Cell Polysaccharides of the Bacillus Calmette-Guérin (BCG)

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Many studies have been made on the cell polysaccharides of *Mycobacterium tuberculosis*, but they have concerned mainly the polysaccharides from the human strain (*1–6*). Immunologically, the polysaccharides of *M. tuberculosis* are known to be haptene; that is, they give precipitation with the antiserum. It is known that there is a relation between the polysaccharides of *Pneumococcus* and its types (*7*), and the structure of some of them has been determined (*8*, *9*), while reports concerning the polysaccharides of *M. tuberculosis* vary even with respect to their component sugars as shown in Table I, and only a few reports deal with the structure of these substances (*3*, *4*).

Table I. Sugars found in the Polysaccharides from *M. tuberculosis*.

Strain	Reference	Glucose	Galactose	Mannose	Rhamnose	Arabinose	Hexosamine	Uronic acid
Human	1		+	+		+		
	2	+*		+		+		
	3			+	+	+	+	
	4		+	+		+	+	
	5	+*						
	6	+		+		+	+	
BCG	10		−	+(45.6%)		+(7.7%)	+(2.9%)	
	11		−	−		+(pentose)		+

* glycogen

In order to determine the interrelationship between a type or strain of *M. tuberculosis* and its cell polysaccharides, and to study the chemical aspects of the immune reaction, the authors began this investigation with the polysaccharides of *Bacillus Calmette-Guérin* (BCG).

Experimentals and Results

*Starting Material**—BCG grown for nine days on Sauton medium

* Kindly supplied by Dr. I. Hara.

was killed with acetone and partly defatted with acetone, methanol, and a methanol-chloroform mixture.

Defatting—The cells were further extracted with an ether-ethanol mixture, and with chloroform according to the method of Anderson-Lederer (*12*).

Isolation of Polysaccharides—The defatted cells (100 g.) were first extracted overnight with water at 37°C, and this extraction was repeated until the extract showed no more yellow color (Extract I). The cells were then heated with water at 100°C for 2 hours, but little extract was obtained. The residue was ground with an equal amount of glass powder in a mortar for 30 minutes and then heated with water at 100°C for 2 hours (Extract II). Finally the residue was extracted with *N* NaOH at 37°C for a week (Extract III).

Extracts I and II were concentrated *in vaccuo*, dialyzed against water and lyophilized (Crude Polysaccharides I and II). The alkaline Extract III was acidified (pH 3.0) with *N* HCl, the precipitates formed were centrifuged off, and after neutralization the supernatant was dialyzed against water, and Crude Polysaccharide III was precipitated with 3 volumes of methanol.

Each crude polysaccharide fraction was dissolved in water and adjusted to pH 3.0 and the precipitates were centrifuged off. After neutralization, the supernatant was deproteinized by the Sevag method (*13*). The aqueous layer was dialyzed and then lyophilized. Thus Polysaccharides I, II and III were obtained.

Each polysaccharide fraction was subjected to paper electrophoresis in borate buffer of pH 9.2. After electrophoresis for five hours at a potential gradient of 15 volt/cm using Toyo filter paper No. 50, the locations of polysaccharides were detected by staining with fuchsin solution after periodate oxidation, according to the method of Köiw *et al.* (*14*). As shown in Fig. 1, Polysaccharides I and II consisted of one electrophoretic component each, while Polysaccharide III consisted of three components. Polysaccharide III was then fractionated with methanol into three fractions; IIIa (methanol concn. 50%), IIIb (75%) and IIIc (80%).

The fractions thus obtained were examined by paper electrophoresis under the same conditions as described above. As shown in Fig. 1, IIIa and IIIc consisted of one component each, but IIIb still contained two components. However, the yield of IIIb was so small that it was difficult to fractionate and no further fractionation was attempted. In these polysaccharide fractions, the following chemical and serological properties were studied.

Yields and Qualitative Tests of Polysaccharide Fractions—Results are summarized in Table II.

