SPECIFIC AND NON-SPECIFIC POLYSACCHARIDES OF TYPE IV PNEUMOCOCCUS*

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The recent separation of Group IV pneumococcus into a number of types with well defined specific properties (1) suggested a comparison of the specific polysaccharide of one of the new subdivisions with the highly type-specific and chemically distinct carbohydrate haptens of Type I, II, and III pneumococcus (2). It seemed of interest to determine whether the substance responsible for the specificity of one of the new types would resemble the specific polysaccharides of Type I, II, and III pneumococcus, or whether it would be as different from these as each of the three is from the others.

Type IV pneumococcus was accordingly chosen for study, since at the time it appeared to be one of the most important of the new subdivisions of Group IV (1). The cultures and homologous antiserum were kindly furnished by Dr. Georgia Cooper of the New York City Board of Health Laboratories, and the writers wish to express their heartiest thanks both to Dr. Cooper and to Dr. William H. Park for their interest and cooperation.

EXPERIMENTAL

It was evident from the very first that the isolation of the Type IV specific polysaccharide would be a very difficult matter since the hapten required far more alcohol for its precipitation than did the corresponding substances of Type I, II, and III pneumococcus. Not

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only, therefore, were the nitrogenous impurities of protein origin more difficult to remove, but also when this was accomplished the product was found to be contaminated by at least two substances which had not been encountered in the work on Type I, II, and III pneumococcus polysaccharides—a serologically inactive substance somewhat related to chitin, and the "C substance" first described by Tillett and Francis, and later studied by Tillett, Goebel, and Avery* (3). Since the chemical and physical properties of both of these products were very similar to those of the Type IV specific polysaccharide, removal of these and the other principal impurities was accomplished only after the sacrifice of much material in preliminary experimentation.

The none too satisfactory method finally adopted was as follows:

20 l. lots of autolyzed Type IV pneumococcus cultures in 0.3 per cent glucose-meat infusion-phosphate broth were autoclaved and concentrated on the water bath to 1 to 1.5 l. The resulting syrup was precipitated with alcohol up to 5 l. and allowed to stand overnight. The supernatant liquid was decanted and the precipitate centrifuged and taken up in the minimum amount of water. The solution was centrifuged and the supernatant and washings were treated with 20 per cent of alcohol and kept until the concentrate from a number of lots had accumulated.

The concentrate from about 80 l. of culture was reprecipitated with four volumes of alcohol and the precipitate taken up in water, diluted to 5 l., acidified with a few cubic centimeters of acetic acid, stirred mechanically, and precipitated with aqueous tannic acid solution containing 200 gm. of the acid per liter, adding the acid only as long as the precipitate was dark and heavy. 600 to 1000 cc. usually sufficed. After thorough stirring the mixture was allowed to stand overnight in the cold. The turbid supernatant was centrifuged in the cold until merely opalescent, while the main precipitate was ground in a mortar with water weakly acidified with acetic acid, filtered on a large Buchner funnel, and sucked dry. The filtrate and the main solution were concentrated *in vacuo* to 400 cc. and precipitated with 350 cc. of alcohol, bringing down a heavy syrup which contained little active material. The supernatant was drawn off and precipitated with alcohol up to 2 l. and allowed to stand. The supernatant was decanted and the precipitate centrifuged, yielding three layers, of which the middle one contained most of the specific substance. This was taken up in 250 cc. of water, and, after the addition of 50 gm. of sodium acetate, precipitated with five volumes of alcohol. The precipitate was dissolved in 1 l. of water, treated with 75 gm. of sodium acetate, acidified with

