This substance has not to our knowledge been previously reported and is herein designated as γ-acetate.

Zone III above, located 60–105 mm. from the column top, was eluted with acetone, evaporated to a sirup and crystallized from ethanol; yield 810 mg. (total yield 3.40 g., including product crystallized directly), m.p. 191–193° cor., $[\alpha]^{25}D$ −5.5° (*c* 3.9, chloroform). These data are in agreement with those published for β-gentiobiose octaacetate. X-Ray powder diffraction data, identical with those of known β-gentiobiose octaacetate, were: 14.03[19]–10,[20] 11.91–10, 10.05–100, 8.96–30, 7.14–25, 5.61–1, 5.48–15, 4.98–20, 4.79–20, 4.45–25, 4.27–25, 4.00–1, 3.78–30, 3.53–20, 3.41–10.

After several days, an additional crop of crystals separated from the mother liquor material of the above zone III; yield 60 mg., m.p. 211–215° cor. Upon recrystallization from ethanol, the constants were: m.p. 227–230° cor., $[\alpha]^{31}D$ +35.3° (*c* 1.7, chloroform). Although these constants are in poor agreement with published values (229.5° and +42°),[26] indicating the presence of impurities, the X-ray powder diffraction data are identical with those of a known sample of α-cellobiose octaacetate: 11.48[19]–15,[20] 10.37–10, 8.71–10, 8.35–2, 7.02–1, 5.41–100, 5.04–50, 4.74–15, 4.38–15, 4.27–20, 4.02–15, 3.77–15, 3.59–10, 3.44–5, 3.29–10. This substance is thus identified as α-cellobiose octaacetate.

Zone IV, 15–60 mm. from the top of the column, was recovered and crystallized in a similar manner; yield 550 mg., m.p. 184–188° cor. After further purification by Magnesol chromatography, as described above, the constants were: m.p. 191–192° cor., $[\alpha]^{25}D$ +51.0° (*c* 4, chloroform). X-Ray powder diffraction data were identical with those of a known sample of α-gentiobiose octaacetate; 11.85[19]–1,[20] 10.05–90, 8.67–100, 7.69–1, 7.08–10, 6.73–1, 5.51–1, 5.44–5, 5.12–25, 4.93–25, 4.73–10, 4.43–20, 4.32–5, 3.92–25.

β-Cellobiose Octaacetate.—Fraction II material from the carbon column of a second run yielded 15 g. of acetylated substance. This sirup was dissolved in benzene and chromatographed on three columns (250 × 75 mm., diam.) of Magnesol–Celite (5:1 by wt.) using 4000 ml. of benzene-*t*-butyl alcohol as developer. The second zone from the bottom, 120–145 mm. from the top of the column, yielded 250 mg. of material, m.p. 160–165°. This material was thrice crystallized from ethanol; yield 50 mg., m.p. 197–199° cor. unchanged on admixture with a known sample of β-cellobiose octaacetate, $[\alpha]^{25}D$ −15.7° (*c* 4.0, chloroform).[26] X-Ray powder diffraction data, identical with those of a known sample of β-cellobiose octaacetate, were: 15.75[19]–15,[20] 12.23–50, 9.43–100, 8.02–2, 7.36–5, 5.40–50, 5.11–10, 4.34–40, 4.65–15, 4.42–10, 4.21–25, 4.12–25, 3.91–20.

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(26) C. S. Hudson and J. M. Johnson, THIS JOURNAL, **37**, 1276 (1915).

[CONTRIBUTION FROM THE RESEARCH AND DEVELOPMENT BRANCH, FITZSIMONS ARMY HOSPITAL]

Chromatographic Isolation of Polysaccharides from *Mycobacterium tuberculosis*

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A method for the isolation of polysaccharides is presented, making use of chromatographic adsorption. By using this procedure, 12 different polysaccharide fractions were isolated from the autolysate from a human strain of *Mycobacterium tuberculosis*. Additional polysaccharides, not present in the autolysate, were extracted from the tubercle bacilli, using an electrolytic current. These were isolated into nine different polysaccharide fractions by chromatography. Of the 21 distinct polysaccharide fractions obtained, 19 have the ability to bind antibodies *in vitro*.

