A "SOLUBLE SPECIFIC SUBSTANCE" DERIVED FROM GUM ARABIC.*

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The unforeseen identification of the so-called "soluble specific substances" (1) of Pneumococcus as polysaccharides (2) has led to an accumulation of evidence that analogous sugar derivatives play an important part in the immunological relationships of micro-organisms of the most diverse types (3). Thus, specifically reacting substances with the properties of carbohydrates have also been isolated from the Friedländer bacillus, the tubercle bacillus, the typhoid-colon group, and yeast; and evidence has been obtained of the existence of similar substances in Streptococcus, the anthrax bacillus, and other pathogenic microbes. This wide distribution of specifically reacting polysaccharides made it seem not improbable that there might occur among higher plant forms other sugar derivatives with specific properties. A number of water-soluble gums of plant origin were therefore tested against anti-pneumococcus sera of Types I, II, and III, and of these gums several were found to give the precipitin reaction. Since occasional samples of gum arabic (gum acacia) precipitated Type II (and Type III) antiserum at as high a dilution as 1:25,000 (*cf.* Table I, 47_2), but did not precipitate normal horse serum or Type I antiserum, this gum was chosen for further study.

It was soon found that the ordinary methods of fractional precipitation from neutral, acid, or alkaline solutions yielded products differing

* An abstract of this paper was presented at the Annual Meeting of the American Chemical Society in Philadelphia, in Sept., 1926. The paper itself was submitted for publication at the same time, but was withdrawn until more positive analytical data on the calcium aldobionate were available. In the meantime Cretcher and Butler have also published a note (*Science*, Aug. 3, 1928) indicating the presence of an aldobionic acid among the products of hydrolysis of gum arabic and have a paper in press giving further details.

little in their specific reactivity. Since it was known that the soluble specific substance of Pneumococcus was comparatively resistant to strong acid in the cold (*2b*, p. 305), fractional hydrolysis of this type was resorted to in the hope that the non-specific portions of the gum would prove the more easily hydrolyzed. This appeared to be the case, as the polysaccharide fraction recovered was found to possess a degree of specificity comparable with that of the bacterial specific substances (*cf.* Table I, 47_{10BIV}, 56). From the fact that 50 per cent of material 100 to 150 times as active as the original gum was recovered it is evident, however, that the process involves more than a mere hydrolysis of accompanying inert material. This point will be taken up more fully in the discussion.

The gum arabic purified in this way resembled in its physical properties the arabic acid so frequently described as the principal constituent of the gum (4). On hydrolysis, however, it yielded mainly galactose and an acid fraction consisting of at least 2 substances. Investigation of these acids has indicated that they are possibly disaccharide (aldobionic) acids of the type recently described as the principal product of hydrolysis of the soluble specific substance of Type III pneumococcus (*3a* and *3b*). Such a relationship would be of considerable theoretical interest in a study of the chemistry of bacterial specificity. The large quantities of starting material available should facilitate the investigation of aldobionic acids to an extent impossible in the case of the polysaccharides elaborated by pathogenic bacteria.

EXPERIMENTAL.

1. Isolation of Reactive Material from the Original Gum.

200 gm. of Squibb's powdered gum acacia were dusted slowly into 1 liter of chilled 1:1 hydrochloric acid. The mixture was stirred until solution was complete and allowed to stand at room temperature for 2 days. A small amount of dark, insoluble material was centrifuged off and the clear liquid precipitated with about 3 volumes of chilled alcohol. After the gum had settled the supernatant was poured off and the precipitate macerated with fresh alcohol. After several hours this was decanted and the gum was dissolved in water, centrifuged if necessary, and reprecipitated with redistilled acetone in the cold. After several hours the precipitate was stirred with fresh acetone, ground up under acetone when thoroughly hardened, filtered, washed with acetone until free from hydrochloric acid, and dried *in vacuo* over calcium chloride and crushed sodium hydroxide.

The yield was 70 to 80 gm. This product (Table I, 56) corresponded closely to that obtained in 97.5 gm. yield by a single acid treatment of 24 hours (47_{10}) and to preparation 47_{10BIV} obtained in 64 gm. yield by 2 single acid treatments of 24 hours each. It was sometimes necessary to redissolve the gum in water a second time and reprecipitate with acetone in order to remove all chlorine ion.

