CHARACTERIZATION OF INTRACELLULAR, GLUCOSIDIC POLYSACCHARIDE PRODUCED BY *BRUCELLA SUI*S

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It has been demonstrated by Gary *et al.* (1957) that polysaccharide production by *Brucella suis* can be influenced by specific alterations of the nutritional environment. Their results indicated that the cellular polysaccharide content could be varied significantly by changing the concentrations of casein hydrolyzate or amino acid supplements in the medium, relatively high concentrations reducing the synthesis of polysaccharide. A medium was found in which the cells propagated contained approximately 20 per cent (dry weight) cellular polysaccharide. Preliminary studies (Gary *et al.*, 1955) indicated that the polysaccharide was a levorotatory glucose polymer. Subsequent results are described in this presentation.

MATERIALS AND METHODS

Brucella suis strain PSIIK was employed in this investigation. Cells were cultivated, harvested and prepared for the isolation of polysaccharide as follows. Each of a number of Roux bottles, containing 150 ml of agar medium, was inoculated with 2.5 ml of an actively growing, liquid, seed culture (Gary *et al.*, 1957). The agar medium consisted of 0.5 per cent casein partial hydrolyzate, 1 per cent yeast autolysate (yeast-75, Vico Products Co.), 5 per cent glucose (added aseptically prior to solidification of medium), 2 per cent agar and 0.0005 M sodium thioglycolate. After 4 days incubation at 37 C, the cells were washed from the agar surfaces with distilled water, pooled, and killed at 70 C for 15 to 20 min. The cells were collected by centrifugation, washed twice with distilled water and finally suspended in a small volume of distilled water. They were then frozen at -20 C and dried *in vacuo* over CaSO₄ overnight. The dried cells were ground into a fine powder with a mortar and pestle and stored in a glass stoppered weighing bottle. This procedure was repeated until 100 g of dried cells were collected.

Total carbohydrate content of the dried cells or of the isolated polysaccharide was determined

as glucose by the anthrone reaction as previously described (Gary *et al.*, 1957). Reducing sugar content of the polysaccharide was measured according to the procedure of Horvath and Knehr (1941). Total nitrogen was determined by standard Kjeldahl digestion followed by the colorimetric determination of nitrogen by nesslerization (Umbreit *et al.*, 1949). Protein was measured with the Folin phenol reagent of Lowry *et al.* (1951).

Hydrolytic products of the isolated polysaccharide were identified by one-dimensional paper chromatography on Whatman no. 1 filter paper using the ascending technique with water-saturated phenol as the solvent phase. Chromatograms were developed at room temperature (20 to 25 C) and the spots located with the aniline hydrogen phthalate spray of Partridge (1949) and identified by comparison of R_f values with known carbohydrates chromatographed under identical conditions.

The infrared spectral analyses were performed by drying material on silver iodide disks with a heat lamp and measuring the spectra with a Perkin-Elmer, model 21, double-beam recording spectrophotometer with a blank silver iodide disk in the reference beam. Optical rotation was measured with a Rudolph, model 80, polarimeter in a 20 cm capillary tube at 25 C.

Moisture content was determined gravimetrically after drying samples to constant weight at 100 C for 2 hr. Ash was measured by combustion of samples in platinum crucibles at 565 C in the presence of dilute H₂SO₄ (2:1) according to the procedure described by Bates (1942). Periodate oxidation of polysaccharide was followed as described by Hehre (1951). Sodium *m*-periodate was prepared from sodium *p*-periodate according to the procedure of Hill (1928).

Respiratory activity (oxygen utilization) was measured at 37 C employing conventional manometric technique (Umbreit *et al.*, 1949).

All pH values were measured with a Beckman pH meter using standard glass electrodes.

RESULTS

Isolation of polysaccharide. Dried cells (95 g) were placed in a large sintered glass (coarse (C) porosity) funnel which was attached to a suction flask. Four L of a petroleum ether-ethanol (1:1) solution were allowed to percolate through the cells under a slight vacuum. The cells were then washed with excess amounts of ethyl ether, dried by vacuum aspiration and placed in an 80 C oven for 3 hr.

Ninety-four grams of the lipid-extracted cells which contained 14.4 per cent cellular carbohydrate were digested with stirring in 2 L of *N* NaOH at 35 C for 1.5 hr. This mixture was care-

fully neutralized with 12 *N* HCl and allowed to stand at 5 C overnight. Protein was precipitated from the cell digest by trichloroacetic acid added to a final concentration of 5 per cent, preliminary experiments having shown that the polysaccharide, if bound to other cellular components, was readily released in this way. The protein was centrifuged off for 40 min at 1000 × *G* and finally for 20 min at 8000 × *G*. The residue was washed with cold trichloroacetic acid and the washings combined with the original supernatant. The yellow, opalescent liquid was then dialyzed against cold, running tap water for 18 hr until the pH increased to 6.6. The

Figure 1. Fractionation and purification of crude polysaccharide isolated from *Brucella suis*. All residues were collected (unless stated otherwise) by centrifuging 20 min at 3000 × *G*.

