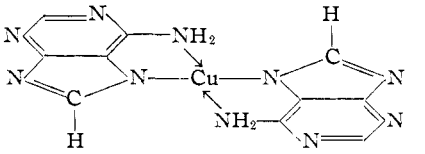
teresting reactions, solubilization of cellulose and other carbohydrates may result from the formation of copper(II) complexes similar to that of ribose. The reaction apparently is reversible in nature since a back-titration with 0.1 *N* perchloric acid exhibits no hysteresis.

On the basis of the foregoing discussion the following structure is proposed as probably representing the copper–adenine complex in aqueous solution



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[CONTRIBUTION FROM THE INSTITUTE OF MICROBIOLOGY, RUTGERS, THE STATE UNIVERSITY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY, AND THE UNIVERSITY OF ALABAMA]

Precipitation of the Specific Polysaccharide of *Cryptococcus neoformans* A by Types II and XIV Antipneumococcal Sera[1]

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The type specific polysaccharide of *Cryptococcus neoformans* A, which is known to contain xylose, galactose, mannose and glucuronic acid, has been shown to be a mixture of at least two polysaccharides, since the proportion of galactose in the mixture was increased by fractional precipitation with Type XIV antipneumococcal serum. Since neither cetyltrimethylammonium bromide nor Type II antipneumococcal serum effected any fractionation, it is concluded that both polysaccharides contain glucuronic acid.

The specific polysaccharides of *Cryptococcus neoformans*, Types A, B and C, although serologically different, are quite similar in composition and contain mainly mannose and xylose, with smaller amounts of glucuronic acid and galactose.[5] A contaminating galactan had also been separated from the immunologically reactive material, so that it became of interest to determine, if possible, whether or not the galactose and perhaps also the glucuronic acid were derived from impurities. Since precipitation with an appropriate antiserum has been shown to be a powerful means for the fractionation of otherwise difficultly separable mixtures of polysaccharides, for instance, gum arabic[6] and lung galactan,[7] advantage was taken of the cross precipitation of the specific polysaccharide of Cryptococcus A in Types II[5] and Type XIV[8] antipneumococcal[9] sera. An attempt also was made to fractionate the polysaccharide with cetyltrimethylammonium bromide, cetavlon, since this reagent has been shown useful in the separation of acidic from non-acidic polysaccharides.[10] However, the original polysaccharide, the cetavlon-precipitated fraction and the cetavlon-soluble fraction all exhibited the same cross reactivity with Type XIV anti-Pn serum and qualitative paper chromatographic analysis of the hydrolysates showed no differences in composition. Recent preliminary experiments suggest that it may be possible to separate the mixture into two components by electrophoresis. The results are of interest in showing once more both the possibilities and the limitations of the methods used.

Since only about 300 μg. of polysaccharide was recovered from either of the specific precipitates, even though relatively large amounts of antiserum were used, and glucuronic acid was present, differential colorimetric reactions appeared to be the method of choice. Moreover, preliminary hydrolysis to the free sugars is not required, an especial advantage in the presence of glucuronic acid.

Inasmuch as the original polysaccharide is known to contain only xylose, mannose, galactose and glucuronic acid, xylose and total hexose were estimated by the basic cysteine reaction,[11] in which mannose gives 0.8–0.9 as much color as galactose. The ratio of mannose to galactose was then determined with the help of the secondary cysteine reaction,[12] in which mannose gives 0.10–0.11 as much color as galactose. Solution of two simultaneous equations then gives the content of each sugar.

Glucuronic acid was determined separately by the carbazole reaction.[13]

Experimental

Materials and Methods.—The Type II anti-Pn horse serum was kindly supplied by the Bureau of Laboratories,

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(5) E. E. Evans, *J. Immunol.*, **64**, 423 (1950); E. E. Evans and J. W. Mehl, *Science*, **114**, 10 (1951); E. E. Evans and J. F. Kessel, *J. Immunol.*, **67**, 109 (1951); E. E. Evans and R. J. Theriault, *J. Bacteriol.*, **65**, 571 (1953); E. E. Evans, L. J. Sorensen and K. W. Walls, *ibid.*, **66**, 287 (1953).

(6) M. Heidelberger, J. Adams and Z. Dische, THIS JOURNAL, **78**, 2853 (1956).

