THE ANTIGENIC COMPOSITION OF CRYPTOCOCCUS NEOFORMANS

IV. The Use of Paper Chromatography for Following Purification of the Capsular Polysaccharide[1]

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The capsular polysaccharide of *Cryptococcus neoformans* has been shown to be serologically reactive (Neill *et al.*, 1950; Evans and Kessel, 1951), and three types have been differentiated on the basis of precipitin reactions (Evans and Kessel, 1951) and the agglutination and quellung reactions (Evans, 1950). An analysis of the polysaccharides from types A, B, and C by paper chromatography revealed that they yielded similar hydrolysis products (Evans and Mehl, 1951). Drouhet *et al.* (1950) reported that the capsular substance from their strain of *C. neoformans* was composed of units of glucuronic acid, mannose, and xylose. This was in agreement with the results from this laboratory although a fourth spot migrating at a rate identical with galactose also was detected (Evans and Mehl, 1951), and an additional spot ("glucose") may be seen in the chromatograms of Drouhet *et al.* (1950).

The purpose of this communication is to present evidence that the earlier preparations actually contained two carbohydrate fractions. Separation of the crude polysaccharide into two fractions has been accomplished by the use of heavy metal precipitation and fractional alcohol precipitation. A method is described for following this separation by performing a paper chromatographic analysis of the fractions after hydrolysis. The chemical and serologic properties of three polysaccharide preparations are described.

MATERIALS AND METHODS

Isolation of the capsular polysaccharide. The 1523 strain of *C. neoformans*, type B, was used for preparation of polysaccharide throughout the investigation. This strain was isolated from a patient with central nervous system cryptococcosis at the Los Angeles County General Hospital.

In keeping with terminology introduced previously, the capsular polysaccharide will be referred to as "S" with the antigenic type designated as SA, SB, or SC. The present study is limited to the isolation and properties of SB.

To avoid alcohol precipitable material from the medium, a dialysate of neopeptone was prepared as described previously (Evans and Kessel, 1951). The dialysate was diluted with distilled water to a tyrosine content of 0.6 mg per ml as measured colorimetrically with Folin-Ciocalteau reagent. Two per cent glucose and one mg per liter of thiamin were added and the pH adjusted to 7.0 with NaOH if necessary. Ten liters of this medium dispensed in two liter flasks were inoculated heavily with neopeptone dialysate seed cultures and placed on a New Brunswick rotary action shaker adjusted to a speed of 180 rpm. After four days of shaking at room temperature, sufficient phenol was added to yield a final concentration of one per cent, and the cells stored at room temperature for 24 hours.

The bulk of the cells were removed by centrifugation, and the supernatant fluid was rendered cell-free by filtration through Whatman no. 54 paper coated with Hy-flo filter aid. The absence of cells was determined by a microscopic examination after centrifugation for one hour at a relative centrifugal force of 1,600. The clear supernate was reduced to 1/10 volume by perevaporation, and 100 grams of sodium acetate crystals per liter were added. The polysaccharide was precipitated by slowly adding 2.5 volumes of absolute ethyl alcohol with constant mechanical stirring. The precipitate was dissolved in two liters of water, and the alcohol precipitation procedure was repeated twice. Finally the material was dissolved in two liters of water and filtered through Whatman no. 54

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paper coated lightly with filter aid to clarify the solution. To the water clear filtrate 2.5 volumes of absolute alcohol were added in the absence of electrolyte. Saturated sodium acetate solution was added dropwise with constant mechanical stirring until a flocculent white precipitate was secured. This fraction, designated "crude SB", was washed repeatedly with 90 per cent alcohol, absolute alcohol, and anhydrous ether on hard filter paper.

The crude SB was desiccated to constant weight over calcium chloride *in vacuo*. A yield of approximately 300 mg per liter of culture was secured. Crude SB was a white amorphous powder which formed extremely viscous clear solutions in water and which did not pass through a dialyzing membrane. In a concentration of one per cent, the polysaccharide was negative to biuret reagent and failed to give tests for starch or glycogen with iodine. At a concentration of one mg per ml, the following tests for protein and amino acids were negative: Millon's, Folin phenol, and ninhydrin. In the same concentration, the polysaccharide gave strongly positive Molisch tests as well as positive tests for pentose (Bial) and hexuronic acid (Dische). After hydrolysis for one hour with N-H_2SO_4 a positive Benedict test for reducing sugar was obtained.

