

POLYSACCHARIDE OF *COCCIDIOIDES IMMITIS*

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ABSTRACT

PAPPAGIANIS, D. (University of California, Berkeley), E. W. PUTMAN, AND G. S. KOBAYASHI. Polysaccharide of *Coccidioides immitis*. J. Bacteriol. **82**:714-723. 1961.—Soluble polysaccharide from mycelia or culture filtrates of *Coccidioides immitis* was found to consist mainly of mannose, but also included small quantities of galactose and another reducing sugar. Isolation of the polysaccharide by ethanol precipitation provided accompanying nitrogenous material. There was 3 to 4% nitrogen, present in amino acids, but in a nondialyzable (possibly protein or peptide) form. The average molecular weight of the complex was 31,700. Attempts to separate the nitrogenous and polysaccharide materials by chemical methods or by moving boundary electrophoresis were unsuccessful; ultracentrifugation showed a single peak. Despite these findings, there appeared to be several antigenic species present, as indicated by multiple lines of precipitation in double diffusion and quantitative precipitin tests. In the latter, polysaccharide-containing fractions gave precipitates without measurable carbohydrate, suggesting separability of nitrogenous and polysaccharide components.

Culture filtrates (coccidioidins) from the pathogenic fungus *Coccidioides immitis* are known to give precipitin and complement fixation reactions with serum from persons with coccidioidomycosis, and are used to detect dermal hypersensitivity to the fungus. Hirsch and D'Andrea (1927) reported that a serologically active component from *C. immitis* culture filtrates was precipitated with alcohol, and

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assayed 20 to 40% reducing sugars after hydrolysis. An osazone identified as glucosazone was prepared from the hydrolyzate. Hassid, Baker, and McCready (1943) also identified the major component of the alcohol-precipitated fraction of coccidioidin as glucose. Galacturonic acid was identified as a component by Hassid et al., who also encountered another reducing sugar which was not identified. The ratio of the galacturonic acid, glucose, and unknown sugar was approximately 1:6:3, respectively.

Both earlier groups of workers found nitrogen present with the alcohol-precipitated polysaccharide. Hirsch and D'Andrea reported that their material contained 3 to 4% nitrogen, whereas Hassid et al. described three preparations, one with 3.23% total nitrogen, the second with 2.3%, and the third with 1.73%. The third preparation, after acetylation and alkaline deacetylation, retained 1.12% and 0.95% nitrogen, respectively. These data suggested that the nitrogen existed in organic combination with the polysaccharide, especially since previous tests for protein had all given negative results. The possible immunological importance of the nitrogenous components accompanying the polysaccharide of coccidioidin was implied in unpublished studies by Seibert, which indicated that skin-test activity accompanied an electrophoretically mobile "protein."

In attempting to identify the "unknown" sugar reported by Hassid et al., one of us (E. W. P.) hydrolyzed a sample of the polysaccharide and by paper chromatography found that mannose was the major reducing sugar component rather than glucose. At this time there was no indication of the presence of uronic acid. We have extended the study of the composition and immunological properties of coccidioidin polysaccharide and report some of our findings herein.

MATERIALS AND METHODS

Culture filtrates (coccidioidins) were prepared from cultures of *C. immitis* grown in the chemi-

cally defined medium of Smith et al. (1948). Two strains of *C. immitis*, strain Silveira and strain 46, which have been previously described (Pappagianis, Smith, and Kobayashi, 1956), were used to produce the coccidioidins. Strain 46 is the same strain used by Hassid et al. (1943) in their investigations on the polysaccharide of *Coccidioides* and has been maintained in laboratory cultures since that time. For the preparation of coccidioidin, the cultures were grown either in 2.8-liter "low form" culture flasks with 700 ml of medium or in Povitzky bottles with 1 liter of medium. Incubation varied from 8 weeks to 6 months at 34 C. Polysaccharide was also studied in autolysates of young (3 days old) mycelia harvested from shaking culture in 2% glucose-1% yeast extract broth (Pappagianis et al., 1957).