* The writers wish to express their gratitude to these workers for the private communication which led to the recognition of this substance in the crude product

acetic acid, and precipitated with 50 gm. of thorium nitrate dissolved in water and made up to 100 cc. The mixture was centrifuged in the cold and the supernatant was run through a Sharples supercentrifuge for further clarification. The grayish, opalescent solution was then made alkaline with ammonia, and the copious precipitate, which came down readily on centrifugation, was washed with 1 l. of water. The water-clear supernatant and washings were acidified faintly with acetic acid and concentrated to small bulk *in vacuo*. The concentrate was dialyzed in a collodion bag,* with occasional concentration *in vacuo*, until practically free from nitrates, and was then again brought down to about 75 cc. *in vacuo*, treated with 75 cc. of glacial acetic acid, and then cautiously with alcohol in the cold until any precipitate formed just began to flock out. The precipitate (A) was collected by centrifugation and the supernatant further precipitated in the cold with alcohol, with the addition of acetone if necessary. The precipitate (B) was taken up in 20 to 30 cc. of water, centrifuged if necessary, and again treated in the cold with an equal volume of glacial acetic acid and small amounts of alcohol until the precipitate formed settled on centrifugation and left a clear supernatant. The precipitate was added to (A) and the supernatant precipitated with excess alcohol (B).

Since (B) represents crude Type IV polysaccharide its further purification will be taken up first. The precipitate is dissolved in about 30 cc. of water, concentrated partially *in vacuo* to remove alcohol, made up to the original volume, and treated with 3 cc. of 30 per cent aqueous sodium nitrite and 1 cc. of glacial acetic acid, with frequent shaking during the course of 1 to 2 hours. In dilute solution the "C substance" is destroyed by nitrous acid as was also found by Tillett, Goebel, and Avery, but much survives this treatment in the presence of relatively much Type IV polysaccharide. For further purification an equal volume of glacial acetic acid is added, and after removal of a very small initial precipitate with alcohol as before, alcohol is added up to 250 cc. The precipitate is centrifuged off sharply, taken up in a little water and reprecipitated twice with glacial acetic acid and redistilled alcohol, after which it is taken up in 20 to 30 cc. of water, poured into twenty volumes of redistilled acetone containing a little glacial acetic acid, centrifuged if necessary, collected on a hardened filter, and dried *in vacuo* over calcium chloride, paraffin, and crushed sodium hydroxide. If colored impurities persisted down to this point the appearance of the product could often be improved by solution in a little water, making up to 200 cc. with 6 per cent cupric acetate solution, precipitating in the cold with 200 cc. of chilled 20 per cent sodium hydroxide solution, centrifuging, taking up, with cooling, in a little water and acetic acid until permanently acid, centrifuging from the precipitate of copper acetate and washing this with a little ice-cold water, adding an equal volume of acetic acid to the supernatant and washings, and making up to 1 l. with alcohol.

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* Considerable amounts of "C substance" pass through the bag at this stage, but not rapidly enough to afford a convenient method for its quantitative removal from the Type IV substance.

All of the copper can be removed by repeated precipitation with acetic acid and alcohol, and the final product is isolated as a curdy, snow-white mass, as indicated above. The yields varied from 0.1 to 0.5 gm.

The A fraction contained both inactive polysaccharide and "C substance." It was dissolved in about 25 cc. of water, centrifuged if necessary, made up to 125 cc. with glacial acetic acid, and treated in the cold with alcohol in small portions until the heavy turbidity produced first showed signs of flocking. On centrifuging in the cold a precipitate (A_1) and a clear supernatant were obtained, the latter yielding "C substance" on precipitation with acetone (A_2). This was centrifuged off sharply, dissolved in 15 cc. of water, treated in the cold with 50 cc. of glacial acetic acid, then with redistilled alcohol to the first permanent turbidity. Traces of precipitate containing more A_1 were centrifuged off, and the precipitation completed with redistilled alcohol up to 250 cc. After letting stand in the cold the precipitate was centrifuged off, redissolved in a few cubic centimeters of water, and poured into 200 cc. of redistilled acetone, yielding a stringy precipitate which was collected and dried as in the case of the B fraction. The yields varied from 0.2 to 0.5 gm.

