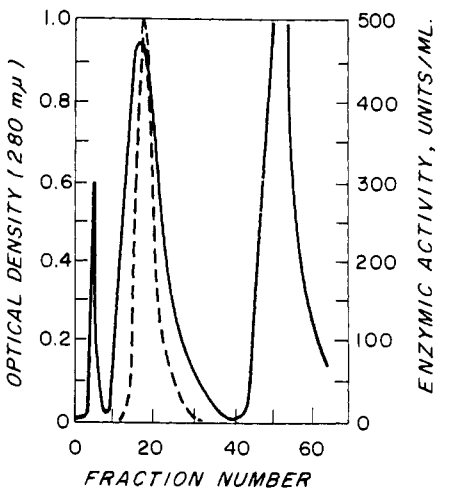

FIG. 2.—Chromatography on DEAE-cellulose of partially purified β-galactosidase. A solution of the enzyme (12 ml) was placed on the column (1 × 40 cm) and washed with water. A gradient was established at tube number 10 consisting of 100 ml of water in the mixing flask and 0.05 M phosphate buffer, pH 7.6 (100 ml), in the reservoir. Fractions (8 ml) were collected and examined for ultraviolet absorption (solid line) and enzymic activity (broken line). Finally, a large, inactive protein peak was eluted with 0.1 M phosphate buffer, pH 7.6.

to carry out trial experiments using small portions of the fraction from stage 2. It may be noted that attempts to fractionate the neuraminidase and β-galactosidase by DEAE-cellulose chromatography without prior ammonium sulfate fractionation were unsuccessful. The activities were eluted together at low salt concentrations.

Stage 4a: Ammonium Sulfate Fractionation of the Supernatant from Stage 3.—Solid ammonium sulfate (245 g/liter) was added with stirring to the pooled supernatants from stage 3. The precipitate which formed was removed by centrifugation after 1 hour and discarded. The supernatant (0.40 saturation) was adjusted to 0.65 saturation by the addition of solid ammonium sulfate (168 g/liter) and the precipitate was collected after 1 hour by centrifugation. It was dissolved in cold water and dialyzed against 0.005 M potassium phosphate buffer, pH 6.8, for 24 hours. The dialyzed solution may be lyophilized at this point with no loss of activity.

Stage 5a: CM-Cellulose Chromatography of the Fraction from Stage 4a.—CM-cellulose ion-exchange resin was washed thoroughly with water and with 0.01 M phosphate buffer, pH 6.8, packed under pressure into a column (1 × 40 cm), and washed with a large excess of the buffer at 4°. A portion of the fraction from stage 4a was applied to the column which was developed at 100 ml/hour with a salt gradient from 0.01 M to 0.10 M phosphate, pH 6.8. Fractions of 10 ml were collected and examined for neuraminidase activity using the standard assay, and for ultraviolet absorption at 280 mμ. The elution profile shown in Figure 1 was satisfactorily reproducible when up to 250 mg of protein was placed on the column, indicating that the neuraminidase was well separated from the bulk of the contaminating protein of the fraction from stage 4a. The recovery of activity was usually 60–70%.

The active fractions were pooled and lyophilized. The white powder was dissolved in cold water, dialyzed against 0.01 M phosphate buffer, pH 6.8, for 24 hours, and stored in ice. This preparation was used for the experiments to be reported.

Stage 4b: Elution of β-Galactosidase from DEAE-Cellulose.—The pellet of DEAE-cellulose from stage 3 was suspended in 100 ml of cold water and the suspension was stirred for 30 minutes. The mixture was centrifuged and the pellet was re-extracted three more times with water. The pellet was then washed in the same manner with increasing concentrations of phosphate buffer, pH 7.6, from 0.01 M to 0.05 M in increments of 0.01 M. The supernatants of each buffer concentration were filtered after extraction through fluted Whatman 12 filter paper to remove traces of resin, and the clear, light-brown filtrates were assayed for enzymic activity. The active fractions (usually those eluted with 0.04 M buffer, pH 7.6) were pooled and contained up to 80% of the β-galactosidase activity of the starting fraction.

Stage 5b: Ammonium Sulfate Fractionation of the Filtrates from Stage 4b.—The concentration in ammonium sulfate of the combined filtrates was adjusted to 0.55 saturation by addition of solid ammonium sulfate (350 g/liter). The mixture was centrifuged after 1 hour at 4°, and the precipitate was dissolved in the minimum amount of cold water. The solution was dialyzed against 0.005 M phosphate buffer, pH 6.8.