Preparation of polysaccharide. Following removal of the mycelial mats by filtration, the culture filtrates were passed through a Seitz filter, and aqueous Merthiolate was added to a final concentration of 1:10,000. They were then concentrated to about one-tenth their original volume under reduced pressure or by pervaporation in Visking sausage casing. After this reduction in volume, they were dialyzed against running tap water for 48 to 72 hr, and, as necessary, again reduced to one-tenth the original volume. The dialyzed concentrates were acidified to pH 3 with 1 N HCl, which precipitated a dark brown material. The supernatant liquids, which were also brown in color, were adjusted to pH 7 with 0.5 N KOH. Precipitation of alcohol-insoluble material was carried out by addition of 95% ethanol to the neutral solution, either by addition of five volumes of ethanol or by fractional addition of alcohol in increments of 10%. The resultant precipitate was washed with 95% ethanol, then with acetone, and dried at 45 C under reduced pressure.

Aqueous solutions of crude polysaccharides from strains Silveira and 46 were brown at 3.3% concentration. They were passed through a column of the cation exchange resin Dowex 50 (35 ml bed volume). The effluent solution, which was still brown, was then passed over the acid-adsorbing resin Duolite A-3, for the purpose of removing acidic substances, e.g., uronic acids. Following treatment with both resins, the neutral effluent was treated with five volumes of ethanol, and the precipitate removed by centrifugation. The precipitate was washed with ethanol and

acetone and dried as before. The fractions adsorbed on the cation exchange resin were eluted with 2 N HCl, and those on the acid-adsorber were eluted with 2 N NaOH. The composition of the latter appeared to be similar to the alcohol-precipitated material from the neutral effluent and were not extensively studied. Therefore, subsequently prepared crude polysaccharides were not treated with ion exchange resins.

In the fractional alcohol precipitation, slight or no precipitate was obtained until a concentration of 40% ethanol was attained. Precipitates were also produced at 50, 60, 70, and 80% ethanol concentrations.

Analytical methods. Carbohydrate was measured by Fairbairn's (1953) modification of the anthrone method. In some of these determinations, glucose was employed as a standard until mannose was demonstrated to constitute the bulk of reducing sugar present. Reducing sugars were estimated by the Nelson (1944) and Somogyi (1952) colorimetric procedure, using either glucose or mannose as a standard.

The micro-Kjeldahl and Dumas methods were employed for nitrogen determinations. The Folin-Ciocalteu method was used in the estimation of the nitrogenous components accompanying the polysaccharide, with crystalline bovine serum albumin (Armour) as a standard (Lowry et al., 1951).

Acid hydrolysis was carried out by treating 50 mg of alcohol-precipitated material with 1 ml of 1 N HCl in a sealed test tube placed in water at 100 C. Hydrolyzates to be used for paper chromatography were evaporated to dryness under reduced pressure over KOH, redissolved, and dried again three times to remove HCl. Acid-hydrolyzed samples used in other assays were neutralized with 1 N NaOH.

In one instance hydrolysis was effected by exposing a solution of the ethanol-precipitated material to Dowex 50 in the H⁺ form. This proved to be slow and the hydrolyzate has not been further studied.

Paper chromatograms were developed with descending solvent systems using *n*-butanol-acetic acid-water, and water-saturated phenol or benzene-*n*-butanol-pyridine-water (Zeitseva and Afanasyeva, 1957). Reducing sugars were detected by spraying with a saturated solution of *p*-anisidine hydrochloride in *n*-butanol, and

amino acids were shown by spraying with 0.2% ninhydrin in 95% ethanol.

Candida krusei [(*Candida monosa*), (*Torula monosa*), Lodder and van Rij, 1952], and galactose-adapted *Saccharomyces fragilis* were used to ferment monosaccharides released by acid hydrolysis.

Quantitative precipitin reactions were carried out by addition of 0.2 ml of antigen dilution to 0.2 ml of antiserum. The resulting buttons of precipitate were disrupted into a very fine suspension by repeated aspiration and expulsion through a 26 gauge needle, and washed by centrifugation in ice-cold saline. This was repeated three times.