The A_1 fraction was dissolved in 25 cc. of water, centrifuged, and the solution treated with nitrous acid as in the case of the B fraction to remove as much "C substance" as possible, and precipitated with an equal volume of acetic acid and enough alcohol to yield a clear supernatant on centrifugation. The precipitate (A_{1a}) was now almost inactive serologically, while the supernatant, precipitated with more alcohol, yielded a small fraction containing all three substances. A_{1a} was redissolved in a few cubic centimeters of water and made up to 200 cc. with glacial acetic acid, giving a copious precipitate which was redissolved, reprecipitated with acetic acid and redistilled alcohol, dissolved once more in water, and isolated by pouring into redistilled acetone as in the case of the other fractions. Usually 1.0 to 2.5 gm. of this inactive polysaccharide was obtained.

Isolation of "C Substance" from Type I Pneumococcus Broth.—16 l. of Type I pneumococcus broth were worked up according to a recently published short method for the isolation of the specific polysaccharide (4). It was found that the supernatant from the first isoelectric precipitation gave strong precipitin reactions with Type I, II, and III pneumococcus antisera. The supernatant and the acetic acid washings of the SSS I were neutralized and concentrated to small bulk on the steam bath. The "C substance" was then precipitated by adding alcohol until the supernatant was free from reactive material. The precipitate was centrifuged off and dissolved in 0.01 N acetic acid. A small amount of Type I SSS remained undissolved. The solution was precipitated with alcohol and the precipitate tested against Type I, II, and III sera. Large amounts of SSS I seemed still to be present, and the precipitate was accordingly dissolved in water and the solution treated with saturated barium hydroxide solution until no further precipitate formed. The precipitate, consisting mainly of the salt of SSS I, was centrifuged off and the supernatant was freed from barium. The volume was now about 100

cc. The "C substance" was precipitated with alcohol, the precipitate dissolved in about 5 cc. of water and reprecipitated with five volumes of glacial acetic acid. The precipitate was centrifuged off and the supernatant precipitated with alcohol. The acetic acid precipitate consisted largely of SSS I and inactive material, while that precipitated from the acetic acid supernatant with alcohol gave strong reactions with Type III antiserum and a slight test with Type I antiserum. The acetic acid precipitation of the latter fraction was repeated four times, or until the more soluble fraction, dissolved in 5 cc. of water, no longer contained material insoluble in five volumes of glacial acetic acid. After repeated precipitation with redistilled alcohol and redistilled acetone it was filtered off and dried. The yield was 60 mg.

Isolation of the "C Substance" from Type III Pneumococcus Broth.—It was found that the alcoholic supernatants obtained in the isolation of the specific carbohydrate of Type III pneumococcus (5) gave strong precipitin reactions with Type I pneumococcus antisera and with Type III antisera that had been completely absorbed with Type III specific carbohydrate. The separation of the "C substance" from the dark nitrogenous impurities in the supernatants from the first two precipitations proved to be difficult. However, the supernatant from the first alkaline alcoholic precipitation (the third precipitation in the series) contained a considerable amount of the "C substance" and was accordingly used as a source of material. Upon acidifying with acetic acid a precipitate formed that gave a positive precipitin reaction while the supernatant was nearly negative. The precipitate was centrifuged off and then taken up in water. A small amount of insoluble material was centrifuged off and the supernatant precipitated with alcohol. This was repeated as long as a residue insoluble in water was obtained. Four alcoholic precipitations were required. The final precipitate was taken up in 1.5 cc. of water and 5 cc. of 10 per cent trichloracetic acid were added. A precipitate formed and was centrifuged off. The supernatant was precipitated with alcohol. The precipitate was taken up in 5 cc. of water and precipitated with five volumes of glacial acetic acid. The "C substance" in the supernatant was precipitated with alcohol and the fractional precipitation with glacial acetic acid repeated twice as above. The substance was then freed from acetic acid by repeated precipitations with redistilled alcohol and was then precipitated by pouring into redistilled acetone. The yield was 100 mg. from 24 l. of broth culture.