Although the nitrogen content of the crude SB was low, a chloroform-water extraction (Sevag, 1934; Evans and Kessel, 1951) was employed to remove traces of protein.

In an attempt to demonstrate whether the crude SB might contain two polysaccharides, fractionation by the use of heavy metal salts was attempted. It was found that all or part of the polysaccharide precipitated with uranium acetate and lead acetate, but not with copper acetate, barium hydroxide, or silver nitrate. Further investigation disclosed that reducing sugar after hydrolysis was demonstrable both in the supernate and in the precipitate of the lead treated crude SB.

Accordingly, one gram of chloroform extracted crude SB was dissolved in one liter of water and precipitated by stirring in 62.5 ml of saturated lead acetate solution. This ratio of crude SB to lead acetate concentration was determined by trial experiment to be approximately optimal. The lead precipitated material (SB_1) was placed in one liter of water. Solution was effected by adding 10 ml of acetic acid and 100 grams of sodium acetate followed by constant stirring for one hour. Two volumes of absolute ethyl alcohol were added to precipitate the material.

Following a third such precipitation, the SB_1 fraction was dissolved in 500 ml of water, filtered to remove lint, and precipitated by adding 1,250 ml of absolute alcohol with constant stirring plus a few drops of saturated sodium acetate solution. The material was dried with alcohol and ether followed by vacuum desiccation as with the crude SB fraction.

The supernate (R_1) from the lead precipitation was isolated by adding 30 ml of saturated sodium acetate solution, 6 drops of acetic acid, and 2.5 volumes of alcohol. Although the material turned opalescent, no flocculation occurred after an hour of constant stirring, and it was necessary to refrigerate the material overnight to complete the precipitation. The precipitate (R_1) was washed with alcohol and either and dried *in vacuo*.

As an alternative to the chemical procedure for removing the lead ion from SB_1, electrodialysis was employed. A three cell electrodialysis chamber in which the cells were separated by sheets of cellophane was used. Platinum electrodes were inserted in the outer cells, and the lead precipitate of SB_1 was added to the center compartment. Each cell was filled with 100 ml of water and 6 drops of acetic acid added to provide electrolyte. Direct current (25 ma) at an initial potential gradient of approximately 8 volts per cm was supplied for a period of 5 to 12 hours during which time lead precipitated out at the cathode and the SB_1 passed into solution. The possibility of employing electrophoretic analysis of SB_1 was suggested by its tendency to migrate toward the anode membrane where it piled up as a clear gelatinous mass. Following this treatment the SB_1 was removed from the center cell and precipitated from solution by the same procedure used for chemically purified SB_1.

Separation of crude SB into fractions SB_2 and R_2 was accomplished by precipitation with one volume of absolute ethyl alcohol in the presence of ten per cent sodium acetate and one per cent acetic acid. Earlier attempts to precipitate the capsular polysaccharide with one volume of alcohol were unsuccessful due to the omission of the acetic acid. The precipitation with one vol-

ume of alcohol was repeated twice after which the material was removed from solution and desiccated by the same procedure used for the other samples.

Chemical analyses. Nitrogen was determined by a modification of the micro-Kjeldahl procedure employing steam distillation. Reducing sugar was determined by the Hagedorn-Jensen method (Kabat and Mayer, 1948) after hydrolysis in 2 N H_2SO_4 for 4, 6, 8, and 10 hours. Relative viscosity of 0.1 per cent solutions of polysaccharide in 0.9 per cent salt solution was determined by the use of an Ostwald-Fenske viscosimeter tube in a 25 C bath.

Paper chromatography. Samples of crude SB, SB_1, SB_2, and R_1 were hydrolyzed by placing 70 to 100 mg in a test tube and adding 1 ml of 2 N H_2SO_4. The tubes were sealed (or covered with a marble for shorter lengths of time) and placed in a boiling water bath. Preliminary hydrolyses were conducted for 3 hours after which the material was neutralized with solid barium carbonate. In addition, samples of crude SB, SB_1, and SB_2 were hydrolyzed four hours and neutralized with barium carbonate. The unhydrolyzed material was precipitated then from the supernate by adding 8 volumes of absolute alcohol. The precipitate was removed by centrifugation, and all alcohol was removed from the supernate by evaporation *in vacuo*. The alcohol precipitate was hydrolyzed 12 additional hours with 2 N H_2SO_4 and neutralized with barium carbonate. Spots of the various hydrolyzates were placed on strips of Whatman no. 1 filter paper as described earlier (Evans and Mehl, 1951). Chromatograms were irrigated by a descending method as in the previous article, and reducing sugars were located by spraying with 0.5 N silver nitrate solution in 5 N ammonium hydroxide. The paper strips were heated in an oven at 85 C until the spots of reducing sugar were clearly visible. Solvents used were: (a) *n*-butanol (400 ml) saturated with water (500 ml) and acetic acid (100 ml); (b) ethyl acetate (300 ml) saturated with water (300 ml) and acetic acid (100 ml); (c) acetone (400 ml) plus 40 ml of one per cent acetic acid; and (d) acetone (400 ml) plus 40 ml of water.