RESULTS

Properties of the 80% alcohol precipitate. Both strains of *C. immitis* yielded finely divided precipitates by the addition of 80% ethanol. These were pale buff in color, readily soluble in

water, gave a positive precipitin reaction with human and monkey antisera, and 10 μ g induced vigorous reactions in coccidioidin-positive human subjects.

In early analyses using glucose as the standard, the anthrone determination showed values of 37.5% carbohydrate for preparation Silveira-1 and 39.5% for preparation 46-1. After chromatographic demonstration of mannose as the principal sugar component, anthrone determinations with a mannose standard gave values of 60 to 70% polysaccharide.

Chromatographic examination of acid hydrolyzates provided evidence that mannose was the main carbohydrate component present (Table 1). There were present also two other faint reducing sugar spots, one representing a slightly slower moving sugar than mannose, and the second with an R_F approximating the position of rhamnose in butanol-acetic acid and near dihydroxyacetone in phenol-water. The latter was not

TABLE 1. Chromatographic excursions of reducing substances released by acid hydrolysis from soluble polysaccharide of *Coccidioides immitis*

Expt	Hydrolyzed sample and known sugars	R_F																																																	
		Phenol-water					Benzene-pyridine or butanol-water																																												
1	T-259* Maltose Glucose Galactose Mannose Rhamnose diOH acetone	0.31	0.39	0.43	0.45	0.62	0.75	0.23	0.26	0.33	0.54																																								
												0.31	0.39	0.43	0.45	0.62	0.75	0.22	0.26	0.32	0.54																														
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																																0.31	0.39	0.43	0.45	0.62	0.75	0.22	0.26	0.32	0.54										
																																										0.31	0.39	0.43	0.45	0.62	0.75	0.22	0.26	0.32	0.54
2	P-257† Maltose Glucose Galactose Mannose Rhamnose diOH acetone	0.31	0.39	0.43	0.44	0.44	0.75	0.21	0.26	0.30	0.52																																								
												0.31	0.39	0.43	0.44	0.44	0.75	0.22	0.26	0.32	0.52																														
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																																										0.31	0.39	0.43	0.44	0.44	0.75	0.22	0.26	0.32	0.52
3	SF-2† Maltose Glucose Galactose Mannose Rhamnose diOH acetone	0.31	0.39	0.43	0.42	0.45	0.76	0.22	0.26	0.33	0.54																																								
												0.31	0.39	0.43	0.42	0.45	0.76	0.22	0.26	0.32	0.54																														
																						0.31	0.39	0.43	0.42	0.45	0.76	0.22	0.26	0.32	0.54																				
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* Nondialyzable fraction from autolysate of *C. immitis*.

† Ethanol-precipitated polysaccharides.

further studied and appeared to be present in very low concentration. After removal of mannose by fermentation with *C. krusei*, chromatographic development indicated that galactose constituted the slower moving reducing sugar. Another acid-hydrolyzed sample was assayed for reducing sugar, then fermented for 2 hr each with *C. krusei* and galactose-adapted *S. fragilis*. *C. krusei* removed 98.5% of the reducing sugar (representing mannose), while exposure to *S. fragilis* further consumed 0.24% of the original reducing sugar. A quantitative paper chromatogram was employed for comparison with the fermentation data. By this latter method, 900 μg of mannose and 74.2 μg of galactose were obtained from a sample containing 1,000 μg of reducing sugar. These values indicated that galactose was a minor component (<10%) and mannose constituted the bulk (90 to 98.5%) of the polysaccharide. The presence of mannose was confirmed by visual identification of the crystals of mannose phenylhydrazone, which resulted from reaction with phenylhydrazine at room temperature.

Supporting evidence that glucose was absent or present in very minute amounts was obtained by exposing a hydrolyzate of the preparation Silveira P-257 to glucose oxidase. The reducing sugar content of the hydrolyzate was virtually unchanged by the oxidase (Table 2), while the control glucose was depleted by 99%. In two preparations there was 1.2 and 1.26% total phosphorus, respectively.

The crude polysaccharide preparations generally contained 3 to 4% nitrogen, as determined by the micro-Kjeldahl technique. In two preparations containing 2.40 and 3.25% nitrogen by the micro-Kjeldahl method, the nitrogen content by the Dumas method was 3.03 and 2.95%, respectively. It is considered unlikely, therefore, that there is nitrogen present in a ring structure which might escape assay by the micro-Kjeldahl procedure.