(7) M. Heidelberger, Z. Dische, W. Brock Neely and M. L. Wolfrom, *ibid.*, **77**, 3511 (1955).

(8) M. Heidelberger, S. A. Barker and B. Bjorklund, *ibid.*, **80**, 113 (1958).

(9) Herein designated anti-Pn.

(10) B. C. Bera, A. B. Foster and M. Stacey, *J. Chem. Soc.*, 3788 (1955); J. E. Scott, *Chem. and Ind.*, 168 (1955); A. S. Jones, *Biochim. Biophys. Acta*, **10**, 607 (1953); M. Stacey and S. A. Barker, "Biochemistry of Nitrogen," *Acad. Sci. Fennica*, 262 (1955).

(11) Z. Dische, *J. Biol. Chem.*, **181**, 379 (1949).

(12) Z. Dische, L. B. Shettles and M. Osnos, *Arch. Biochem.*, **22**, 169 (1949).

(13) Z. Dische, *J. Biol. Chem.*, **167**, 189 (1947).

TABLE I

CROSS REACTIVITY OF *Cryptococcus* A POLYSACCHARIDE WITH ANTIPNEUMOCOCCAL SERA

Cryptococcus A added, mg./ml. serum	Antibody nitrogen precipitated per ml. Anti-Pn II 513, μg.	Anti-Pn XIV 635, μg.
0.03		19
.1	113	19
.3	67	

Basic Cysteine Reaction.—Duplicate 0.50-ml. portions of the extracts or solutions were mixed with 0.50 ml. of distilled water and analyzed according to the basic cysteine reaction[11] together with suitable standards made up in the appropriate concentrations of TCA and with the original *Cryptococcus* A polysaccharide as a control in each case. The data and calculations are shown in Table II.

Secondary Cysteine Reaction. Procedure A.—Triplicate aliquots, 0.40 ml. each, of the concentrated anti-Pn XIV extract were treated according to the secondary cysteine

TABLE II

ABSORBANCIES, BASIC CYSTEINE REACTION

Substance analyzed	A_{390}[a]	A_{422}	$A_{390} - A_{422}$	A_{412}	A_{368}	$A_{412} - A_{368}$
2 mg. % xylose	480, 480	37, 28	443,452	134	132	0
5 mg. % mannose	175	175	0	210, 213	60, 64	150, 149
5 mg. % galactose				360, 379	175, 194	185, 185
10 mg. % OPS[b]	530, 530	201, 225	329, 305	314, 325	151, 157	163, 168
Double-diluted SPS[b] Pn XIV	245, 249	90, 96	155, 153	154, 149	74, 71	80, 78
	A_{390}	A_{416}	$A_{390} - A_{416}$	A_{412}	A_{368}	$A_{412} - A_{368}$
2 mg. % xylose	464, 445	70, 40	394, 405	84, 98	88, 96	0
5 mg. % mannose	209, 200	208, 205	0	222, 222	32, 38	190, 184
5 mg. % galactose				238, 238	30, 36	208, 202
12 mg. % OPS	740, 750	352, 342	388, 408	415, 410	180, 190	235, 220
SPS Pn II	340, 338	164, 160	176, 178	192, 194	52, 64	140, 130

SPS Pn XIV Double-diluted	Xylose, $\frac{154}{448} \times 2 = 0.69$ mg. %; hexose,[c] $\frac{79}{150} \times 5 = 2.63$ mg. %
OPS (10 mg. %)	Xylose, $\frac{317}{448} \times 2 = 1.42$ mg. %; hexose, $\frac{166}{150} \times 5 = 5.53$ mg. %
SPS Pn II	Xylose, $\frac{177}{400} \times 2 = 0.88$ mg. %; hexose, $\frac{135}{187} \times 5 = 3.61$ mg. %
OPS (12 mg. %)	Xylose, $\frac{398}{400} \times 2 = 1.99$ mg. %; hexose, $\frac{228}{187} \times 5 = 6.09$ mg. %

[a] A = optical density × 1000; subnumeral = wave length in mμ. [b] OPS = original polysaccharide, SPS = serum precipitated polysaccharide. [c] Calculated as mannose equivalent. Absorbancy ratio, mannose:galactose, Pn XIV, 150:185 = 0.81; mannose:galactose, Pn II, 187:205 = 0.91.

New York City Department of Health, and the Type XIV anti-Pn horse serum by the Division of Laboratories, New York State Department of Health.