Quantitative precipitin test. Antiserum was prepared as described earlier (Evans, 1950). To detect possible degradation of the polysaccharide during isolation procedures, fractions SB_1 and SB_2 were compared to crude SB on the basis of the quantity of antibody nitrogen which each was able to precipitate from type B antiserum. Preliminary antigen dilution titrations accompanied by tests on the supernates for excess antigen or antibody were conducted in order to determine the location of the zones of antibody excess, equivalence, and antigen excess. For the quantitative test, accurately measured amounts of antigen were added to 1 ml quantities of antisera nos. 1523-15 and 1523-47. Tests were set up in duplicate and incubated for two hours at room temperature followed by refrigeration at 0 C for 48 hours. Following this, the precipitates were washed twice with cold salt solution and analyzed for nitrogen by a modification of the micro-Kjeldahl procedure employing steam distillation. Proof of the identity of fractions SB_1 and SB_2 with the capsule surrounding intact cells was obtained by performing capsular "quellung"[2] tests with supernatant serum from the quantitative tests. This demonstrated the ability of SB_1 and SB_2 to remove antibodies responsible for the capsular quellung reaction.

RESULTS

The value of paper chromatography for following fractionations of this type can be seen in figure 1. Hydrolysis of crude SB revealed 4 spots, and after the crude material was fractionated, the spot migrating similarly to galactose was found in fraction R_1, while the other three (xylose, mannose, and glucuronic acid) were found in fraction SB_1 (and in SB_2). Since fraction R_1 contained trace amounts of SB_1 and since R_1 also was relatively insoluble as isolated in this study, it has not been investigated for serologic reactivity. Studies of the R_1 and R_2 fractions have not been completed yet but will be reported at a later time. Fractions SB_1 and SB_2 were found to be completely soluble and to form clear viscous solutions at higher concentrations (0.5 to 1 per cent). Relative viscosities of 2.03, 2.16,

[2] The term "quellung" has been used for convenience and also because of the resemblance of the reaction to capsular reactions of the pneumococcus and other bacteria. Since the term "quellung" implies a swelling of the capsule, it should be noted that there does not appear to be any marked increase in size of the cryptococcus capsule when mixed with antiserum, but only a clarification of its outline (Neill *et al.*, 1950; Evans, 1950).

and 2.22 were obtained for crude SB, SB_1, and SB_2, respectively. It is apparent that purification resulted in a slight increase of relative viscosity. This provides evidence against degradation which might have occurred during isolation procedures. Fractions SB_1 and SB_2 precipitated approximately the same amount of total antibody nitrogen from antisera 1523-15 and 1523-47 as did the crude SB. Antibody nitrogen values for additional points in the quantitative precipitin curve are also in table 1.

Supernate tests performed on qualitative reactions covering the entire range of the precipitin curve revealed equivalence zones for SB_1 and SB_2 in which neither antigen nor antibody was detectable. With crude SB, a zone was demonstrable in which antibody was absent but with a positive test for antigen. The identity of SB_1 and SB_2 with the intact capsule was demonstrated by the fact that SB_1 and SB_2 absorbed all quellung reactivity from an antiserum. In the zones of equivalence and antigen excess the