No positive biuret reaction was obtained with 10 to 20 mg of crude polysaccharide from coccidioidin filtrates or autolysates of mycelium, and the crude material was not precipitated in hot 10% trichloroacetic acid. These results are in agreement with those reported by Hassid et al. (1943). The crude alcohol-precipitated material was also ninhydrin-negative and gave no reaction with bromeresol green or mercuric chloride.

TABLE 2. *Glucose oxidase activity on hydrolyzed polysaccharide of Coccidioides immitis*

Time, min	Micrograms reducing sugar after exposure to glucose oxidase			
	P-257*	Glucose	Mannose	Enzyme control
0	93	98	105	2
10	93	64	105	
20	90	50	105	
30	92	38	105	3
40	92	25	105	
50	91	22	105	
60	92	17	105	3
90	92	9	105	
120	92	5	100	

* Polysaccharide P-257 hydrolyzed in 1 N HCl at 100 C.

methyl orange when spot-tested on paper. When hydrolyzed and chromatographed, however, six ninhydrin-reactive spots were observed, with mobilities corresponding to R_F values of amino acids. In phenol-water and butanol-acetic acid solvent systems, the color obtained with ninhydrin and the R_F values suggested the presence of the following or chromatographically similar amino acids: aspartic acid, serine, glycine, threonine, alanine, tyrosine, and proline. Furthermore, when tested with the Folin-Ciocalteu reagent, the crude material contained from 15 to 20% reactive peptide material. This is in the range one might expect from the micro-Kjeldahl nitrogen values of 3 to 4% if these are considered to represent protein or derivatives of proteins.

Several methods were tried but were ineffective in separating the carbohydrate from the amino acid-containing components of the crude material: Sevag's (1934) chloroform-amyl alcohol method for extraction of proteins from polysaccharide; treatment with crystalline trypsin; treatment with papain; treatment with formamide (Fuller, 1938); and extraction with 90% phenol (Kabat and Mayer, 1948). Treatment with 40% NaOH at room temperature, or hot 30% KOH (Hassid and Abraham, 1957), reduced the nitrogen content to 0.5% or less; such alkali-treated preparations when hydrolyzed with acid were ninhydrin negative. When used as antigens in the precipitin test with positive human and monkey sera, the alkali-treated samples were inactive, and did not evoke a dermal reaction in coccidioidin-positive humans.

TABLE 3. *Composition of fractions precipitated by ethanol from the nondialyzable constituents of coccidioidin*

	Percentage ethanol at which precipitate collected					
	40	50	60	70	80	P-257*
"Protein,"† %	46.1	14.0	21.5	33.1	51.9	18.8
Polysaccharide,‡ %	32.8	75.5	65.0	66.3	51.0	64
Yield, g	0.47	2.1	0.84	0.07	0.21	
Yield, %	12.8	57.0	22.8	1.19	5.7	83

* Polysaccharide precipitated by direct addition of ethanol to 80% concentration.

† "Protein" measured with Folin-Ciocalteu reagent.

‡ Polysaccharide as mannose.

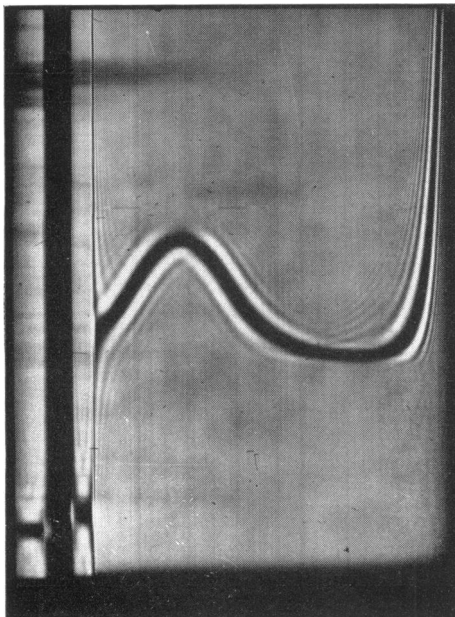


FIG. 1. *Ultracentrifugal pattern of a 1% solution of coccidioidin polysaccharide P-257 in water. Frame shown was taken after 112 min at 59,780 rev/min.*