Stage 6b: DEAE-Cellulose Chromatography.—DEAE cellulose ion-exchange resin, treated as previously described, was packed under pressure into a column (1 × 40 cm) and washed thoroughly with cold water. Portions of the fraction from stage 5b (5–15 ml, containing 20–200 mg protein) were passed down the column at a flow rate of 40 ml/hour. The column was then developed with a salt gradient using 100 ml of water in the mixing chamber and 100 ml of 0.05 M phosphate buffer, pH 7.6, in the reservoir. Fractions (8 ml) were collected and examined for β-galactosidase activity and ultraviolet absorption at 280 mμ. The active fractions (Fig. 2) were pooled and lyophilized. The purification obtained in this step was not extensive and it is probable that this chromatography may be conveniently omitted from the fractionation scheme with little change in specific activity of the final product.

Stage 7b: Gel Filtration on Sephadex G-100.—The lyophilized powder from stage 6b was dissolved in 5 ml

TABLE I

PURIFICATION OF NEURAMINIDASE AND β-GALACTOSIDASE OF *Diplococcus pneumoniae*

Stage[a]	Neuraminidase			β-D-Galactosidase		
	Total Activity (units × 10^{-3})	Specific Activity (units/mg)	Yield (%)	Total Activity (units × 10^{-3})	Specific Activity (units/mg)	Yield (%)
1	335	25	100	138	10	100
2	340	45	101	100	13	73
3	303	81	89			
4a	180	470	59			
5a	100	11,200	30			
4b				84	90	61
5b				54	250	39
6b				43	540	31
7b				37	2000–5100	27

[a] Starting volume of clarified growth medium = 5 liters.

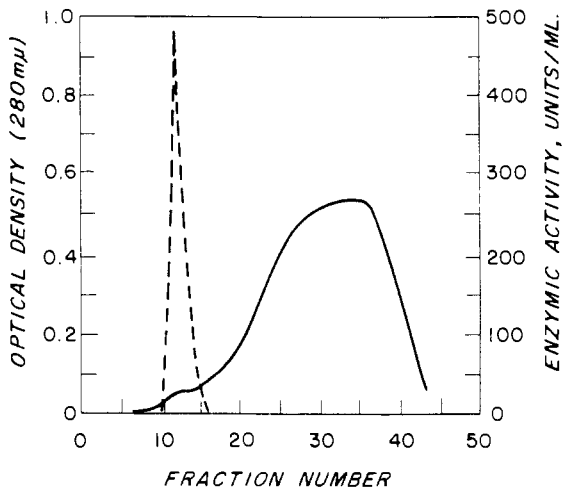

FIG. 3.—Gel filtration of partially purified β-galactosidase on Sephadex G-100. A solution of enzyme (5 ml) from stage 6b was put on the column (2.2 × 50 cm) and eluted with 0.01 M phosphate buffer, *p*H 6.8, containing 0.4 M sodium chloride. Fractions (5 ml) were analyzed for ultraviolet absorption at 280 mμ (solid line) and β-galactosidase activity (broken line) using the standard conditions.

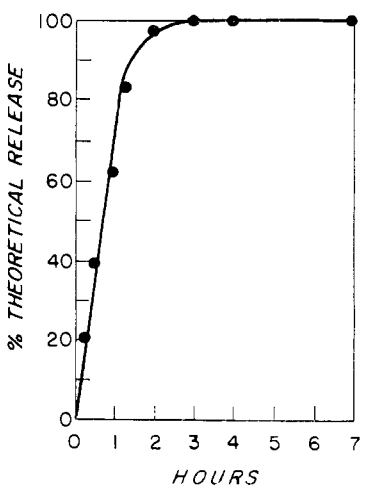

FIG. 4.—Release of *N*-acetylneuraminic acid from α_1-acid glycoprotein by the purified neuraminidase. The glycoprotein was incubated with the enzyme in the standard assay mixture and portions of the mixture were analyzed for free *N*-acetylneuraminic acid by the method of Warren (1959).

of cold water. The solution was passed through a column of Sephadex G-100 (2.2 × 50 cm) which had been previously equilibrated with 0.01 M phosphate buffer, *p*H 7.6, containing 0.4 M sodium chloride (Ada, 1963). The high salt concentration was used in order to reduce protein interactions which might occur and prevent efficient separation of the mixture. The column was run at 20 ml/hour, and fractions of 5 ml were collected and assayed for enzymic activity and ultraviolet absorption at 280 mμ.

Active fractions (tubes 10–14, Fig. 3) were quantitatively assayed for protein, and those fractions of specific activity greater than 2000 units/mg were stored individually on ice. The peak tubes had specific activities of approximately 5000 units/mg of enzyme protein.