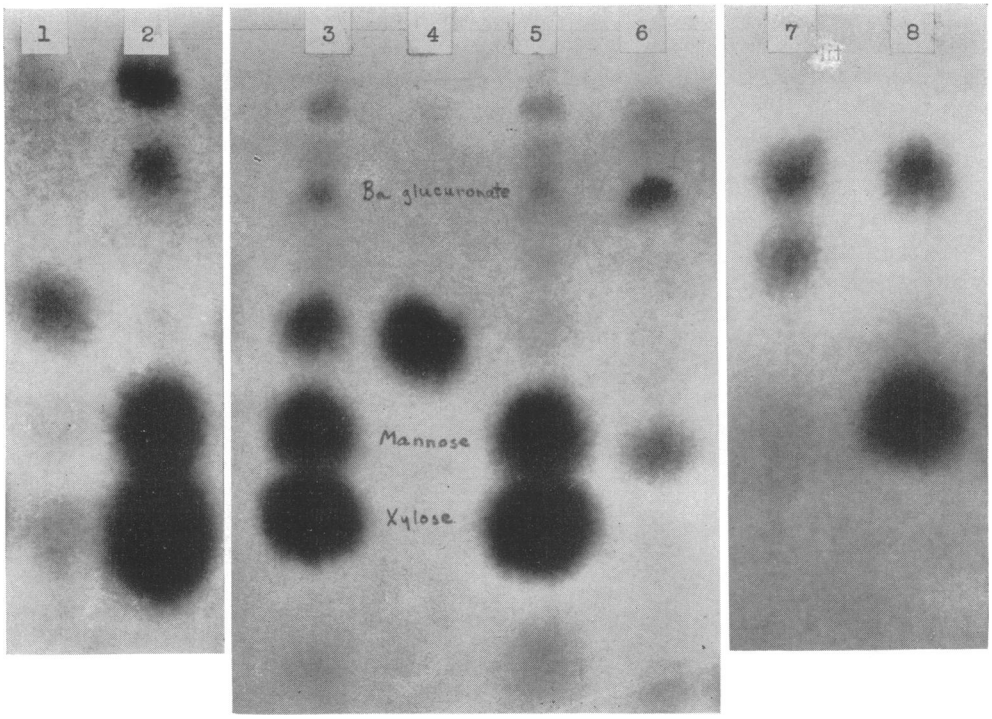

Figure 1. Paper chromatograms of polysaccharide fractions after hydrolysis. Solvents used: 1 through 6, butanol-acetic acid; 7 and 8, ethyl acetate-acetic acid. Although all reference sugars used are not shown, the components of SB_1 and SB_2 are labeled. A faint spot below xylose in no. 3 and no. 5 may represent the lactone of glucuronic acid. The faint spots above barium glucuronate have not been identified, but presumably represent partial hydrolysis products.

1. Fraction R_1; 3 hour hydrolysis
2. Fraction SB_1; 3 hour hydrolysis
3. Crude SB; 3 hour hydrolysis
4. Galactose
5. Partially purified SB_2 (crude SB after a single precipitation with one volume of ethyl alcohol in the presence of sodium acetate and acetic acid). A trace of R_2 remains. Hydrolysis time: 4 hours.
6. Fraction SB_1; 16 hour hydrolysis. Note the stronger spot of barium glucuronate and the absence of xylose.
7. Fraction SB_1; 16 hour hydrolysis. The mannose spot was located much closer to glucuronic acid in this solvent.
8. Barium glucuronate; the faster moving spot at the bottom is glucuronolactone.

supernates from quantitative precipitin tests displayed no quellung reactivity against type B cells as contrasted to control sera.

Although the presence of uronic acid was reported earlier (Evans and Mehl, 1951), definite evidence for the presence of glucuronic acid was not secured un prolonged hydrolysis was employed following the technique of Drouhet *et al.* (1950).

however, is seen in the fact that tests for antigen and antibody on the supernates from quantitative precipitin tests have revealed a zone in which neither antigen nor antibody is present.

Purified SB may be prepared by precipitation with one volume of absolute ethyl alcohol in the presence of one per cent acetic acid and ten per cent sodium acetate, or an alternate method is the use of lead precipitation. Lead may be

TABLE 1

Properties of crude SB, SB_1, and SB_2

ANALYSIS	FRACTION		
	Crude SB	SB_1	SB_2
Kjeldahl N (per cent)*	0.05	<0.01	<0.01
Ash (per cent)	2.71	3.72	2.89
Reducing sugar after hydrolysis (per cent as glucose)*	76	75	75
Relative viscosity of 0.1 per cent solution in 0.9 per cent salt solution at 25 C	2.03	2.16	2.22
Hydrolysis products identified by paper chromatography	glucuronic acid mannose xylose galactose	glucuronic acid mannose xylose	glucuronic acid mannose xylose
Ability to absorb quellung reactivity from type B antiserum	+	+	+
Antibody nitrogen precipitated (mg) mg antigen added:			
0.10†	0.27	0.26	0.25
0.30†	0.41	0.38	0.40
0.50‡	0.60	0.60	0.58
Total antibody nitrogen per ml of antiserum (mg)			
serum 1523-15	0.62	0.58	0.60
serum 1523-47	0.76	0.77	0.77

* Corrected for ash.
† Serum 1523-15.
‡ Serum 1523-47.