Efforts have been made to separate the carbohydrate and nitrogenous components by electrophoresis. In borate buffer at pH 9, the more mobile of two components observed appeared to be enriched in polysaccharide. However, the nitrogenous material was not reduced sufficiently to consider this an effective method of separation. Preliminary efforts with paper electrophoresis were discouraging without a useful color indicator, though paper-curtain electrophoresis with collection of fractions may be effective.

The yield of precipitates at alcohol concentrations of 40 to 80% is shown in Table 3. Of a

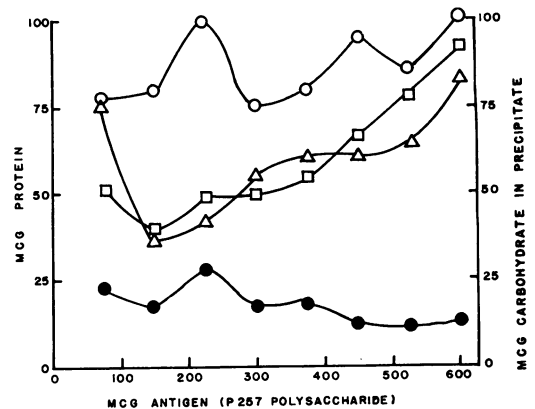


FIG. 2. *Carbohydrate and protein analysis of precipitates formed from ethanol-precipitated fractions of coccidioidin with human serum. P-257 was the crude coccidioidin polysaccharide from which ethanol-precipitated fractions were prepared. ●, carbohydrate in precipitate from 40% ethanol fraction (as mannose); ○, protein in precipitate from 30% ethanol fraction; □, protein in precipitate from 30% ethanol fraction (no carbohydrate in precipitate); △, protein in precipitate from 70% ethanol fraction (no carbohydrate in precipitate); 0.2 ml of human serum per determination.*

total of 3.65 g of precipitate, 56% was precipitated with 50% ethanol, and 93% was precipitated with 40, 50, and 60% ethanol.

In Table 3 are also listed the amounts of Folin-Ciocalteu reactive material (FCR) and polysaccharide as determined in the various precipitates. It is evident that a wide variation occurs in the ratio of polysaccharide to FCR in the precipitates at different alcohol concentrations. The lowest amount of FCR was found in the 50% alcohol precipitate, i.e., 14% FCR; and the highest yield of FCR, 51.9%, in the 80% alcohol fraction. In terms of the aforementioned problem

of attempting to gain a more complete separation of the nitrogenous components from the polysaccharide, none of the selected concentrations of alcohol was sufficiently effective.

The inability thus far to separate carbohydrate and FCR indicated the possibility of a close association between the components, as in mucopolysaccharides. However, the different ratios of polysaccharide to FCR obtained at

different concentrations of ethanol suggested a heterogeneity of the molecular size of the component precipitated. Although no attempt was made to measure the relative molecular weights of these fractions, a molecular weight estimation was carried out on the crude material prepared by precipitation with 80% ethanol, i.e., without fractional addition of alcohol. Using the osmometric method of Bull (1941), an average molecu-