Introduction

The isolation of pure and chemically unaltered polysaccharides from *Mycobacterium tuberculosis* is an important problem in immunology and serology. The methods used by previous workers were fractional precipitation methods.[1,2] However, these methods have yielded fractions containing varying small amounts of nitrogenous materials. In most cases these are believed to be protein and nucleic acid impurities that are adsorbed to the polysaccharides. The chromatographic separation excludes coprecipitation, overlapping solubility and incomplete recovery, all common to fractional precipitation methods, and is most likely to separate adsorbed impurities from the polysaccharides. Also, none of the eluants used in this paper are likely to chemically alter the polysaccharides.

In an effort to obtain polysaccharides from the tubercle bacillus, not present in the autolysate, a method for electrolytic extraction of the bacilli was devised. This method was chosen because it avoided the use of heat or chemicals that might alter the structure of the polysaccharides within the cells.

(1) F. B. Seibert, *Bibliotheca Tuberculosea, Separatum Fasc.*, **3**, (1950).

(2) M. Stacey and P. W. Kent, "Advances in Carbohydrate Chemistry," **3**, 311 (1948).

Experimental

Preparation of Autolysate.—A strain of *M. tuberculosis*, isolated in this Laboratory from a tuberculous patient, was grown on Long medium[3] for ten weeks at 37.5°. The bacilli were then killed by adding 90% phenol to form a 2% phenol suspension. The suspension was incubated for two additional weeks. The cells were removed by centrifugation and stored at 4°.

The supernatant was Seitz-filtered and concentrated from 900 to 150 ml. by vacuum distillation in the cold. It was electrodialyzed from a cellophane bag against continuously changing distilled water until no current was observed at 120 v. d.c. During the electrodialysis the *p*H dropped from 7.0 to 5.0, and a sizable precipitate accumulated, consisting mainly of proteins and nucleic acids. The supernatant contained a mixture of polysaccharides which in this paper is called the polysaccharide autolysate. This autolysate was concentrated to 5 ml. volume in preparation for chromatography. Sterile techniques were used.

Electrolytic Extraction of Cells.—The cells were washed thoroughly with distilled water and dried for two days by vacuum desiccation; the dry weight was 14.2 g. An aqueous suspension was then prepared by adding 135 ml. of distilled water. This was placed in a stoppered chamber containing two carbon electrodes and a sterile cotton escape vent for evolved gases; the apparatus was thermostated at 0°. A potential of 120 v. d.c. (higher potentials presently are under investigation) was applied for eight hours; the current rose to 350 ma. The cells were centrifuged from the supernatant and resuspended in 135 ml. of distilled water. The electrolysis was repeated four additional times, until the current dropped to 20 ma. The supernatants were combined, filtered, concentrated from 775 ml. to 150 ml. and electrodialyzed. Again a precipitate, mainly proteins and

(3) E. R. Long and F. B. Seibert, *Am. Rev. Tuberc.*, **13**, 393 (1926).