The properties of the fractions are summarized in Tables I and II, while Table III gives comparative data for the specific polysaccharides of Type I, II, III, and IV pneumococcus and for the inactive and "C" substances.

Pentose was determined in the different preparations by a modification of the Pervier and Gortner method (6) further adapted as a semi-micro-method by the use of smaller apparatus and tubes of narrower bore.

The sample, dissolved in 100 cc. of 12 per cent HCl, was heated to boiling over a small flame and distilled in a slow current of steam for 6 hours, about 500 cc. of distillate being collected. The concentration of HCl in the distillate was adjusted to 3 per cent, 2 cc. of 20 per cent potassium bromide solution was added and the

TABLE I

Summary of Properties of Type IV, "C," and Non-Specific Polysaccharide Fractions

Preparation	$[\alpha]_D$	Acid equivalent	Total N	NH_2 N	Acetyl N	Reducing sugars on hydrolysis (as glucose)	Pentose	Phosphorus	Ash (as Ca)	C	H
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Type IV											
68 B	+33.9°	3200	5.3						0.1		
71 B	+34.1°	3330	5.4	0.4				<0.1	0.3		
72 B	+31.3°	1403	4.7			76.4	14.6				
75 B	+29.2°	2310	5.9		5.9	75.5	12.0		0.1		
76 B	+37.7°	1250	5.2	0.1	5.6		13.3		0.1		
78 B	+17.0°	1380	5.9			67.0	5.4	<0.1	0.0	45.9	6.7
79 B	+35.0°	1420	5.6			68.0	7.9		0.2		
"C" substance											
76 A_2	+49.3°	1250	6.2	0.7	3.7	37.0	5.0		0.9		
78 A_2	+33.0°	1000	6.6	1.1	3.7	40.0	3.1		1.1	42.1	6.7
80 (from Pneumococcus I)	+35.9°	690	5.8					4.0	<1.0		
81 (from Pneumococcus III)	+51.2°	1270	5.8	0.8	3.7	30.4		4.0	<1.0		
Inactive fraction											
75 A_{1a}	0°	5600	5.7	0.0		58.5			0.0		
76 A_{1a}	+20.2°	3970	6.0		5.6	52			0.1	46.9	6.7

titration made with 0.01 N potassium bromate solution, using starch-iodide solution as an outside indicator. A definite blue color 2 minutes after the addition of the last portion of bromate was taken as the end point. A blank was run upon an equal volume of 3 per cent hydrochloric acid containing the same concentration of potassium bromide, titrating it to the blue color taken as the end point in the

TABLE II

Specificity Tests with Antipneumococcus Sera, Types II and IV

0.5 cc. serum 2:3, 0.5 cc. dilution.

Preparation	Serum II	Serum IV	Preparation	Serum II	Serum IV	Preparation	Serum II	Serum IV
75 A_{1a} 1:200	++±	±	76 A_{1a} 1:1000	++±	+±	78 A_{1a}		
1000	±	−	10,000	+	±	Not tested		
10,000	−	−	100,000	−	−			
						A_2 1:10,000	++	++++
A_2 1:1000	++	++	A_2 1:1000	−		100,000	++±	+±
10,000	+±	±	10,000	++		500,000	++	− (+)
100,000	+	−	100,000	+++	++±	1,000,000	+±	
500,000	−	−	500,000	++±	− (+)	1,500,000	+	
			1,000,000	+±		2,000,000*	+	
B 1:10,000	+±		1,500,000	+				
100,000	±	++±	2,000,000	+		B 1:1000	±	
1,000,000		+				10,000	+	
1,500,000		±	B 1:1000	++		100,000	− (−)	++++
			10,000	+		1,000,000		+ (+)
A_2 with nitrous			100,000	− (−)	++++	1,500,000		− (+)
acid, neutral-			1,000,000		+ (++)	2,000,000		− (±)
ized, diluted			1,500,000		± (+)			
to 1:10,000	±		2,000,000*		+ (+)	79 B 1:1000	+	
						10,000	+±	
B treated simi-						100,000	± (+)	++±
larly, 1:10,000		++++				500,000	− (−)	+ (+±)
						1,000,000		± (+)
						1,500,000		± (+)
						2,000,000*		± (+)