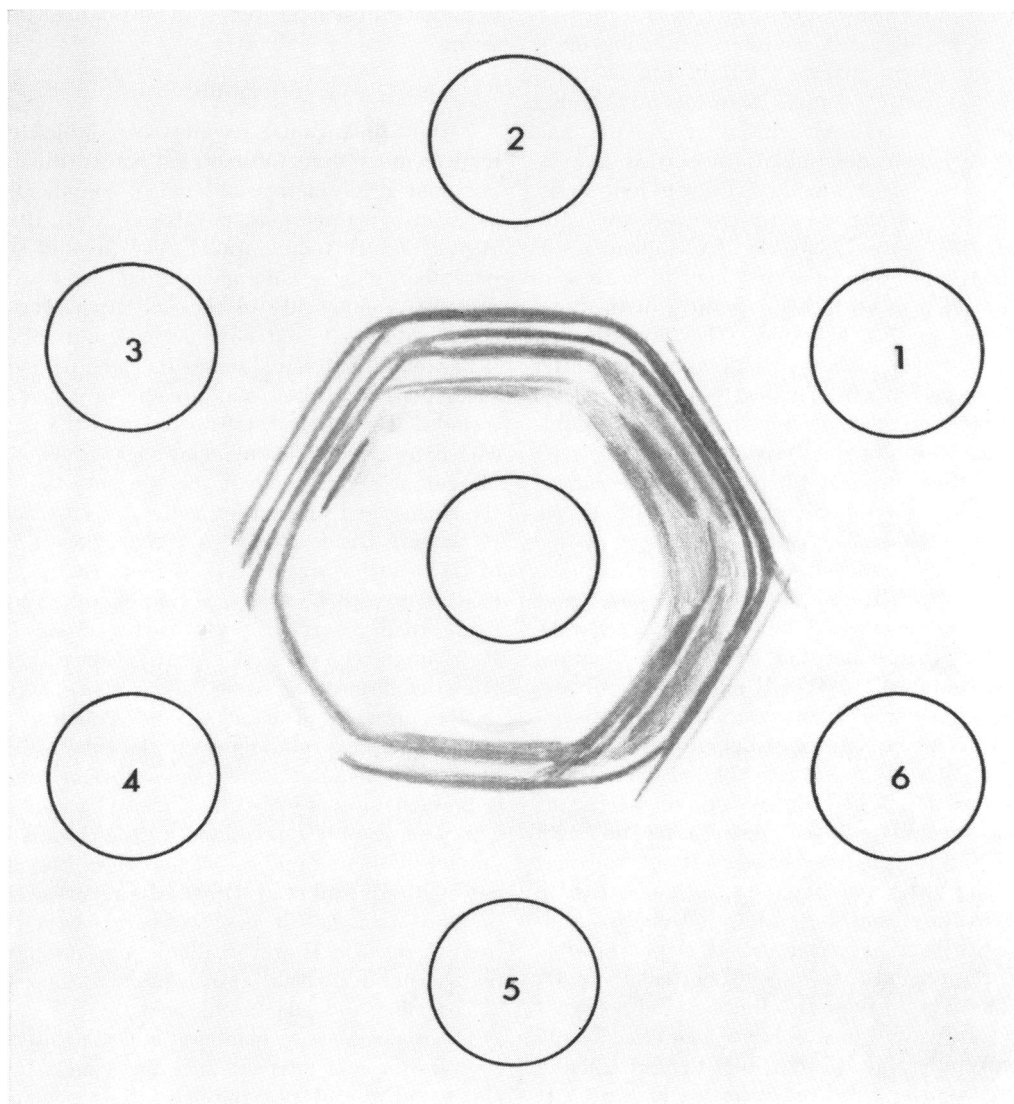


FIG. 3. Agar diffusion precipitation of crude *Coccidioides* antigen with human serum. Each peripheral well contained 500 g of polysaccharide (as mannose). Well no. 1, crude T-16; no. 2, calcium phosphate gel-precipitated T-16; no. 3, crude T-259; no. 4, T-259 treated with Solka Floc; no. 5, crude T-16 supernatant after prolonged storage; no. 6, supernatant from calcium phosphate precipitation of T-16.

lar weight of 31,700 was calculated. Hassid et al. (1943) reported a molecular weight of 6,760 for the acetylated derivative of their polysaccharide.

The ultracentrifugal pattern of the crude material obtained with 80% ethanol is indicated in Fig. 1. As a 1% solution in water, the polysaccharide sedimented as a single component in the ultracentrifuge, with a sedimentation coefficient of approximately 2.2 Svedberg units, a value not incompatible with the osmometric average molecular weight.