The fractionation scheme gave final solutions of neuraminidase and β-galactosidase which were water clear, and which could be stored indefinitely on ice or as lyophilized powders without loss of activity. The preparations were free of each other and of *N*-acetylglucosaminidase activity, and did not release free amino groups on incubation with α_1-acid glycoprotein. The overall purification and yields of neuraminidase and β-galactosidase are summarized in Table I. The most highly purified preparations of neuraminidase and β-galactosidase had values in the range of 1.10–1.20 for the ratio of ultraviolet extinction at 280 and 260 mμ.

Unsuccessful attempts were made to eliminate the small amounts (2–3%) of nucleic acid present in these fractions by precipitation with streptomycin sulfate, protamine sulfate, and $MnAc_2$, and by absorption on charcoal. None of these treatments improved the values obtained for the ultraviolet ratio.

RESULTS

Preliminary Experiments

The crude culture medium was tested for various enzymic activities. Samples of medium were incubated at 37° for 24 hours with lactose, melibiose, maltose, methyl α-D-mannopyranoside, phenyl 2-acetamido-2-deoxy-α- and β-D-glucopyranoside, several β-D-galactopyranosides, and α_1-acid glycoprotein. Examination of the digests by paper chromatography indicated that only the last three substrates were acted upon by the enzyme mixture. Paper chromatography of the digest of the α_1-acid glycoprotein showed the presence of free *N*-acetylneuraminic acid, galactose, and 2-acetamido-2-deoxyglucose; no mannose was detected. These results show that the organism elaborates neuraminidase, β-galactosidase, and β-*N*-acetylglucosaminidase activities but no α-galactosidase or α- or β-mannosidase activity. Recently an α-galactosidase has been purified from cultures of *Diplococcus pneumoniae* type 6 (Li *et al.*, 1963).

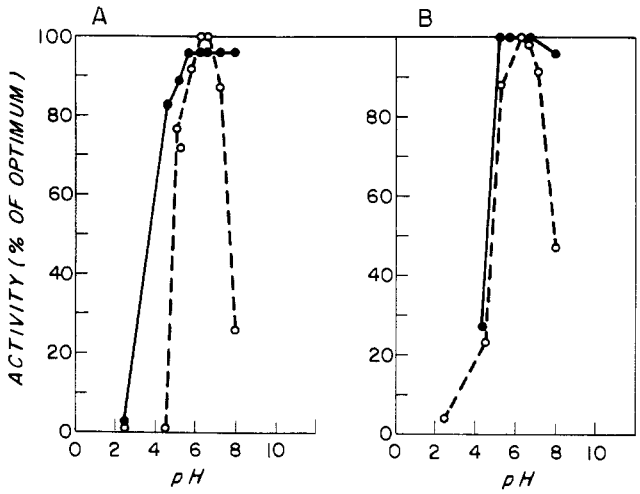

FIG. 5.—*p*H Stability and *p*H optimum of the neuraminidase (A) and of the β-galactosidase (B). *p*H Stability (●); the purified enzymes were incubated in buffer at different *p*H values for 1 hour at 37°, then suitable portions of the mixtures were assayed under the standard conditions described in the text. *p*H Optimum (○); the enzymes were assayed in phosphate-citrate buffers of different *p*H values.

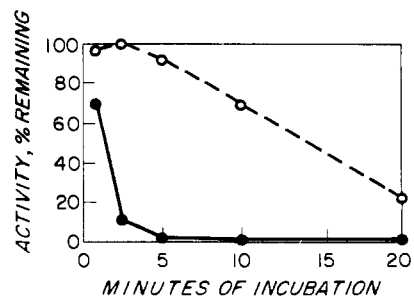

FIG. 6.—Heat stability of the purified neuraminidase (○) and of the β-galactosidase (●). The enzymes were incubated at *p*H 6.8 in a water bath maintained at 55°. At different times, portions of the solutions were withdrawn, cooled to 37°, and assayed under standard conditions.

Neuraminidase

Nature of the Reaction.—The rate of release of *N*-acetylneuraminic acid from the α_1-acid glycoprotein by the enzyme eluted from a CM-cellulose column proceeded smoothly up to about 90% hydrolysis of the *N*-acetylneuraminic acid of the glycoprotein (Fig. 4). Prolonged incubation after 100% release of *N*-acetylneuraminic acid caused no detectable decrease in the amount of free *N*-acetylneuraminic acid present, indicating the absence of *N*-acetylneuraminic acid aldolase in the enzyme preparation. The nondialyzable product remaining after exhaustive degradation contained quantitatively the galactose, mannose, 2-acetamido-2-deoxyglucose, and fucose of the starting substrate.