Readings in parentheses obtained after centrifuging at low speed.
All serum and dilution controls negative even after centrifuging.
* Highest dilution tested.

pentose determinations. The amount of pentose was calculated as 0.01 cc. of N bromate x 0.7505 = milligrams of pentose.

The method was checked by determinations of pentose in gum arabic and upon pure recrystallized xylose.

10.0 mg. xylose distilled 6 hours required 14.5, 14.6 cc. 0.01 N $KBrO_3$. Blank 1.0 cc.

13.55 x 0.7505 = 10.17 mg. pentose.

28.3 mg. gum arabic required 15.2 cc. 0.01 N $KBrO_3$. Blank 1.0 cc.

14.2 x 0.7505 = 10.65 mg. pentose = 37.6 per cent pentose.

14.3 mg. gum arabic required 8.0 cc. $KBrO_3$. Blank 1.0 cc.

7 x 0.7505 = 5.25 mg. pentose = 36.7 per cent pentose.

On some samples pentose and -uronic anhydride were determined at the same time by the method of Bowman and McKinnis (7) using the modified method of Pervier and Gortner for determining the pentose.

The sample, dissolved in 12 per cent hydrochloric acid, was distilled in a current of steam and carbon dioxide-free air. The furfural and steam were condensed in an ice-cooled receiver and the CO_2 was absorbed in 0.02 N barium hydroxide solution after passing through an absorption bulb containing silver nitrate to remove traces of hydrochloric acid.

The apparatus and method were checked upon a sample of pure, crystalline aldobionic acid from gum arabic (8).

100 mg. of $C_{12}H_{20}O_{11} \cdot 2H_2O$ required 28.15 cc. 0.02 N $Ba(OH)_2$. Blank, 2.30 cc. 28.15 − 2.3 = 25.85 x 0.44 = 11.32 mg. CO_2 or 45.28 per cent -uronic anhydride. Calculated for $C_{12}H_{20}O_{11} \cdot 2H_2O$ = 44.9 per cent.

Pentose: 650 cc. distillate; 300 cc. aliquots titrated. Required, 10.2, 9.7 cc. 0.01 N $KBrO_3$. Blank 0.50 cc. 9.7 x 0.75 = 7.27. 9.2 x 0.75 = 6.90. Mean. 7.08 mg. pentose. $7.08 \times \frac{650}{300}$ = 15.34 mg. pentose, or 46 per cent -uronic anhydride. No CO_2 was evolved in the case of 78 B and 79 B.

The acetyl groups were determined by either of two methods: (1) by distilling from a 10 per cent sulfuric acid solution in a current of steam, maintaining the volume constant and titrating the distillate; (2) by distilling from a phosphoric acid solution containing 3 cc. of 85 per cent phosphoric acid. The flask was heated in an oil bath at 140° and the solution evaporated nearly to dryness between additions of 25 cc. portions of water. This method had the advantage of giving smaller blanks and smaller volumes of distillate for titration.*

* Private communication from Prof. Hans T. Clarke.

The acetic acid in the distillate was identified by converting it into the silver salt with silver carbonate, isolating and identifying the crystalline silver acetate.

Silver acetate from SSS 73:* 0.0294 mg. gave 0.0250 gm. AgCl. Found: 64.0 per cent Ag. Calculated: 64.7 per cent Ag.