Acid hydrolysis. A solution containing 0.8% of crude polysaccharide P-257 in 1 N HCl was heated at 100 C, samples were taken at 30-min intervals, neutralized with 1 N NaOH, and reducing sugar determinations carried out by the Nelson-Somogyi method. Prior to hydrolysis, about 10% of the reducing groups of the polysaccharide were available for reduction of reagent, and during the first hour of heating in acid there was an increase to 80% of the total reducing groups liberated. The liberation of reducing sugars was virtually complete after heating for 180 min. This pattern was paralleled by liberation of reducing sugars from soluble polysaccharide of autolyzed mycelium.

Quantitative precipitin reactions were carried out using the fractions precipitated with 40, 50, and 70% ethanol. These results have been plotted in Fig. 2. The 40% EtOH fraction, which contained 46% FCR and 33% polysaccharide, gave a precipitate composed of protein polysaccharide. On the other hand, the 50% EtOH fraction, which contained 14% FCR and 75.5% polysaccharide, reacted with antiserum to give a precipitate with no measurable carbohydrate. Similarly, the fraction obtained with 70% EtOH, which contained 33.1% FCR and 66.3% polysaccharide, gave a precipitate in the precipitin reaction with no detectable polysaccharide. It is apparent that the latter two fractions, although rich in carbohydrate, contributed only nitrogenous constituents to the precipitate. It is not possible from the present data to conclude that the polysaccharide from these two fractions did not react with antibody, since soluble complexes could have resulted; but it is true that the formation of precipitate succeeded in separating at least part of the nitrogenous components from the polysaccharide. Some of these nitrogenous components can therefore be considered as not bound to carbohydrate in a mucopolysaccharide type

complex. Using lyophilized, pooled, precipitin-positive human serum or the globulin fraction therefrom, 10 to 32% of the polysaccharide added in the antigen could be found in the antigen-antibody precipitate. But the addition of more antigen usually resulted in further precipitation, and appears only to amplify the concept of heterogeneity of antigen components. This is pictorially supported in Fig. 3, in which crude polysaccharide preparations show multiple lines in the gel-diffusion reaction with human precipitin serum.

DISCUSSION

Certain findings are recorded here which extend or differ from the reported composition of immunologically active material of coccidioidin, as given by earlier workers (Hassid et al., 1943; Hirsch and D'Andrea, 1927). The previous report that glucose and galacturonic acid were principal components of the polysaccharide of coccidioidin could not be confirmed, but rather it was found that mannose was the main reducing sugar present, with a small amount of galactose included. Hassid et al. (1943) reported the presence of an unidentified reducing sugar which was present at about one-half the concentration of the glucose and was not fermented by *T. monosa* (*C. krusei*). Our results indicate that about 1.5% of the reducing sugar was not removed by fermentation with *C. krusei*, a considerably lower figure than reported by the earlier group for their unknown reducing sugar. [Goldschmidt (1958) confirmed our report (Pappagianis, 1955) of the presence of mannose and galactose in coccidioidin, but also reported the presence of glucose. He found that an unknown sugar which is present appears to be monomethyl mannose. This was possibly represented by the chromatographically more rapid component which we also detected.] McNall et al. (1960) also reported the presence of mannose in a polysaccharide extracted from *C. immitis*. However, this was an insoluble polysaccharide which also contained glucose, glucosamine, and galacturonic acid.

The appearance of mannose and galactose in coccidioidin is of interest, since these sugars are also found in a polysaccharide of *Mycobacterium tuberculosis* and tuberculin (Asselineau and Lederer, 1951; and several other earlier works; see Burger, 1950). Cross-reactivity to coccidioidin and tuberculin has been reported, but appeared

unlikely (Salvin, 1959), although some chemical similarity of the polysaccharides may contribute like actions in the similar pathogenesis of coccidioidomycosis and tuberculosis. Salvin and Smith (1959) reported the presence of mannose and galactose in a protein carbohydrate complex of *Histoplasma capsulatum*. The known occurrence of cross-reactions in serological and dermal hypersensitivity tests for coccidioidomycosis and histoplasmosis (Smith, 1943; Smith et al., 1949; Campbell and Binkley, 1953) may have its basis in similarity of the polysaccharide from these two fungi.