*p*H *Optimum and Stability.*—The effect of *p*H upon the activity and stability of the enzyme is shown in Figure 5. The same *p*H optimum (6.5) was found when 0.1 M Tris–acetic acid buffers, *p*H 5.3–8.3, were substituted for the phosphate-citrate buffers. No inhibition of neuraminidase activity by the acetate ion of the buffer at *p*H 6.5 was observed. The *p*H optimum of hydrolysis of *N*-acetylneuraminyllactose by the enzyme was 6.5, and the shape of the curve was identical with that obtained for the α_1-acid glycoprotein. The enzyme is completely stable in incubation at 37° for 1 hour in buffers from *p*H 5.6 to 8.0. The effect on neuraminidase activity of heating at 55° for various times is shown in Figure 6. Over 90% of the activity was retained after heating for 5 minutes, after which time the enzyme was rapidly inactivated.

Effect of Various Substances.—The following substances, present in the assay mixture at the final concentration indicated, had no effect on the enzyme activity (Table II): 2mM $MgCl_2$, 2 mM NaCl, 2 mM EDTA, 20 mM EDTA. The enzyme was completely inhibited by 2 mM $HgCl_2$ and, to some extent, by $CdCl_2$, $CaCl_2$, and $MnAc_2$. A slight activation (20%) was given by 2 mM KCl and 2 mM cysteine.

Activity toward Various Substrates.—The velocity of release of *N*-acetylneuraminic acid by the enzyme at several different concentrations of α_1-acid glycoprotein was determined. A Lineweaver-Burk plot (1934) of the results gave for the Michaelis constant (K_M) a value of 3.5×10^{-4} M of *N*-acetylneuraminic acid terminal residues of the glycoprotein and for the maximum theoretical velocity (V_{max}) a value of 1.5 μmoles of *N*-acetylneuraminic acid released per minute per mg of enzyme protein.

TABLE II

EFFECT OF ADDED SUBSTANCES ON THE ACTIVITY OF THE PURIFIED NEURAMINIDASE AND OF THE β-GALACTOSIDASE[a]

Substance	Final Concn (mM)	Neuraminidase Activity (% of control)	Galactosidase Activity (% of control)
Hg^{2+}	2.0	0	9
Cd^{2+}	2.0	80	37
Mg^{2+}	2.0	100	105
Ca^{2+}	2.0	80	105
Mn^{2+}	2.0	61	115
EDTA	2.0	87	13
EDTA	20.0	96	0
EDTA + Mg^{2+}	2.0, 50.0		76
EDTA + Ca^{2+}	2.0, 50.0		76
EDTA + Mn^{2+}	2.0, 50.0		71
Na^+	2.0	100	101
K^+	2.0	118	98
Cysteine	2.0	120	98

[a] Reagents were added, to the final concentrations indicated, to the neuraminidase or β-galactosidase in Tris-succinate buffer, *p*H 6.5 or 6.3, respectively. After 15 minutes at 37°, a solution of α_1-acid glycoprotein (0.05 ml) or of *o*-nitrophenyl β-D-galactopyranoside (1.0 ml) was added and the solutions were assayed for enzymic activity under the standard conditions described under Materials and Methods. In the reactivation experiments performed with β-galactosidase, the enzyme was incubated with 2 mM EDTA for 15 minutes at 37°. Cation was then added to a final concentration of 50 mM and the enzymic activity was determined immediately as under standard conditions.

A similar experiment was carried out using *N*-acetylneuraminyllactose as substrate. The corresponding kinetic constants were K_M 18×10^{-4} and V_{max} 3.1.

Behavior on Sephadex G-100.—Preparations of *Diplococcus pneumoniae* neuraminidase at various stages of purification were examined, for filtration behavior, on Sephadex G-100. The different columns were equilibrated and eluted with water or with 0.05 M phosphate, *p*H 6.5. In all cases the neuraminidase activity was detected, together with β-galactosidase activity (if present in the starting material), in the breakthrough peak. No difference in the position of elution of the neuraminidase was obtained, when the column was equilibrated and developed with 0.01 M phosphate, *p*H 6.8, containing 0.4 M NaCl. This buffer system is de-

TABLE III

ACTIVITY OF β-GALACTOSIDASE TOWARDS VARIOUS SUBSTRATES[a]

Substrate	Range of Substrate Concentration ($\times 10^{-3}$ M)	K_M ($\times 10^{-3}$ M)	V_{max} (moles hydrolyzed/ min per mg protein)	Relative Rate of Hydrolysis (%)
o-Nitrophenyl β-D-galacto-pyranoside	1.0–7.0	4.5	3.9	100
-Nitrophenyl β-D-galacto-pyranoside	0.2–4.8	1.9	1.6	41
2-Acetamido-2-deoxy-4-*O*-(β-D-galactopyranosyl)-D-glucose	0.7–6.5	4.4	2.4	61
p-Nitrophenyl β-D-fuco-pyranoside	1.0–4.8	8.3	0.01	0.3

[a] Activity was measured at several different concentrations of each substrate (at least five concentrations). The methods of assay and evaluation of K_M and V_{max} are described in detail under Materials and Methods. Relative rates of hydrolysis are referred to the values for *o*-nitrophenyl β-D-galactopyranoside. The enzyme used for the experiments had specific activity of 5100 units/mg enzyme protein. All experiments were conducted at 37°.