Hydrolysis of the Inactive Polysaccharide ($76A_{1a}$).—1.0 gm. of substance was dissolved in 50 cc. of normal hydrochloric acid, boiled under a reflux for 3 hours, treated with acid-washed norit, and filtered. $[\alpha]^{D}_{24}$ was $+43.8°$ ($\alpha_D + 1.75°$, $l = 2$). The solution was concentrated to small bulk *in vacuo*, chilled, and seeded with glucosamine hydrochloride, 0.15 gm. of the salt separating. For analysis it was recrystallized from water.

0.0743 gm., volume 5.0 cc., $l = 1$; initial α, $+1.37$, $[\alpha]^{23}_{D}$ $+92.2°$; final α, $+1.04°$, $[\alpha]_D$ $+70.0°$. For glucosamine hydrochloride: Initial $[\alpha]^{20}_{D}$ $+100°$; final, $+72.5°$ (9).

The filtrate from what is thus almost certainly glucosamine hydrochloride appeared to contain a mixture of nitrogenous sugar derivatives, but yielded no definite products on fractionation. The alcohol insoluble portion gave analytical figures in agreement with the dihydrochloride of a diaminotrisaccharide, an intermediate product analogous to the acid-resisting triglucosamine hydrochloride encountered by Karrer and Hofmann in the hydrolysis of chitosan by snail juice (10).

Like chitin and chitosan, the inactive polysaccharide is partially hydrolyzed by snail juice. It yields an insoluble product and a small proportion of soluble reducing sugars. The snail juice, however, did not destroy the specificity of the Type IV and "C" polysaccharides in spite of their chemical relationship to chitin.

Hydrolysis of the Type IV Specific Polysaccharide.—1 gm. of a mixture of several active fractions was dissolved in 50 cc. of normal hydrochloric acid and boiled under a reflux for 2 hours. The specific rotation, which was originally $+33.5°$, dropped to 12°, and the reducing sugars,† calculated as glucose, rose to 65 per cent. After another

* This was a preparation of Type IV specific substance precipitated by alcohol from alkaline solution, and washed with alcohol in order to ensure absence of adsorbed acetic acid.

† By the Schaffer-Hartmann micro-method, *J. Biol. Chem.*, 1921, **45**, 363.

hour's boiling $[\alpha]_D$ was $+7°$ and the reducing sugar content 64 per cent. The solution was decolorized with norit and concentrated repeatedly *in vacuo*, first with the addition of methyl alcohol and finally with methyl alcohol and benzene in order to drive off the last traces of water. The resulting spongy mass was extracted twice with hot absolute ethyl alcohol, the total volume being 250 cc., and was almost entirely soluble. Neither fraction yielded glucosamine hydrochloride. On concentration of the solution to small bulk *in vacuo* it deposited 0.25 gm. of a solid. $[\alpha]_D^{25}$ was $+27.8°$ (0.1134 gm. substance dried *in vacuo* at 61°, α, $+0.63°$, $l = 1$, 5.0 cc.) and no mutarotation was observed.

Micro-Kjeldahl on 0.5 cc. of this solution: 2.62 cc. N/70 HCl used; N, 4.6 per cent.

Micro-Volhard on 0.25 cc.: 0.85 cc. N/50 $AgNO_3$ used. Cl, 10.6 per cent.

Reducing sugars: 0.5 cc. made up to 20.0 cc., 5 cc. samples, 0.8, 0.8 mg. glucose. As glucose, 28.2 per cent. After hydrolysis in 1.5 N HCl for 2 hours, 1.63 mg. glucose. As glucose, 57.5 per cent.

Since it seemed possible that more or less methyl glucoside had been formed after the addition of methyl alcohol to the original concentrate which contained an excess of hydrochloric acid, a portion was boiled for 2 hours with 1.5 normal hydrochloric acid, and it was indeed found that the reducing sugar content, calculated as glucose, rose to 57.5 per cent. This would be the equivalent of one reducing group in a substance of molecular weight 313, while the minimum molecular weight on the basis of the nitrogen percentage is 305, and on the basis of the chlorine 335. The material might thus consist chiefly of the hydrochloride of an aminodihexose or an aminohydrochloride derivative of a disaccharide composed of one molecule of pentose and one of hexose.