For the quantitative estimations of antibody nitrogen,[14] reaction mixtures were allowed to stand in a bath at 0° for 2–3 weeks before they were centrifuged, washed twice with chilled saline and analyzed (Table I).

Precipitates which were to be analyzed for their content of sugars were given the second washing with 1.2% sodium sulfate, as NaCl interfered with some of the color reactions used. The gum in the washed precipitates was dissociated from the antibody with 5% trichloroacetic acid (TCA) solution.[6]

As a result of a small-scale preliminary analysis, 70 ml. of C-absorbed[15] anti-Pn XIV horse serum, No. 635, was mixed with a solution of 7 mg. of *Cryptococcus* A polysaccharide in 0.9% saline and allowed to stand in a bath at 0° for some weeks. The mixture was centrifuged and the precipitate was washed and dissociated as above with 5% TCA. The supernatant, 6.3 ml., which contained the polysaccharide, was poured off and concentrated *in vacuo* to 3.7 ml. and a final TCA concentration of 7.7%. Three aliquots of 0.40 ml. each were removed for the secondary cysteine reaction, and the remaining solution, 2.50 ml., was diluted with an equal volume of water (referred to subsequently as the diluted extract).

Ten ml. of anti-Pn II horse serum, No. 513, was mixed with 1 mg. of *Cryptococcus* A polysaccharide in 0.9% saline and treated in the same manner as before. The total volume of 5% TCA extract was 5.0 ml., evaporation having been omitted in this case.

TABLE III

ABSORBANCIES, SECONDARY CYSTEINE REACTION

Substance	A_{605} Procedure A 1st anal.	A_{605} Procedure A 2nd anal.	A_{605} Procedure B
Pn XIV, SPS	119		
10 mg. % galactose	569		
Pn XIV, SPS, double-diluted			72
2.5 mg. % galactose			190
10 mg. % xylose			20
OPS 50 mg. %, no cysteine	86	95	
OPS 50 mg. %, with cysteine	270	296	
7 mg. % xylose	5		
8.3 mg. % glucurone	12		
2 mg. % galactose	69		
5 mg. % galactose	190	233	
10 mg. % galactose	416		
50 mg. % mannose, no cysteine		70	
50 mg. % mannose with cysteine		324	
Pn II SPS			88
OPS 48 mg. %, no cysteine			154
OPS 48 mg. %, with cysteine			426
Mannose, 45 mg. %, no cysteine			104
Mannose, 45 mg. %, with cysteine			403
2 mg. % xylose			57
0.83 mg. % galactose			98
2.50 mg. % galactose			179
Blank			59

(14) M. Heidelberger and F. E. Kendall, *J. Exptl. Med.*, **61**, 559 1935); R. Markham, *Biochem. J.*, **36**, 790 (1942); E. A. Kabat and M. Mayer, "Experimental Immunochemistry," C. C. Thomas. Springfield, Ill., 1948.

(15) Serum which had first been precipitated with pneumococcal C-substance, the somatic, group-specific polysaccharide.

TABLE IV

CALCULATIONS FOR HEXOSES

Substance	Basic cysteine	Secondary cysteine	x = mannose, mg. %	y = galactose, mg. %
SPS Pn XIV	$0.81x + y = 4.25$	$0.11x + y = 2.0$	3.2	1.6
OPS control for above	$0.81x + y = (5.53)(0.81)$	$0.11x + y = (4.6)\frac{(10)}{(50)}$	5.1	0.36
SPS Pn II	$0.91x + y = (3.61)(0.91)$	$0.11x + y = 0.62$	3.3	.24
OPS control for above	$0.91x + y = (6.09)(0.91)$	$0.11x + y = 4.4\frac{(12)}{(48)}$	5.5	.5

reaction,[12] except that all volumes were reduced to 50% of those recommended, and tubes were heated 16 hr. at 50° after the addition of cysteine, instead of 8 hr.

Secondary Cysteine Reaction. Procedure B.—The analysis was repeated, modifying the procedure so that a larger volume of a more dilute extract could be used. 1.8 ml. of the double-diluted anti-Pn XIV extract was mixed with 0.20 ml. of 100 mg. % mannose and triplicate 0.60-ml. aliquots of the mixture were treated with 2.7 ml. of H_2SO_4 (6:1) at 0°, then heated 3 minutes at 100° and cooled to room temperature, after which cysteine–HCl was added as in procedure A.