TABLE 1
Chemical properties of polysaccharide fractions isolated from Brucella suis

Fraction	Isolation	Carbohydrate		Protein	Total Nitrogen	Ultra-violet Absorption*	pH (0.5% conc.)	Moisture	Ash	Total†
		Total	Reducing							
	Precipitated by:	%	%	%	%	OD		%	%	%
I	2 vol acetone‡	76.4	0.47	21.2	3.66	0.432	8.7	3.7	3.2	104.5
II	1 vol acetone‡	91.7	0.24	8.0	1.65	0.260	7.9	4.7	1.8	106.2
III	Fraction II treated with Amberlite IR-120	101.0	0.11	4.2	0.81	0.096	6.7	4.4		109.6

* Optical density of absorption spectrum measured at 260 m μ wave length with Beckman DU spectrophotometer, 1.5 mg per 3 ml in 1 cm silica curvette.

† Sum of values for total carbohydrate, protein, moisture and ash.

‡ With 2 volumes ethanol (see figure 1).

dialyzed solution (approximately 3 L) was concentrated to 1 L by pervaporation overnight in a cellophane tube, 2 volumes of 95 per cent ethanol were added and the solution was stored at 5 C overnight. The precipitate was removed after centrifugation for 20 min at 3000 $\times$ G and discarded. The supernatant was treated with excess cold acetone until no further precipitation occurred. The white, flocculent, acetone precipitate was collected after centrifugation for 20 min at 3000 $\times$ G and dried *in vacuo* over CaSO₄. Both the original ethanol precipitate and the supernatant remaining after precipitation with acetone contained negligible amounts of carbohydrate. The material precipitated by acetone weighed 10.7 g and contained 68 per cent carbohydrate.

Purification of polysaccharide. The crude polysaccharide was dissolved in water and the solution centrifuged to remove a small amount of insoluble material. The supernatant was re-treated with cold ethanol and acetone as previously described and the residue finally fractionated and purified as shown in figure 1. This procedure resulted in 3 water soluble fractions. Fraction II, isolated in largest quantity, was highly soluble in water (to 25 per cent w/v), pure white in appearance, and was gummy in nature when wet. This "gummy" characteristic was lost during passage through the IR-120 (Rohm and Haas) resin column (fraction III). Fraction I obtained upon the addition of the second volume of acetone was a white flocculent sediment. Infrared spectral analyses revealed that fractions I and II were qualitatively similar but differed with respect to the concentration of

contaminating substances. This was verified by the chemical analyses described below.

Characterization of polysaccharide. The chemical compositions of fractions I, II, and III are presented in table 1. The decrease in protein, total nitrogen, pH and ultraviolet absorption (maximum at 260 m μ) accompanying purification suggest that nucleoprotein might have been the principal contaminant. Essentially all components of each fraction were accounted for as indicated by the total recovery values of 104.5 to 109.6 per cent.

Fraction III was found to be composed essentially of reducing sugar units, a value of 95.9 per cent being obtained after hydrolysis in 2 N H₂SO₄ at 100 C for 2 hr. Chromatograms of the hydrolyzed product (neutralized with BaCO₃) revealed only a single spot with an R_f value identical with that of glucose, and suggested that the polysaccharide was a polymer composed of glucose units. Moreover, a modified Dische's (Brody, 1953) reaction and the anthrone reaction with the polysaccharide resulted in absorption spectra identical with that of glucose. A comparison of the infrared spectra of dried cells and the purified polysaccharide (fraction III) is presented in figure 2. The absence of strong absorption peaks at 8.7 and 9.75 μ (Levine *et al.*, 1953) is evidence that fraction III is unlike glycogen. Furthermore, all fractions failed to react with iodine.

The specific rotations of fractions II and III, based on the carbohydrate ("glucose") concentration of each fraction, were $[\alpha]_D^{25} = -14.7^\circ$ and -16.9° , respectively. The specific rotation for fraction III after hydrolysis in 1 N H₂SO₄

at 100 C for 2 hr was + 44.1° and increased to + 50.6° after standing at 25 C overnight, which further substantiates glucose as the sole structural unit of the polysaccharide.

Figure 2. Infrared spectra of dried cells and polysaccharide (fraction III) from *Brucella suis*.

The periodate oxidation values of fractions II and III (table 2) closely agree. These values when compared to the values obtained for the known polysaccharides indicate that the glucose units of the polysaccharide fractions isolated from *B. suis* are not linked by 1,6-glucosidic linkages. The dissimilarity of the unknown polysaccharide to starch and glycogen was demonstrated further by the failure of fraction II to serve as a substrate for α - and β -amylases (Nutritional Biochemicals Corp.).