DISCUSSION

Owing to the relatively small amounts of the polysaccharides in the Type IV cultures and their relatively great solubility in alcohol—at least before their isolation—these substances were far more difficult to separate from accompanying protein degradation products than the specific polysaccharides of Type I, II, and III pneumococcus. Once this separation had been fairly well effected, there remained the problem of separating each of three nearly similar polysaccharides from the

mixture. That this was not rigorously accomplished is evident from the varying data on successive preparations presented in Table I and the cross reactions with immune serum noted in Table II. It will be seen that each preparation of a polysaccharide of Type IV pneumococcus was contaminated with more or less of the accompanying carbohydrates, but in spite of this, marked differences in the chemical and specific properties were discernible. The serologically inactive fraction has the lowest optical rotation and the highest carbon content of the three, is the weakest acid, is the least soluble in alcohol or acetic acid, and differs from the Type IV and "C" substances in yielding, on hydrolysis, crystals with the optical rotation of glucosamine. The Type IV specific substance, on the other hand, differs from the others in being the poorest in nitrogen and the richest in reducing sugars on hydrolysis, and in occupying an intermediate position as regards optical rotation, carbon content, and acidity. The species-specific, or "C" polysaccharide, is the highest in optical rotation and in total and amino nitrogen, and the poorest in reducing sugars yielded on hydrolysis, in its carbon content, and in its proportion of acetylated nitrogen.* It differs from the fully acetylated Type IV substance in being broken down by nitrous acid, and differs from all the hitherto investigated specific polysaccharides of Pneumococcus in containing phosphorus.

At the suggestion of Dr. Thomas Francis, Jr., of the Hospital of The Rockefeller Institute for Medical Research the "C" substance was analyzed for phosphorus, and 4 per cent of this element was actually found. The "C" substance is thus the first phosphorus-containing specific polysaccharide to be encountered. The phosphorus is firmly bound in organic combination, as no test for phosphate can be obtained with the molybdate reagent until the solution of the polysaccharide has been heated with acid. At 100° phosphoric acid is only slowly split off by normal hydrochloric acid or sodium hydroxide.

From Table I it is seen that the "C" substance, whether derived from Type I, III, or IV pneumococcus broth, showed a higher optical rotation, higher nitrogen, and in two cases a higher reducing sugar content on hydrolysis than reported by Tillett, Goebel, and Avery. These

* The acetyl groups are considered to be attached to nitrogen rather than to oxygen, since they survived treatment with strong alkali during the purification process.

workers prepared their material from "R"-pneumococcus cells, and the discrepancies in the two sets of data are perhaps due to the presence of impurities derived from the cells on the one hand, and from the broth on the other. The definite, though small, fraction of the nitrogen now found to be reactive as amino nitrogen, is, however, better in keeping

TABLE III

Comparison of the Type-Specific, Species-Specific, and Non-Specifically Reactive Polysaccharides of Pneumococcus

Polysaccharide	$[\alpha]_D$	Acid equivalent	Total N	Amino N	Acetyl N	Hydrolysis products	
			per cent	*per cent*	*per cent*	*calculated as glucose, per cent*	
Type I	+300°	310	5.0	2.5	0	28	(Galacturonic acid) (Amino sugar derivative)
Type II	+74°	1250	0.0			70	Glucose
Type III	−33°	340	0.0			75	Aldobionic acid, glucose
Type IV*	+30°	1550	5.5	0.1	5.8	71	(Amino sugar derivative) Acetic acid
Species-specific ("C" substance)†	+42°	1050	6.1	0.9	3.7	36	(Amino sugar derivative) Phosphoric acid Acetic acid
Inactive†	+10°	4540	5.9	0.0	5.6	55	(Glucosamine) (Amino sugar derivative) Acetic acid

* Average values, omitting 68 B and 71 B. Calculated for $C_6H_9O_4NHCOCH_3$: N, 6.9 per cent; for $C_6H_{10}O_5 \cdot (C_6H_9O_4NHCOCH_3)_2$: N, 4.9 per cent.