The product so obtained still contained about 0.3 per cent of nitrogen, or the entire amount in the original gum. 60 gm. of preparation 47_{10BIV} were therefore dissolved in 300 cc. of water and stirred ½ hour with 5 cc. of 30 per cent sodium nitrite solution and 25 cc. of acetic acid. About 1.5 volumes of acetic acid were then added, precipitating most of the gum. After 2 hours the deposit was drained, dissolved in about 200 cc. of water, and reprecipitated with acetic acid. It was finally treated with successive portions of redistilled alcohol and acetone, ground up, washed thoroughly with acetone, and dried as before. The yield was 42.3 gm. This product (Table I, 51E) contained less than 0.1 per cent of nitrogen and reacted with Type II anti-pneumococcus serum at a dilution of 1:5,000,000. It also precipitated Type III anti-pneumococcus serum. The fraction of the gum not thrown down by the acetic acid resembled the precipitated portion in all its properties, but it contained 0.5 per cent of nitrogen and was somewhat less reactive with Type II serum.

The purified gum is a white powder, readily soluble in water. It possesses marked acidic properties and rotates the plane of polarized light weakly to the left, somewhat more strongly on neutralization. It gives a positive naphthoresorcin test. When hydrolyzed it yields 68 per cent of reducing sugars, calculated as glucose, but since about one-third of the products of hydrolysis appear to be disaccharide or polysaccharide acids (see below), the actual yield of reducing sugars is higher. The pentose content, 19 per cent, calculated from the yield of furfural on distillation with hydrochloric acid,[1] is about one-half that of the original gum, so that much of the portion hydrolyzed in the method of preparation consisted of pentose or pentosan. Part of the remaining material which reacts as pentose is accounted for by the sugar acid fraction. The principal hexose component of the purified gum is galactose.

[1] A modification of Pervier and Gortner's method (5) was used. Instead of titrating at an acidity of 4 per cent and plotting the end-point with the aid of a bromine electrode, galvanometer, and stop-watch, it was found simpler to use an outside spot indicator of starch iodide solution. At an acidity of 3 per cent, the end-point is taken as the first burette reading at which a spot test is still obtained after 2 minutes. Large drops should be withdrawn for the test.

2. Attempts at Further Purification of the Specific Fraction.

The specific fraction of the gum is incompletely precipitated by barium hydroxide in large excess. The precipitate soon turns yellow, and as will be seen from Table I the recovered gum (51B) shows practically the same properties as the original material.

Uranyl nitrate also precipitates the gum incompletely when the excess acid is neutralized, but in this case also no purification results.

Partial adsorption of specific gum on "Type C" aluminium hydroxide (6) resulted only in a product with the properties of the starting material (Table I, 54D).

Fractionation of the specific product with hydrochloric acid and alcohol gave 3 portions with practically identical properties.

3. Precipitation of the Specific Gum by Means of Type II Pneumococcus Antibodies.

An attempt was made to determine whether the specific gum could be precipitated by Type II pneumococcus antiserum and recovered from the precipitate. This was of importance not only in establishing the polysaccharide as actually analogous or not to the specific polysaccharides of bacterial origin, but also in determining whether the reaction with Type III antiserum was caused by an accompanying impurity or was an inherent property of the specific polysaccharide itself. Thus, an accompanying substance which did not precipitate Type II serum, but yielded a precipitate with Type III serum, should be largely eliminated in effecting a specific precipitation with Type II serum.

3 liters of Type II pneumococcus antibody solution, prepared essentially by Felton's method (7), were precipitated by a slight excess of neutralized 1:1000 saline solution of preparation 47_{10}. The amount of precipitate was small, and only 0.07 gm. of specific gum was recovered by the method already described in detail in the case of the soluble specific substance of Type II pneumococcus (2*c*, p. 737).

Except for an unavoidably high nitrogen content (0.6 per cent) and a higher specific reactivity toward both sera the product (Table I, 47_{10}A) resembled the starting material in its analytical and physical properties. It still gave a precipitate with barium hydroxide in excess, gave the brown red color characteristic of galactans with orcin

TABLE I.

Properties of Gum Arabic and Derived Specific Fractions.