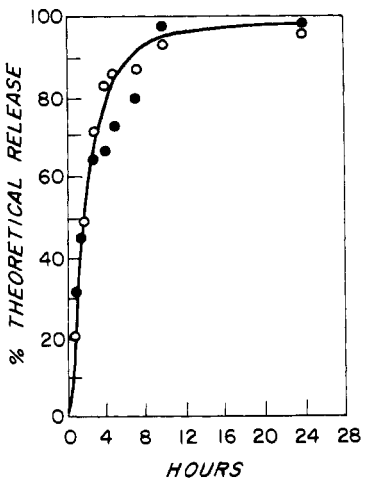

FIG. 7.—Stoichiometry of the hydrolysis of *o*-nitrophenyl β-D-galactopyranoside by β-galactosidase from stage 7b. The enzyme (0.5 ml, 180 units) was incubated, in 0.01 M phosphate-citrate buffer, *p*H 6.3 (0.5 ml), with 10 mM *o*-nitrophenyl β-D-galactoside (1.0 ml) at 37°. The release of the phenol (○) was followed spectrophotometrically in alkaline solution, *p*H 10.8, at 430 mμ. At intervals aliquots (0.1 ml) of the mixture were pipetted into cuvets containing carbonate buffer, *p*H 10.8 (0.9 ml). The yellow color was measured at once at 430 mμ and the amount of the phenol was estimated from a standard curve. Samples (0.05 ml) were also taken and spotted directly on paper for quantitative chromatographic analysis of free galactose (●) as described under Materials and Methods. Buffer salt did not interfere in the chromatography at the concentration used in the assay. The *p*H of the incubation mixture was constant at 6.3.

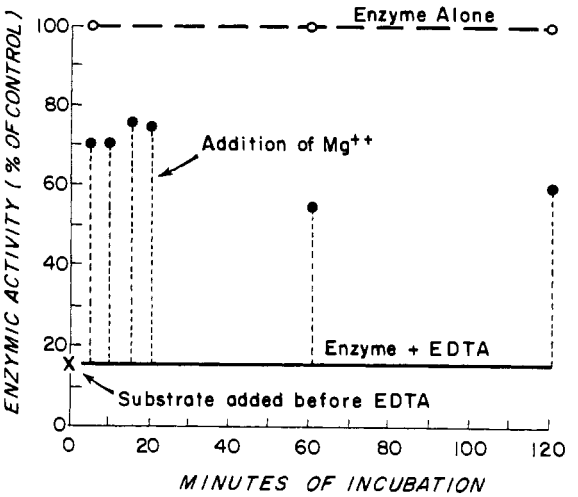

FIG. 8.—Effect of EDTA on β-galactosidase. The enzyme was incubated at 37° in Tris-succinate buffer, *p*H 6.3, either alone (○) or in the presence of 2 mM EDTA. At various times, up to 120 minutes, samples of the mixture were made 50 mM in Mg^{2+} by addition of 0.1 M $MgCl_2$. The volume was adjusted to 1.0 ml and then 10 mM *o*-nitrophenyl β-D-galactopyranoside (1.0 ml) was added. The mixture was then assayed for β-galactosidase activity (●). Similar enzyme-EDTA mixtures were also assayed for enzymic activity without addition of Mg^{2+}; the level of activity found is indicated on the graph. The effect of the addition of EDTA to the enzyme after the addition of substrate is also indicated (×).

signed to interfere with protein aggregation, which would increase the apparent molecular weight of the neuraminidase (Ada, 1963).

β-Galactosidase

Nature of the Reaction.—The stoichiometry of β-galactosidase action with *o*-nitrophenyl β-D-galactopyranoside was essentially quantitative at the concentration (5 mM) of substrate examined (Fig. 7). For each mole of *o*-nitrophenyl β-D-galactopyranoside hydrolyzed, 1 mole of galactose and 1 mole of *o*-nitrophenol were released.

*p*H *Optimum and Stability.*—The rate of hydrolysis of *o*-nitrophenyl β-D-galactopyranoside was examined in phosphate-citrate buffers of different *p*H (Fig. 5). The optimum activity is in the *p*H range 6.3–6.5. The effect of *p*H on the stability of the enzyme was studied by incubation for 60 minutes at 37°. The enzyme was stable in the *p*H range 5.3–8.0. No difference in *p*H optimum or initial rate of velocity was found in Tris-acetate buffers.