† Average values.

with the fact first reported by Tillett, Goebel, and Avery that the substance is destroyed by nitrous acid. According to the analytical data, one nitrogen atom in every seven is subject to attack by nitrous acid. while four in every seven are protected by acetyl groups.

If the phosphorus in the "C" substance is present in its usual form of combination with sugars, that is, as phosphoric acid attached to two

hydroxyl groups, with one acid group free, and if this represents the total acidity of the substance, 4 per cent of phosphorus would correspond to an acid equivalent of 775. The higher values found in three cases are possibly to be explained by the presence of basic ash.

The differences between the three polysaccharides isolated from Type IV pneumococcus are summarized in Table III, which also shows the corresponding data for the Type I, II, and III specific substances. The differences between the latter have already been discussed (11), but now that more material is available it is seen that the specific polysaccharides of Pneumococcus fall into two sharply defined groups: on the one hand, the Type II and Type III substances, which are nitrogen-free, and on the other, the Type I, Type IV, and "C" substances, which contain nitrogenous sugars. The last two examples in this group, with their content of acetylated nitrogen, are more closely related to chitin than is the Type I substance. In the nitrogenous group, the Type I substance differs sharply from the others in its high optical rotation, its pronounced amphoteric character, its insolubility at the isoelectric point, its freedom from acetyl groups,* and in its high proportion of nitrogen susceptible to attack by nitrous acid. The "C" substance differs from the other members of both groups in its phosphorus content, but resembles the Type I substance in that its specificity is destroyed by nitrous acid, and is somewhat similar to the Type IV substance in that a part of the nitrogen is acetylated. The Type IV specific substance, on the other hand, differs from the Type I and "C" substances in containing only acetylated nitrogen and in yielding as high a percentage of reducing sugars on hydrolysis as do the nitrogen-free Type II and Type III substances.

It is plain that the study of the Type IV specific polysaccharide has brought to light a carbohydrate of a type new amongst those with specific properties, but somewhat resembling chitin in its general structure. It has, moreover, again shown that in the closely related pneumococcus types thus far studied, each polysaccharide responsible for type specificity is radically different from the others in structure, composition, and properties. The study has also shown the presence in the culture of a serologically inactive polysaccharide, chemically similar to the Type IV substance, but even more closely related to

* Unpublished experiments.

chitin. Whether this fraction is derived from the broth or is a degradation product of the bacterial "skeletal" substance is not known; but this uncertainty does not apply to the Type IV specific substance, which is undoubtedly derived from the bacteria themselves. Thus the finding of one, or possibly two substances similar in structure to chitin, but not identical with it, may point toward the cause of the still existing uncertainty as to whether or not chitin is a constituent of the bacterial cell wall (12). Finally, owing to the presence of the "C" substance or species-specific polysaccharide in the crude material, it has been possible to extend the observations of Tillett, Goebel, and Avery (*loc. cit.*) and to contrast the properties of this substance with those of the type-specific polysaccharides of Pneumococcus.

SUMMARY

1. Three nitrogen-containing polysaccharides have been isolated from autolyzed cultures of Type IV pneumococcus: (1) a type-specific carbohydrate differing markedly from those of Type I, II, and III pneumococcus, and representing a type of substance hitherto not observed among specific polysaccharides, (2) a chemically similar carbohydrate without specific function, and (3) the "C" substance, or species-specific polysaccharide of Tillett, Goebel, and Avery.

2. The chemical differences between the specific polysaccharides of Pneumococcus are discussed, and the relationship of the new examples to chitin is pointed out and its bearing indicated on the unsettled controversy as to whether or not chitin occurs in bacteria.

3. The data of Tillett, Goebel, and Avery on the "C" substance have been extended.

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