The chemical properties of crude and purified SB are presented in table 1. Both SB_1 and SB_2 were found to be essentially nitrogen-free and yielded approximately 75 per cent reducing sugar after hydrolysis for 6 or 8 hours in 2 N H_2SO_4. The ash values were slightly high for SB_1 which may be due to traces of lead.

Tests on the homogeneity of SB_1 and SB_2 are not complete and will be reported at a later time. Presumptive evidence for homogeneity, removed from SB_1 by chemical methods or by electrodialysis. No appreciable difference could be detected between SB_1 samples purified by the two respective techniques. Data presented in table 1 are for chemically purified SB_1, however.

DISCUSSION

It has been shown that the crude polysaccharide isolated from *C. neoformans* by earlier

methods actually contains two fractions. These may be separated by precipitation with lead (SB_1 and R_1) or by alcohol precipitation (SB_2 and R_2). Both SB_1 and SB_2 appear to be identical with the capsule on the basis of absorption experiments, and both yield at least three sugars on hydrolysis. By the use of paper chromatography these have been identified as glucuronic acid, mannose, and xylose. Results of hydrolysis for different lengths of time suggest the presence of an aldobionic acid composed of mannose and glucuronic acid in agreement with the observation of Drouhet *et al.* (1950). Fractions R_1 and R_2 have not been studied extensively although fraction R_1 yields a hydrolytic product which migrates at the same rate as galactose on paper chromatograms with four different solvents. The relation of fractions R_1 and R_2 to the antigenic composition of the cell remains to be determined.

The combination of mild chemical fractionation with paper chromatography has been of definite value in this instance, and it is likely that the method will be found applicable to other biological fractionations. As used in this study the method has permitted demonstration that the crude SB polysaccharide was inhomogeneous. It cannot, however, be stated with certainty that the SB_1 and SB_2 fractions are homogeneous at this time and R_1 is obviously contaminated with a trace of SB_1 (figure 1). In all chromatographic work presented in this paper, the polysaccharide was first subjected to acid hydrolysis. Attempts to demonstrate chromatographic separation of unhydrolyzed crude SB have not been successful.

The use of paper chromatography provides a convenient, but not completely foolproof, method for identifying hydrolysis products. It is conceivable that other, less common sugars may have the same migration rates as those employed here. This objection can be overcome partially by employing a variety of solvents, but absolute identification of the sugars reported here will require confirmation by other methods.

An attempt has been made in this laboratory to use only mild procedures in isolating the capsular polysaccharide. Others have extracted polysaccharides from *C. neoformans* with boiling 8 N sodium hydroxide (Foley and Uzman, 1952) or with N potassium hydroxide (Drouhet *et al.*, 1950). The product of Foley and Uzman appears to be different from the capsular polysaccharide obtained in this study since their material was no longer soluble following desiccation and yielded only 0.5 per cent reducing sugar on hydrolysis as contrasted to 75 per cent for SB_1 and SB_2. The use of even weak alkali is apt to degrade serologic reactivity of some polysaccharides (Heidelberger *et al.*, 1950), and since our results indicate that the capsular polysaccharide of *C. neoformans* may be secured without alkaline extraction, such procedures should be avoided. Lack of degradation in our preparations is indicated by their reactivity in antiserum as measured by the quantitative precipitin test and by relative viscosity data. Although slight differences were observed between samples, it is believed these were within the limits of experimental error.

Data presented in this paper have been limited to the capsular polysaccharide from type B designated in this paper as SB. A similar fractionation of SC has been accomplished, and SA is under investigation at the present time.

SUMMARY

Further purification of the capsular polysaccharide of *Cryptococcus neoformans*, type B, has been accomplished by precipitation with heavy metal and by fractional alcohol precipitation.

A method for following separation of the crude polysaccharide into two fractions by paper chromatography of the hydrolyzates is presented.

Fractions SB_1 and SB_2 appear to be identical with the polysaccharide of the intact capsule on the basis of absorption experiments. Both SB_1 and SB_2 are essentially nitrogen-free and yield approximately 75 per cent reducing sugar, as glucose, on hydrolysis. There was no detectable degradation during isolation procedures as measured by the quantitative precipitin test and relative viscosity determinations.

Hydrolysis products of SB_1 and SB_2 have been identified as xylose, mannose, and glucuronic acid by paper chromatography.

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