TABLE I

Fraction no.	Amount, mg.	Extinction coefficient (g./liter-cm.) 260 mμ	280 mμ	Optical rot.	Inhibition react. Hemagg.	Hemolyt.	Hemagg. activity	Hemolytic activity
A1	Trace	....	....	....	....	....	..	..
2	6.5	4.49 ± 0.04	3.23 ± 0.03	Levo	Neg.	Neg.	Neg.	Neg.
3	6.1	1.02 ± .01	0.760 ± .007	Dextro	Part.	Part.	Neg.	Neg.
4	26.0	0.633 ± .002	.490 ± .002	Dextro	Comp.	Comp.	Neg.	Neg.
5	65.3	.575 ± .005	.453 ± .004	Dextro	Comp.	Comp.	Neg.	Neg.
6	21.4	.534 ± .002	.382 ± .004	Dextro	Comp.	Comp.	+	+
7	23.7	.629 ± .002	.437 ± .002	Dextro	Part.	Comp.	2+	2+
8	6.7	.880 ± .007	.605 ± .005	Dextro	Part.	Part.	Neg.	Neg.
9	4.3	.654 ± .008	.453 ± .006	Dextro	Part.	Part.	+	Neg.
10	2.5	1.21 ± .03	.800 ± .016	Dextro	Part.	Part.	Neg.	Neg.
11	4.6	1.20 ± .02	.767 ± .009	None	Comp.	Part.	Neg.	Neg.
12	3.6	1.26 ± .02	.930 ± .013	None	Part.	Part.	Neg.	Neg.
C1	2.4	0.219 ± .005	.159 ± .003	None	Comp.	Comp.	4+	4+
2	0.9	0.351 ± .020	.214 ± .012	Dextro	Comp.	Comp.	3+	4+
3	3.2	2.46 ± .04	1.65 ± .03	Levo	Comp.	Comp.	4+	4+
4	5.0	0.914 ± .009	0.620 ± .006	Levo	Comp.	Comp.	3+	4+
5	3.3	.775 ± .012	.537 ± .008	Levo	Comp.	Comp.	4+	4+
6	7.0	.235 ± .002	.174 ± .001	None	Comp.	Comp.	4+	4+
7	3.1	.880 ± .014	.631 ± .020	Levo	Comp.	Comp.	3+	4+
8	6.0	.178 ± .002	.132 ± .001	None	Comp.	Comp.	4+	4+
9	6.7	.591 ± .004	.443 ± .003	None	Comp.	Comp.	3+	4+

nucleic acids, was formed during electrodialysis. The supernatant, chiefly a mixture of polysaccharides, was separated from the precipitate and concentrated to 5 ml. volume in preparation for chromatography.

Chromatography. Materials.—Only solvents transparent in the ultraviolet range could be used as eluants. A.C.S. methyl alcohol and distilled water were satisfactory; U.S.P. chloroform was satisfactory above 250 mμ. These solvents were redistilled prior to use. Crude hexane was purified by passage through a column of silica gel (28–200 mesh, Fisher) four feet high and 50 mm. in diameter. About two liters could be made ultraviolet transparent in this manner before the column was recharged with fresh silica gel.

Chromatography. Procedure.—The 5-ml. sample of polysaccharide autolysate previously prepared was placed upon a silica gel column 20 mm. in diameter and 20 cm. high. The column had been prepared by adding silica gel to a chromatographic tube half-filled with hexane. The column was water jacketed at 4°. Pressure was maintained at the top of the column to ensure a reasonable flow rate.

Additional hexane was added and the filtrate examined in 10-ml. increments for ultraviolet absorption at 260 mμ using a Beckman DU spectrophotometer. The following sequence of increasingly polar solvents was used to elute the column: hexane, chloroform, methyl alcohol and water. The bands reached an absorption maximum and then fell to complete transmission, at which point the eluant was changed to the next higher one in the polarity scale. The 10-cc. portions comprising a single zone were combined to form four fractions. Of these four, the last two were rechromatographed. The methyl alcohol fraction was placed upon a silica gel column prepared from chloroform and eluted with systematically increasing mixtures of chloroform and methyl alcohol in 5% increments. The water fraction was placed upon a silica gel column prepared from methyl alcohol and eluted with systematically increasing mixtures of methyl alcohol and water, again in 5% increments. All the 10-ml. portions comprising a single zone were combined, lyophilized to dryness, and weighed. Aqueous solutions of known concentration of all fractions were prepared. This same procedure was used to separate the polysaccharides from the bacterial cells.

Chemical and Serological Measurements.—Ultraviolet spectra from 230 to 300 mμ were taken on all fractions. The extinction coefficients at several different wave lengths were calculated.

The optical rotations of all fractions were obtained. Due to the microquantities available, only semi-quantitative specific rotations could be calculated, and only levo, dextro or no rotation, is reported. Anthrone and biuret tests were performed on all fractions.