The anti-Pn II extract was analyzed in the same manner. The data for both sets of reactions are shown in Table III.

Calculation of the Ratio of Galactose to Mannose. Basic Cysteine Reaction. Anti-Pn XIV Precipitated Polysaccharide.—Let x = mg. % mannose and y = mg. % galactose

$$\text{Absorbancy ratio, } \frac{\text{mannose}}{\text{galactose}} = \frac{150}{185} = 0.81$$

Double-diluted anti-Pn XIV extract contains 2.63 mg. % hexose calculated as mannose, therefore the original extract contains $2.63 \times 2 \times 0.81 = 4.25$ mg. % hexose as galactose.

(A) $0.81x + y = 4.25$

Secondary Cysteine Reaction.—Procedure A, Anti-Pn XIV Extract.—

$\frac{119}{569} \times 10 = 2.09$ mg. % hexose, calculated as galactose.

Procedure B, Double Diluted Anti-Pn XIV Extract.—

$\frac{72}{190} \times 2.5 = 0.95$ mg. % hexose, calculated as galactose

Calculated average

$$\frac{2.09 + 2 \times 0.95}{2} = 2.0 \text{ mg. \%}$$

$$\text{Absorbancy ratio } \frac{\text{mannose}}{\text{galactose}} = \frac{\frac{324-70}{10}}{233} = 0.11$$

(B) $0.11x + y = 2.0$ mg. %

Solving (A) and (B) simultaneously

$x = 3.2$ mg. % mannose

$y = 1.6$ mg. % galactose

The equations and solutions thereof for the remaining determinations are shown in Table IV.

Carbazole Reaction.—Duplicate 0.60-ml. portions of the extracts were analyzed by the carbazole reaction[13] with all other volumes reduced to 60% of those specified. The data and calculated percentages of glucuronic acid as glucurone are shown in Table V. Small corrections for the presence of hexoses were made as previously described.[12,13]

TABLE V

ABSORBANCIES, CARBAZOLE REACTION

Substance	A_{535} 1st anal.	A_{535} 2nd anal.	A_{535} 3rd anal.	Glucuronic acid, calcd. as glucurone mg. %
Xylose, 1.5 mg. %		0		
Mannose, 5 mg. %		18	28	
Mannose, 10 mg. %		44		
Glucurone, 0.75 mg. %	51			
Glucurone, 2.5 mg. %		180	183	
OPS 2.5 mg. %	34			0.50
OPS 12 mg. %			209	2.38
SPS Pn XIV, double-dil. ext.		150		1.92
SPS Pn II extract			124	1.42

The data obtained are summarized in Table VI.

TABLE VI

SUMMARY OF RESULTS, CALCULATED TO 100%[a]

Substance	Xylose, %	Galactose, %	Mannose, %	Glucurone, %
SPS Pn XIV	14	16	32	38
OPS control for above	16	4.5	57.5	22
SPS Pn II	15	4	57	24
OPS control for above	19	5	53	23
Fraction of polysaccharide pptd. by				
anti-Pn XIV serum	370 μg. out of 7 mg., or 5%			
anti-Pn II serum	350 μg. out of 1 mg., or 35%			

[a] The concentration of each sugar in mg. % was recalculated to a single polysaccharide concentration. These values were added together, divided by the total and multiplied by 100.

Discussion

It is apparent from Table VI that the Type XIV antipneumococcal serum effects a fractionation of a mixture whereas the Type II antiserum does not. If the mixture were composed of a xyloglucuronogalactan and a xyloglucuronomannan, concentration of the galactan by the use of the Type XIV serum might be effected through 1,3-, 1,6-, or 1,3,6-linked galactose or galactose end groups.[6,16] The heavy cross-reaction of the polysaccharide with the Type II antiserum probably indicates that at least some of the glucuronic acid is present as end groups.[17] If both polysaccharides contain glucuronic acid in similar linkage, no separation would be expected to occur, either by precipitation with cetyltrimethylammonium bromide or Type II anti-Pn serum.

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(16) M. Heidelberger, THIS JOURNAL, **77**, 4308 (1955).

(17) M. Heidelberger and J. Adams, *J. Exptl. Med.*, **103**, 189 (1956).