Preparation No.	$[\alpha]_D$		Acid equivalent	Nitrogen	Reducing sugars on hydrolysis		Highest dilution precipitating pneumococcus antiserum		Ash	
	Free acid	Na salt			Total	Pentose	Type II	Type III		
				per cent	*per cent*	*per cent*			*per cent*	
47_2		−31.0°		0.35	77.4	40	25,000	25,000	1.4	Squibb's gum acacia
47_{10}	−7.5°	−8.8°	1006			15	3,000,000*	1,000,000	0.3	Single acid treatment, 24 hours
$47_{10\text{-BIV}}$	−12.0°	−16.0°	906			19	4,000,000	+	0.03	Two acid treatments
$47_{10}A$		−20.0°	665	0.6	67.3		8,000,000	2,000,000	0.3	Recovered from immune precipitate
51B	−12.0°			0.15			4,000,000	+	0.1	From $Ba(OH)_2$ precipitate
51E	−10.7°	−14.3°	856	0.08	68.0	18	5,000,000	+	0.06	47_{10BIV}, treated with HNO_2 and precipitated with acetic acid
54D	−11.5°	−16.6°	723	0.2	68.5	18	4,000,000	+	0.03	Adsorbed on $Al(OH)_3$
56	−7.5°	−12.5°	866	0.33	68.0	19	3,000,000†	+	0.13	Single acid treatment, 48 hours

* There is apparently relatively little antibody to the specific gum in Type II and Type III antisera. In the former case the precipitate is a transparent jelly, and the tubes containing the higher dilutions must be centrifuged in order to render the deposit more compact and more easily visible. The Type III precipitate is loose and flocculent until centrifuged.

† Highest dilution tested.

and hydrochloric acid, and showed a positive naphthoresorcin test. Not only did it react at a dilution of 1:8,000,000 with Type II antipneumococcus serum, but also at a dilution of 1:2,000,000 with Type

III serum, indicating that it is actually the same substance which precipitates with both sera.

While this experiment seemed fairly conclusive in establishing the purified gum as a true "soluble specific substance," it remained possible that the polysaccharide reacted with some other constituent of the serum than the pneumococcus antibodies themselves.

Accordingly a portion of the Type II antiserum was absorbed by means of a saline suspension of heat-killed Type II pneumococci. Another portion of the serum was treated with small amounts of a 1:10,000 solution of the Type II soluble

TABLE II.

Dilution of preparation 47_{10BIV}	Type II serum unabsorbed	Type II serum absorbed with Pneumococcus II	Type II serum absorbed with soluble specific substance II	Type II antibody solution absorbed with specific gum arabic
1:1,000	++++	−	−	
1:5,000	+++	−	−	
1:25,000	+++	−	−	
1:250,000	++	−	−	
1:1,000,000	−	−	−	
Saline	−	−	−	
Dilution of Type II soluble specific substance, 1:20,000			−	++++
Agglutination of Type II pneumococci			−	++++

Dilution of serum, 2:3. Tubes were not centrifuged. After centrifugation the 1:1,000,000 dilution reacted + in the unabsorbed serum and all the tubes containing serum absorbed by Pneumococcus II contained a trace of scaly precipitate. Otherwise the results were unchanged.

specific substance until no further precipitate could be obtained after 2 hours at 37° and standing over night in the ice-box. The reaction of the purified gum with these sera and untreated Type II serum was then tested, giving the results shown in Table II.

4. Hydrolysis of the Specific Polysaccharide.

A. 41 gm. of preparation 51E were dissolved in water, treated with 85 cc. of concentrated sulfuric acid, diluted to 3 liters, and boiled for 4 hours, a preliminary experiment having shown a maximum reducing power after this time. The sulfuric acid was removed quantitatively with barium hydroxide, and to the filtrate, concentrated *in vacuo* to about 300 cc., basic lead acetate solution was

added in slight excess. The precipitate was suspended in water and treated with acetic acid in small amounts until only a small amount of yellow precipitate remained. The filtrate from this was again precipitated with an excess of basic lead acetate. The lead salt was decomposed with hydrogen sulfide and the solution concentrated repeatedly *in vacuo* to a syrup in order to eliminate acetic acid. The residue was taken up in hot water, boiled with acid-washed Norite, and concentrated to dryness, finally in a high vacuum. The yield of crude sugar acid was 5.5 gm. A determination of the reducing power by the Shaffer-Hartmann micromethod (8) gave a value of 44.6 per cent, calculated as glucose.

0.3329 gm., made up to 15 cc. with H_2O: α_D, 0.39°, $l = 2$. $[\alpha]_D = +8.8°$.

1 cc. of the same solution required 2.62 cc. 0.02 N NaOH for neutralization to phenolphthalein. Acid equivalent, 427. Calculated for $C_{12}H_{20}O_{12}$, 356.