Four serological procedures, the hemolytic[4] and hemagglutination[5] reactions and the hemolytic and hemagglutination inhibition reactions,[6] were performed on each fraction, using a pooled serum from seven patients with active tuberculosis.

Results and Discussion

The results are summarized in Table I. The polysaccharide fractions obtained from the autolysate are numbered A_1 through A_{12}, in the order of elution from the column; those obtained from the electrolysis of the bacterial cells are numbered C_1 through C_9.

All of the 21 fractions gave typical polysaccharide absorption curves in the ultraviolet.[7] These curves showed no maximum absorption and were characteristic in indicating a diminishing absorption above 230 mμ. There was no change in inflection at 260 mμ or at 280 mμ. This indicated the complete absence of nucleic acid[8] and protein,[7] the chief contaminants found by previous workers.

The anthrone tests were positive in all cases except $A_{1,2}$ and $C_{2,3}$. This could be attributed to the inability of these four to form a furfural structure as an intermediate in the anthrone reaction.[9]

The absorption coefficients at several different wave lengths were chosen as the principal physicochemical means of differentiating and identifying the various polysaccharides. This method is extremely sensitive and gave accurate values with the micro-quantities available. All successive fractions coming off the column exhibited different coefficients except $A_{10,11}$. However, A_{10} was dextrorotatory and A_{11} gave no appreciable rotation.

The sum of the partial contributions of the individual polysaccharide fractions is equal to the total absorption coefficient of the unseparated

(4) G. Middlebrook, *J. Clinical Invest.*, **29**, 1480 (1950).

(5) G. Middlebrook and Dubos, *J. Exptl. Med.*, **88**, 521 (1948).

(6) K. Landsteiner, "The Specificity of Serological Reactions," Harvard University Press, Cambridge, Mass., 1947.

(7) F. Ellinger, *Tabulae Biological*, **12**, 291 (1937).

(8) D. Rapport, *Science*, **112**, 469 (1950).

(9) L. Sattler and F. W. Zerban, THIS JOURNAL, **72**, 3814 (1950).

mixture: $\epsilon = \sum_i \frac{g_i \epsilon_i}{G}$.[10] This relationship holds if the recovery is quantitative and if the molecules are unchanged during the chromatographic separation.

In the case of the autolysate the agreement was remarkably good. The calculated summation of $\sum_i \frac{g_i \epsilon_i}{G}$ from the experimental data at 260 mμ is 0.803 while the absorption coefficient of the unseparated polysaccharides is 0.810. At 280 mμ, the values are 0.585 and 0.592, respectively. The electrodialysis procedure separated quantitatively the autolysate polysaccharides from the other substances, whereas some contaminants were present in the case of the bacterial cells. These contaminants were irreversibly adsorbed upon the column and were not recovered.

(10) A. Weissberger, "Physical Methods of Organic Chemistry," Vol. I, Interscience Publishers, Inc., New York, N. Y., 1949, p. 1298.

The unseparated autolysate and most polysaccharides from the autolysate were dextrorotatory. The unseparated cell material and most of the cell polysaccharides were levorotatory.

The *in vitro* serologic reactions show that 19 of the 21 polysaccharide fractions have the ability to bind antibodies. The antibody-binding abilities of the cell polysaccharides, C_1 through C_9, are uniformly more active than those from the autolysate, as indicated by the hemolytic and hemagglutination reactions.