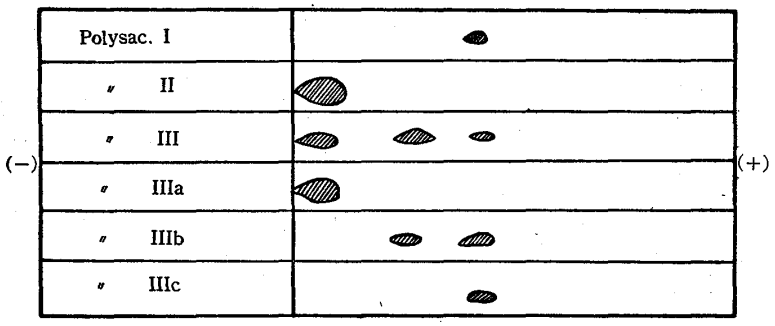

Fig. 1. Paper electrophoresis of polysaccharide fractions in borate buffer pH 9.2 at 15 volt/cm for 5 hours.

Table II. Yields and Qualitative Tests of Polysaccharide Fractions.

Polysaccharide	Yield* %	Biuret	Cu-Folin	Iodine	Naphtho-resorcinol
I	0.17	−	−	reddish brown	−
II	0.73	−	+	−	−
III	1.45	−	−	reddish brown	−
III a	0.41	−	−	reddish brown	−
III b	0.22	−	−	−	−
III c	0.16	−	−	−	−

* Percentage of defatted cell weight.

Chemical Analyses of Polysaccharide Fractions—Optical rotation was measured in aqueous solution. The nitrogen and phosphorous contents were determined by the micro-Kjeldahl and the modified Allen method (*15*), respectively. The amount of reducing sugar was estimated by the method of Hagedorn and Jensen (*16*). Results are shown in Table III. Polysaccharides I, II, III, and the defatted cells were hydrolyzed with N H_2SO_4 at 100°C and the time course of change in reduction value (calculated as glucose) was determined. As shown in Fig. 2, the maximum reduction was reached in five hours.

Table III. Chemical Analyses of Polysaccharide Fractions.

Material	$[\alpha]_D$	N %	P %	Reducing Sugar %*
Defatted cells	—	6.95	0.73	21.0
Polysac. I	+77.8°	1.01	0.45	74.3
II	+63.7°	2.65	0.41	70.7
III	—	1.68	0.54	72.8
III a	+182.0°	0.29	0.24	96.9
III b	+81.5°	2.37	0.74	71.6
III c	− 6.1°	2.79	0.68	66.4

* After five hours' hydrolysis.

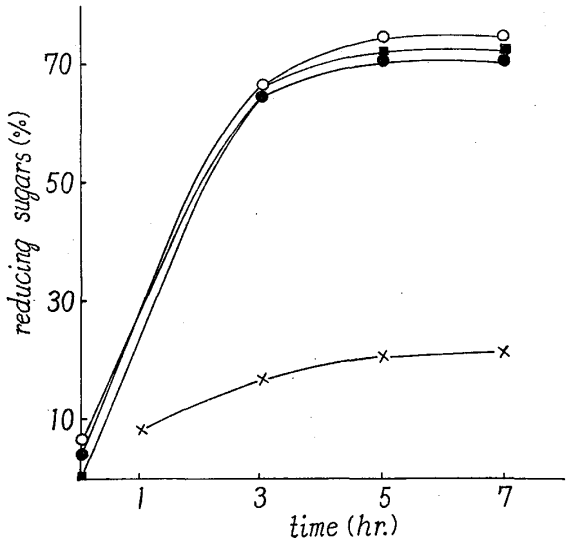

Fig. 2. Change in reduction values with time of hydrolysis.
×——× Defatted cells
○——○ Polysaccharide I
●——● 〃 II
■——■ 〃 III

Polysaccharide IIIa has high dextrorotation, gives a reddish brown color with iodine solution and consists mainly of glucose, as shown in Table V below. This is considered to be the so-called "glycogen" fraction (*5*).

Precipitin' Reaction—Precipitin reaction was carried out using anti-BCG rabbit serum in order to examine the serological activity of each fraction. Polysaccharides IIIa, IIIb and IIIc were also tested with anti-human strain (Aoyama B) rabbit serum (Table IV).