Hirsch and D'Andrea (1927) had described the presence of 3 to 4% nitrogen, determined by Nesslerization; but from the tests conducted by Hassid et al. (1943), the nitrogen could not be established as of protein origin. Part of the nitrogen was considered to be an integral part of the polysaccharide molecule. The present results demonstrated the presence of amino acids which are in some large molecular form, i.e., they are nondialyzable. It is considered possible that they exist in a protein-like material (positive Folin-Ciocalteu reaction; negative ninhydrin and biuret tests), or as peptides, or as amino acids complexed with a polysaccharide chain. In the quantitative precipitin reaction with carbohydrate-rich antigens (50 and 70% ethanol fractions), specific precipitates were obtained which lacked polysaccharide. From this it is concluded that at least a part of the nitrogenous material is not tightly bound to polysaccharide. The present data preclude further conclusions regarding the amount of free nitrogenous substances, or existence of a complex between polysaccharide and nitrogenous materials. One of the difficulties is the inability to distinguish between the antibody protein and the FCR material contributed to the serological precipitate by the antigen.

Another difficulty is posed by the data of the quantitative precipitin reactions. By use of either human or rabbit antisera, it was found that a precipitin reaction yielded a precipitate in which the polysaccharide was insufficient to account for the total polysaccharide added; i.e., the precipitate was formed in the zone of antigen (polysaccharide) excess. Yet when further addition of the antigen was made, more precipitate resulted. This paradox suggests the possibility that optimal concentrations of components of the crude antigen occur at different points, a result in agree-

ment with the findings using the fractions discussed above and with the occurrence of multiple lines of precipitation in gel-diffusion determinations. Smith et al. (1950) had earlier reported that the "precipitin button forms in the presence of excess antigen." However, no further precipitate was formed when more antigen was added to the serum-antigen mixture which had already given a precipitate.

Treatment with strong alkali also freed the polysaccharide of nitrogenous material. However, this digestion rendered the material inactive in precipitin and skin tests. Such rigorous treatment might also have affected the structure of the polysaccharide and in this manner caused inactivation. Hassid et al. (1943) found that skin-test activity was destroyed by exposure of the polysaccharide to 3% KOH in 50% aqueous methanol, although the latter when used to deacetylate the previously acetylated polysaccharide did not remove the precipitin reactivity of the polysaccharide.

Each of the fractions obtained by ethanol precipitation was active in demonstrating dermal sensitivity in both humans and rabbits. With vesiculation occurring at the skin-test sites with two of the fractions, it is possible that some differences exist in the nature of these fractions related to the variable carbohydrate to "protein" ratio. The failure of the crude polysaccharide to give the local Shwartzman reaction in rabbits and its lack of toxicity in mice indicates that it is not in the class of "endotoxins" (Pappagianis and Kobayashi, unpublished data). Other studies (Tarbet and Breslau, 1953; Breslau, 1955) have provided histochemical evidence for the superficial position of "mucopolysaccharide" in the wall of *Coccidioides* endospores. Should this constitute the cellular counterpart of the soluble polysaccharide studied from cultures which participate in precipitin reactions, agglutination of endospores by precipitins constitutes a reaction in vivo worthy of study in connection with "neutralizing" activity.

For the present, there appear to be heterogeneous fragments comprising skin-test and precipitin antigens present in coccidioidin. The variation in activity of various coccidioidins with respect to the foregoing tests and the complement fixation reaction has been emphasized (Smith et al., 1948; 1950), and this is consonant with observed changes in cultures of *C. immitis* after

autolysis (Pappagianis et al., 1957; Pappagianis and Kobayashi, 1958). In unreported experiments we have observed that digestion of casein in skim milk cultures of *C. immitis* may become apparent after 3 to 7 days, with some strains showing complete dissolution of the casein after several weeks. Perhaps gradual proteolytic action leads to modification of the nitrogenous constituents observed in the fractions obtained at different concentrations of alcohol; as Wilkinson (1958) points out, there is little likelihood that extracellular polysaccharides (of bacteria) will be subject to degradation by the cells from which they originate, and this perhaps holds true in cultures of *C. immitis* which appear to have a steady increase in polysaccharide with time (Pappagianis and Kobayashi, 1958).

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