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[CONTRIBUTION FROM THE DEPARTMENTS OF BIOCHEMISTRY AND MEDICINE, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY, AND THE PRESBYTERIAN HOSPITAL]

Chemical Modifications of the Specific Polysaccharide of Type III Pneumococcus[1a,b] and Their Immunological Effects

BY HAROLD MARKOWITZ AND MICHAEL HEIDELBERGER

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Complete acetylation of a degraded preparation of the polysaccharide (S III) eliminated the capacity of the product to precipitate specific antiserum. Progressive deacetylation of the acetyl derivative gave progressively increasing reactivity toward antiserum. The fully deacetylated material reacted as well toward antiserum as the starting product. Undegraded S III could not be completely acetylated. After deacetylation it precipitated only one-half as much antibody as the starting material and hence was degraded by the chemical manipulations. Treatment of S III with diazomethane led to methylation of hydroxyl groups in addition to the usual esterification. After saponification to the acid, the derivative, in which two hydroxyl groups were methylated per cellobiuronic acid repeating unit, precipitated 50% as much antibody with rabbit antiserum as did the native polysaccharide. Conversion of the dimethoxy ester to the amide gave a product which did not react with antiserum. Esterification of S III to the extent of 35% and conversion of the partial ester to the amide gave products which precipitated 86% and 90% as much antibody, respectively, as the native polysaccharide. The results are discussed with reference to modern immunochemical theory.

I. Introduction

Studies on the relation of the structure of natural antigens to the specificity of antigen–antibody reactions[2] have clarified only a few of many problems demanding solution. Investigations with protein antigens, especially, have been difficult to interpret owing to the lability and the as yet unknown fine structure of proteins.

These difficulties are not as great in the case of the carbohydrates. The structure of the specific polysaccharide of the Type III pneumococcus (S III)[3] for example, is known. S III is a polycellobiuronic acid with 1,3-linkages, probably β-, between cellobiuronic acid units (Fig. 1). It has three varieties of functional groups: primary alcohol, secondary alcohol and carboxylic acid groups. Modifications were achieved by acetylation and methylation of the hydroxyl groups and by preparation of methyl esters and amides from the carboxyl groups. The immunological reactivities of the products were quantitatively studied.

(1) (a) A preliminary report was presented at the 122nd Meeting of the American Chemical Society in Atlantic City, N. J., September 14–19, 1952. This work was sponsored by the Commission on Acute Respiratory Diseases of the Armed Forces Epidemiological Board, and supported in part by the Office of the Surgeon General, Department of the Army, and in part by the Harkness Research Fund of the Presbyterian Hospital. (b) Submitted by Harold Markowitz in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

(2) Summarized in K. Landsteiner, "The Specificity of Serological Reactions," Harvard University Press, Cambridge, Mass., 1946.

(3) M. Heidelberger and W. F. Goebel, *J. Biol. Chem.*, **70**, 613 (1926); **74**, 613 (1927); R. D. Hotchkiss and W. F. Goebel, *ibid.*, **121**, 195 (1937); R. E. Reeves and W. F. Goebel, *ibid.*, **139**, 511 (1941).

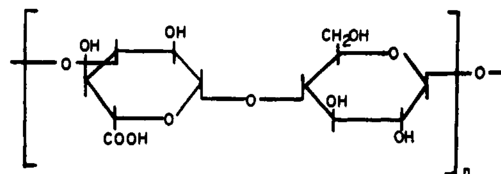

Fig. 1.—The specific polysaccharide of type III pneumococcus.

II. Experimental

Materials and Methods.—S III, lot 186[4] was purified by previously described methods.[5] Fraction 186B, obtained by precipitation with sodium sulfate, contained 5.5% ash as Na and 0.01% N. Preparation 300[4] was similarly purified and contained 5.1% ash as Na and 0.2% N. Sample 104 had undergone degradation, presumably as a result of storage as the free acid for 18 years.

The insoluble S III acid was converted to the soluble sodium salt for serological testing by stirring a suspension with Dowex 50 in the Na form for 4 hours. The mixture was centrifuged and the supernatant, after adjusting to 3% sodium acetate concentration, treated with one volume of cold

(4) Obtained through the courtesy of E. R. Squibb and Sons, New Brunswick, N. J.

(5) (a) M. Heidelberger, C. M. MacLeod, H. Markowitz and A. S. Roe, *J. Exper. Med.*, **91**, 341 (1950); (b) M. Heidelberger, C. M. MacLeod, H. Markowitz and M. M. DiLapi, *ibid.*, **94**, 359 (1951).