Table IV. Precipitin Titres with the Polysaccharide Fractions.

Polysaccharide		anti-BCG rabbit serum	anti-human strain rabbit serum
Crude Polysac.	I	64,000	—
	II	128,000	—
Polysaccharide	I	128,000	—
	II	256,000	—
	III a	64,000	4,000
	III b	1,024,000	512,000
	III c	512,000	256,000

From the results shown in Table IV, it is evident that Polysaccharides I and II are more active than Crude Polysaccharides I and II, and parallel results were obtained when anti-human strain rabbit serum was used instead of anti-BCG rabbit serum.

Component Sugars—a) Qualitative analysis—Polysaccharides were hydrolyzed by *N* H_2SO_4 at 100°C for five hours, neutralized with Am-

berlite IR-4B (OH form) and evaporated to dryness. The residue was taken up in a few drops of water and the reducing sugars were detected by spraying with aniline hydrogen phthalate after multiple ascending paper chromatography with acetone/*n*-buthanol/water (7 : 5 : 2) and paper electrophoresis in borate buffer of pH 9.2. Hexosamine was detected by the Elson-Morgan reaction (*17*). The sugars found in each fraction are given in Table V.

Glucose and galactose have not hitherto been found in BCG polysaccharides. Uronic acid was reported by Bourdillon (*11*), but we were unable to confirm this. There has been no report which refers to the nature of the hexosamine in BCG polysaccharides, and the hexosamine found here seems to be not galactosamine but glucosamine, considering the fact that the hydrolyzate of the polysaccharides shows an absorption maximum at 510 mμ with the Elson-Morgan reaction (*17*, *18*) and that the spot corresponding to lyxose could not be detected after ninhydrin-oxidation on filter paper (*19*).

Table V. Component Sugars of Polysaccharide Fractions.

Polysac.		Glucose	Galactose	Mannose	Arabinose	Hexosamine
I	A	+	−	+	+	+
II	A	+	−	+	+	+
	B	58.5	0	18.5	21.3	1.7
IIIa	A	+	−	−	−	+
	B	99.2	0	0	0	0.8
IIIb	A	+	(+)	+	+	+
	B	4.8	1.3	58.8	28.8	6.3
IIIc	A	(+)	+	+	+	+
	B	5.5	26.5	16.9	49.9	1.2

A: Qualitative analysis.
B: Quantitative analysis (in *per cent*).

b) Quantitative analysis—Estimation of the component sugars of the polysaccharide fractions was performed by the method of Khym and Zill (*20*), using ion exchange chromatography, which has not hitherto been applied to the analysis of bacterial polysaccharides. Each fraction was hydrolyzed with *N* H_2SO_4 and neutralized in the manner described above, concentrated to a small quantity and the solution placed on a Dowex 1 (borate form) column. The column was then eluted with 0.02 *M* sodium borate solution, and a part of every 30 ml. fraction was colorimetrically assayed with anthrone for hexoses (*21*, *22*) and with orcinol for pentoses (*23*). The results are listed in Table V. Hexosamine content was determined by the method of Belcher *et al.* (*17*).

Heidelberger *et al.* (*2*, *24*) have pointed out the relationship between pentose content and serological activity, but from the results shown in Tables IV and V, not only the pentose, but also the hexosamine content

seems to have some connection with such activity. Polysaccharide IIIb, the hexosamine content of which is the highest, is more active than the IIIc fraction, which has the highest arabinose content.

Summary

Defatted BCG cells were extracted by successive treatments with warm water, hot water and alkali solution, and the polysaccharide fractions I, II and III were obtained. Paper electrophoresis showed that Polysaccharides I and II each consisted of one component, while III contained three components. An attempt was made to fractionate this with methanol. The chemical and serological properties of these polysaccharide fractions were studied.

In addition to mannose, arabinose and glucosamine, glucose and galactose were identified in the polysaccharides on hydrolysis. The neutral sugars were determined by using ion exchange chromatography. The polysaccharide fraction having a high serological activity showed a high hexosamine content.

Acknowledgements

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