Heat stability at 55° was studied with the purified β-galactosidase. The enzyme was remarkably heat labile (Fig. 6), being completely inactivated after 3 minutes. A similar instability has been observed for the β-galactosidase of *E. coli* (Cohn and Monod, 1951).

Substrate Specificity and Kinetic Constants.—The kinetics of hydrolysis of several substrates by the purified β-galactosidase are reported in Table III. The initial velocity at several concentrations of each substrate in 0.025 M phosphate-citrate buffer, *p*H 6.3, was determined, and values for K_M and V_{max} were derived graphically. To allow a comparison to be made of the results, the values for V_{max} are given as

μmoles of substrate hydrolyzed per minute per mg of enzyme protein.

Activity Toward Fucosides.—The purified enzyme from stage 7b was examined for its ability to hydrolyze the *p*-nitrophenyl α- and β-glycopyranosides of D- and L-fucose. Only *p*-nitrophenyl β-D-fucopyranoside was hydrolyzed by β-galactosidase, and at a very low rate. The Michaelis constant is 8.3×10^{-3} M and V_{max} is 0.01 μmole of substrate hydrolyzed per minute per mg of enzyme protein (Table III). The *p*H optimum for the reaction is 6.3, the same as that found for the reaction of β-galactosidase with *o*-nitrophenyl β-D-galactopyranoside.

Effect of Added Substances.—The effect of various substances upon the activity of β-galactosidase is presented in Table II. Mg^{2+}, Ca^{2+}, Mn^{2+}, Na^{+}, and K^{+} were without effect on the activity of the enzyme. The enzyme was markedly inhibited by Hg^{2+} and Cd^{2+}, indicating the importance of sulfhydryl groups in the catalytically active protein. Cysteine was found, however, to be without effect on activity. EDTA at 2 mM concentration inhibited about 87% of the activity of the enzyme and 20 mM EDTA completely abolished it. The inhibition by 2 mM EDTA was partially removed on addition of excess Mg^{2+}, Ca^{2+}, or Mn^{2+} prior to assay. The three cations were equally effective in reactivating the inhibited enzyme. Addition of substrate (*o*-nitrophenyl β-D-galactopyranoside) to the enzyme immediately prior to addition of EDTA did not prevent inhibition (87%) of enzymic activity (Fig. 8). Incubation of the enzyme with 2 mM EDTA for various periods before assay resulted in an identical loss of approximately 85% of activity. Addition of Mg^{2+} ions, in excess of the concentration of EDTA, resulted in an immediate partial restoration of activity (Fig. 8). It is notable that 100% inhibition by 2 mM EDTA was not attained even after the longest time examined.

Activity Toward Sialic Acid–free α_1-Acid Glycoprotein.—Since the pure β-galactosidase was shown to be free of neuraminidase, β-*N*-acetylglucosaminidase, L-fucosidase, and protease activities, its action on a derivative of the α_1-acid glycoprotein of human plasma was studied. Incubation of the α_1-acid glycoprotein with neuraminidase results in the release of 100% of the *N*-acetylneuraminic acid from the glycoprotein (Fig. 4). The sialic acid–free glycoprotein, at a final concentration of 10 mg/ml, was incubated with β-galactosidase at *p*H 6.3. The rate of hydrolysis of terminal galactose residue was followed, subtractively, by analysis of the galactose content of the nondialyzable products remaining after different times of reaction (Table IV). The maximum yield of galactose released was approximately 80% of the total galactose content of the substrate. It was shown that the value obtained was a truly limiting one, since on dialysis of the reaction mixture and incubation of the nondialyzable product with fresh β-galactosidase no further galactose was released. The recoveries in the nondialyzable fraction of the other monosaccharide constituents of the substrate were: mannose (85–90%), 2-acetamido-2-deoxyglucose (100%), and fucose (100%). The experiment was repeated at a final substrate concentration of 5 mg/ml. The rate of release of galactose was found to be identical with that found at the higher substrate concentration, and approached the same limiting value. The initial velocity of release of galactose from sialic acid–free α_1-acid glycoprotein was calculated to be approximately 0.4 μmole of galactose released per minute per mg of enzyme at 10 mg/ml concentration of substrate. The purified β-galactosidase also released about 80% of the total galactose of α_1-acid glycoprotein, from which the *N*-acetylneuraminic acid residues had been completely removed by acid hydrolysis in 0.04 N sulfuric acid at 80° for 1 hour (Eylar and Jeanloz, 1962b). In addition, a portion (about 10%) of the L-fucose residues of the glycoprotein is also removed by this acid hydrolysis.[1]

TABLE IV

EFFECT OF PURIFIED β-GALACTOSIDASE ON SIALIC ACID–FREE α_1-ACID GLYCOPROTEIN[a]