Biological activity. Tests for any biological function of fraction II were negative. It did not support the growth of *B. suis* when sterilized by filtration and added to growth media at a final concentration of 0.5 and 1.0 per cent, nor was it oxidized by resting cell suspensions in the Warburg respirometer. This lack of activity can be attributed to the absence of extracellular glucosidic enzymes or to the selective permeability of the cell membrane. Previous findings (Gary *et al.*, 1957) indicated that the polysaccharide produced by *B. suis* was intracellular and was utilized during periods of prolonged incubation.

To determine whether fraction II might be antigenic, a total of 75 mg per rabbit was injected into the ear veins of two albino rabbits over a period of 5 days (25 mg every other day). Sera obtained from the rabbits before injection and 6 days after the final injection did not contain antibodies when assayed by the ring precipitin test.

TABLE 2
Periodate oxidation of polysaccharides

Polysaccharide*			Periodate Consumed†		
Type	Source	Glucosidic linkage	Theoretical <i>mmoles</i>	Determined	
				24 hr <i>mmoles</i>	48 hr <i>mmoles</i>
Starch, soluble.....	Pfanstiehl	1,4-	1.0	1.15	1.17
Glycogen.....	Nutritional Biochem. Corp.	1,4-	1.0	1.39	1.25
Dextran.....	NRRL,‡ B-512	1,6-	2.0	1.82	1.84
Fraction II.....	<i>Brucella suis</i>			1.06	1.06
Fraction III.....	<i>B. suis</i>			0.93	0.94

* Protocol: 110 mg of polysaccharide (except fraction III: 54 mg); 6 ml of 0.275 M sodium *m*-periodate; CO₂-free distilled H₂O to 250 ml volume. Incubated at 26 C in the dark; 50 ml aliquots analyzed according to the procedure of Hehre (1951).

† Calculated per mmole of glucose present in polysaccharide determined by anthrone reaction.

‡ Northern Regional Research Laboratory.

DISCUSSION

The glucose polymer described in this report may be related to the polysaccharide fractions described by Huston *et al.*, (1934) and Favilli and Biancallani (1934), by virtue of its non-reducing and levorotatory characteristics, its failure to react with iodine and its failure to produce antibodies. However, chemical characterization of *Brucella* polysaccharides in the literature has not been extensive. Several investigators have reported the isolation of immunologically inactive polysaccharide fractions. Huston *et al.* (1934) isolated a nondialyzable carbohydrate from *Brucella suis* and *Brucella abortus* which was inactive in precipitation and complement fixation tests, gave a strong Molisch reaction, failed to react with iodine and was levorotatory before hydrolysis and dextrorotatory after hydrolysis. Favilli and Biancallani (1934) isolated from *B. abortus* a similar non-reducing hexose polymer which gave a strong Molisch reaction and possessed a specific rotation of -7.5° , but exhibited a high specific titer in precipitation tests. Hershey *et al.* (1935), upon reinvestigation of this finding, obtained a similar polysaccharide with the exception that it was immunologically inactive.

It is assumed that the polysaccharide isolated from *B. suis* grown on solid media is similar or identical to the polysaccharide produced by this organism when grown in liquid media of the same composition. All data indicate that the polysaccharide described in this report is unlike dextran, glycogen, and starch, particularly on the basis of the shift in optical rotation (levo- to dextrorotatory). This change in rotation during hydrolysis suggests that a β -type linkage is predominant in this polysaccharide. The absence of infrared absorption at 11.9μ (840 cm^{-1}) and the presence of a moderate absorption peak at 11.1 to 11.2μ (900 to 890 cm^{-1}) also indicates (Lawson and Stacey, 1954) the β -configuration for the glucose units. The relative resistance (rate) of the polysaccharide to acid hydrolysis suggests that the glucose units are present as the pyranoside ring structure (Quinnell *et al.*, 1957).

The periodate oxidation values for the polysaccharide fractions agree well with the 1,4- but not with the 1,6-type polysaccharides assayed by Hehre (1951). The results do not negate the existence of a 1,2-glucosidic linkage,

which would result in the utilization of 1 mole of periodate per mole of glucose. A levorotatory bacterial polysaccharide containing 1,2-linkages has been described (McIntire *et al.*, 1942 and Putman *et al.*, 1950).

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SUMMARY

The fractionation of *Brucella suis* cells harvested from a casein hydrolyzate, yeast autolysate, glucose, agar medium by alkaline digestion followed by treatment with ethanol, acetone and an ion exchange resin (Amberlite IR-120, Rohm and Haas), resulted in a neutral polysaccharide which contained glucose as the sole structural unit. The optical activity ($[\alpha]_D^{25} = -16.9^\circ \rightarrow +50.6^\circ$, after hydrolysis), infrared spectrum, periodate oxidation and the inability of the polysaccharide to serve as substrate for α - and β -amylases indicated that this product is dissimilar to glycogen, dextran, starch (and other related glucose polymers) and consists primarily of 1,4- or 1,2-glucosidic linkages. The isolated polysaccharide failed to support growth of *B. suis*, was not oxidized by resting cell suspensions of this organism and was not antigenic by the assay conditions employed.

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