Time of Incubation (hours)	Galactose Remaining (%)
0	100
2.5	66
5	44
10	20
24	22
72	21
96	21

[a] The α_1-acid glycoprotein derivative, from which 100% of the *N*-acetylneuraminic acid residues had been removed by purified neuraminidase, was incubated at 10 mg/ml final concentration with β-galactosidase (5.0 ml, 1300 units) in 0.1 M phosphate-citrate buffer, *p*H 6.3 (5.0 ml) at 37°. The final volume was 20 ml and toluene was added to prevent bacterial growth. At times, samples (2.0 ml) were dialyzed against water overnight in the cold, care being taken to minimize losses in transfer to the dialysis bags. The dialyzed solutions were then hydrolyzed in sulfuric acid for 8 hours. The deionized solutions were then analyzed by paper chromatography for the galactose remaining in nondialyzable form after enzymic degradation of the glycoprotein. After 72 hours incubation the whole of the digest remaining was dialyzed against water and the dialyzed solution was lyophilized. The product was then reincubated with fresh enzyme as described and the mixture was examined after 24 hours for galactose as before.

DISCUSSION

Diplococcus pneumoniae neuraminidase, free of β-galactosidase, β-*N*-acetylglucosaminidase, and protease activity, catalyzes the quantitative release of *N*-acetylneuraminic acid from α-acid glycoprotein as found by other workers (Oncley *et al.*, 1958; Li and Shetlar, 1961; Eylar and Jeanloz, 1962b) and from *N*-acetylneuraminyllactose. The Michaelis constant for α_1-acid glycoprotein is about 3.5×10^{-4} moles of bound sialic acid per liter and for *N*-acetylneuraminyllactose about 18×10^{-4} moles. A value of $1–2 \times 10^{-4}$ moles was found for *Vibrio cholera* neuraminidase (Ada and French, 1959; Schramm and Mohr, 1959) with sheep stromata as substrate. Using neuraminidase from influenza PR 8 strain, Rafelson *et al.* (1963) report a value K_M 1.2×10^{-3} moles for *N*-acetylneuraminyllactose. The *Diplococcus pneumoniae* enzyme requires no metal for activity and retains full activity after treatment with a large excess of EDTA, in agreement with the properties of other neuraminidases (Gottschalk, 1960). However, the inhibition of purified neuraminidases from *Vibrio cholera* and influenza virus by EDTA has been described (Ada *et al.*, 1961; Rafelson *et al.*, 1963).

The molecular weight of the enzyme from *Diplococcus pneumoniae* has not been studied directly, but a lower limit is indicated by the filtration behavior on Sephadex G-100. The apparent exclusion of neuraminidase from the gel particles indicates the enzyme to have a molecular weight greater than about 100,000, which is considerably higher than values, 10,000 and 50,000 respectively, determined for the neuraminidases of *Vibrio cholera* and *Cornybacterium diphtheria* (Ada

[1] A. M. Closse, unpublished results.

and French, 1959; Schramm and Mohr, 1959; Warren and Spearing, 1963).

Diplococcus pneumoniae β-galactosidase, purified some 500-fold from concentrated culture fluids, catalyzes the hydrolysis of various β-D-galactopyranosides. The values for V_{max} decrease in the series *o*-nitrophenyl β-D-galactopyranoside (100%), *N*-acetyllactosamine (61%), *p*-nitrophenyl β-D-galactopyranoside (41%), and *p*-nitrophenyl β-D-fucopyranoside (0.3%). Similar findings have been reported for the enzyme of *E. coli ML 309* (Wallenfels and Malhotra, 1960). In the latter case, the rate of hydrolysis of *p*-nitrophenyl β-D-galactopyranoside was only 13% that of *o*-nitrophenyl β-D-galactopyranoside. *Diplococcus pneumoniae* β-galactosidase attacks β-D-galactosides or β-D-fucosides, but not α- or β-L-fucosides. The lack of specificity with regard to C_6 has also been observed with β-galactosidases from other sources (Monod *et al.*, 1951, Takano and Miwa, 1953; Wallenfels and Malhotra, 1960).

Diplococcus pneumoniae type I β-galactosidase was examined for a transferase activity during reaction with *o*-nitrophenyl β-D-galactopyranoside. However, the reaction was shown to be purely a hydrolytic one at the concentration of substrate used (5 mM), in contrast to the results obtained with the crystalline β-galactosidase from *E. coli ML 309* (Wallenfels *et al.*, 1959). Furthermore, unlike the *E. coli* enzyme (Wallenfels and Zarnitz, 1957; Wallenfels and Malhotra, 1960), no difference either in *p*H optimum or maximum theoretical velocity of reaction were found for *Diplococcus pneumoniae* type I β-galactosidase in the presence or absence of Na^+ ion.

The enzyme is almost completely inhibited by EDTA, however, indicating the involvement in some way of a divalent cation in the catalytic activity of the enzyme. This view is strengthened by the finding that the inhibition is partially reversed on addition of Mg^{2+}, Ca^{2+}, or Mn^{2+} ions in molar excess over EDTA. The possibility that EDTA reacts directly with a catalytically important site of the enzyme, thereby destroying activity, and that the addition of a divalent cation removes the cause of the inhibition has been definitely eliminated in the case of the well-known EDTA inhibition of α-amylases from many sources (Fischer and Haselbach, 1951; Vallee *et al.*, 1959; Yamamoto and Fukumoto, 1959; Fischer and Stein, 1960). It has been shown also that several α-amylases, after treatment with EDTA to remove intrinsically bound (Vallee, 1955) Ca^{2+} ion, are considerably less stable to thermal denaturation and to digestion by trypsin (Stein and Fischer, 1958; Vallee *et al.*, 1959). *Diplococcus pneumoniae* β-galactosidase can be reactivated to only 71–76% of control activity by excess Mg^{2+} ion after incubation with EDTA at 37° for 15 minutes, which may reflect similarly an increased lability to heat inactivation of the enzyme in the presence of EDTA. After preincubation with chelating agent for 120 minutes, addition of Mg^{2+} ion gave about 62% reactivation.

The cleavage of 80% of the galactose units from neuraminidase-treated α_1-acid glycoprotein by crude enzyme preparations (Eylar and Jeanloz, 1962b) has been confirmed using purified β-galactosidase. The reaction rapidly reaches a limiting value, whereupon about 80% of the galactose is released. There is no concomitant degradation of the glycoprotein as shown by the essentially quantitative recovery of the other components of the glycoprotein. As expected from specificity studies with model compounds, the glycoprotein retains all the L-fucosyl residues of the intact α_1-acid glycoprotein. It is of interest that Dr. A. M. Closse in our laboratory found no action of the crystalline β-galactosidase of *E. coli ML 309* (Wallenfels *et al.*, 1959) or of the purified β-galactosidase of calf intestinal mucosa (Wallenfels and Fischer, 1960), on α_1-acid glycoprotein from which *N*-acetylneuraminic acid had been completely removed and fucose partially removed by mild acid hydrolysis.[2] These experiments indicate a wide divergence in the specificity of β-galactosidases from various sources for the hydrolysis of nonreducing terminal galactose residues, which is not so apparent when the relative rates of hydrolysis of low-molecular-weight substrates are compared.

The structural significance of the finding that 20% of the total galactose of α_1-acid glycoprotein remains after β-galactosidase action must await chemical studies on the enzymically modified glycoprotein. It is considered that *N*-acetylneuraminic acid and fucose residues occupy the nonreducing terminal positions of the carbohydrate chains of the glycoprotein, and are linked to galactose units which are in turn joined to residues of 2-acetamido-2-deoxyglucose (Popenoe, 1959; Eylar and Jeanloz, 1962a).

Since the L-fucose residues are probably linked directly to galactose, and in view of the specificity of β-galactosidase on model fucosides, these galactose residues would be unavailable for enzymic hydrolysis. From the fucose and galactose contents of α_1-acid glycoprotein, it would be expected that about 10–15% galactose would be protected in this manner, which is close to the value obtained. However, the possibility that a small amount of galactose is situated in the interior of the carbohydrate chains or is combined in α-linkages cannot be excluded, particularly in view of the uncertainty of the true fucose content of the glycoprotein.

Acknowledgments

We wish to thank Miss L. Wetterlow and the staff of the Massachusetts State Laboratories, Jamaica Plain, Massachusetts, for much advice and help in preparing the bacteria. We are also indebted to Mr. L. Larsen of the same laboratories for providing fraction VI from human plasma, and to Mr. E. Lane for technical assistance in the preparation of the bacteria and the α_1-acid glycoprotein. We are grateful to Drs. M. C. Glick, S. Roseman, and P. G. Walker, and Miss E. Walker for kindly providing substances.

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[2] Similar results have been experienced by other workers with crystalline β-galactosidase of *E. coli*, using high-molecular-weight substrates (M. L. Zarnitz, unpublished results. Reported by E. A. Kabat in Transactions of the Fifth Conference of the Macy Foundation, p. 46, 1959).

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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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Received May 19, 1964

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-

* This is publication XL of the series *Amino Sugars* and No. 376 of the Robert W. Lovett Memorial Group for the Study of Crippling Diseases, Harvard Medical School at the Massachusetts General Hospital, Boston, Mass. This investigation was supported by a research grant from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service (A-3564-2).

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