The filtrates from the first and second precipitations of the lead salt were freed from lead, concentrated to small bulk, boiled with Norite, concentrated to a syrup, and, while still warm, were treated with about 2 volumes of glacial acetic acid and seeded with a few crystals of galactose. Crystallization took place rapidly, and the solid cake which formed over night in the ice-box was crushed, sucked off *in vacuo* on a Buchner funnel, and washed first with chilled 66 per cent acetic acid, then with the glacial acid, and finally with alcohol. The yield was 8.3 gm., with an initial $[\alpha]_D$ of +120.5°.

1 gm. of the crude sugar, oxidized with warm 1:1 nitric acid, began to deposit crystals within a few hours and ultimately yielded 0.46 gm. of mucic acid, melting at 214–215° with decomposition.

0.1009 gm. gave 0.1279 gm. CO_2 and 0.0458 gm. H_2O.

Calculated for $C_6H_{10}O_8$: C, 34.27 per cent; H, 4.80 per cent. Found: C, 34.57 per cent; H, 5.08 per cent.

6.8 gm. of the sugar itself were dissolved in water, boiled with Norite, and recrystallized as before, yielding 5.4 gm. of purified sugar, which from its analysis, rotation, and the isolation of mucic acid in good yield from the crude product, was chiefly galactose.

0.1007 gm. gave 0.1480 gm. CO_2, and 0.0620 gm. H_2O.

Calculated for $C_6H_{12}O_6$; C, 39.98 per cent; H, 6.72 per cent. Found: C 40.08 per cent; H, 6.89 per cent.

0.5513 gm., made up to 10 cc. with H_2O, gave an initial reading of 6.55° and a final value of 4.05°, $l = 1$. $[\alpha]_D$, initial, +118.8°; final, +73.5°.

The galactose isolated in this crop was not as pure as that recovered in later fractions (see below), but whether the impurity was the sugar acid still present in the mother-liquors, or some other sugar, has not been determined.

The filtrate from the initial crop of galactose was repeatedly diluted with water and concentrated *in vacuo* in order to remove acetic acid. It was then diluted to about 350 cc. and again treated with basic lead acetate solution, yielding a heavy

precipitate. This salt was worked up as was the first lead salt, and yielded 3.9 gm. of a crude sugar acid resembling the first product except in its rotation and somewhat lower reducing value, the latter being 39.2 per cent, calculated as glucose.

0.3064 gm., made up to 15 cc. with H_2O: α_D $-0.17°$, $l = 2$. $[\alpha]_D = -4.2°$.

2 cc. of the same solution required 4.53 cc. 0.02 N NaOH for neutralization to phenol red. Acid equivalent, 450. Calculated for $C_{12}H_{20}O_{12}$, 356.

The filtrate from the above lead salt was freed from lead and acetic acid, taken up in a little water, neutralized with barium hydroxide, and again concentrated to a thick syrup. This was boiled with 3 successive 200 cc. portions of 90 per cent alcohol. The insoluble residue, purified over the lead salt, gave 2.0 gm. of a product which appeared to be a mixture of sugar and sugar acid, but was not further investigated. The alcoholic solutions after concentration to a syrup readily yielded 3.3 gm. of galactose, which melted at 161–163° after recrystallization.

0.5522 gm., made up to 10 cc. with H_2O, gave an initial reading of 7.52° and a final value, after addition of 0.5 cc. concentrated aqueous NH_3, of 4.11°, $l = 1$. $[\alpha]_D$, initial, $+136.2°$; final, $+78.2°$.

In Beilstein, 3rd edition, vol. i, p. 911, the rotations given for pure galactose are $[\alpha]_D^{20}$ $+140°$, 80.5°, respectively.

The mother liquors from the galactose contained but 1.7 gm. of sugar, calculated as glucose, and were not investigated.

(*B*).[2] 49 gm. of a product (active with Type II serum at a dilution of 1: 4,000,000) were hydrolyzed as in the previous instance. After removal of the sulfuric acid the concentrated solution was boiled with calcium carbonate and Norite, filtered, concentrated to small bulk, and fractionated with methyl alcohol as in the case of the calcium aldobionate derived from the Type III pneumococcus (*3b*). The partially purified salt thus obtained was further fractionated into three arbitrary portions with the aid of methyl alcohol and acetone.

Fractions	I	II	III	Theory
	per cent	*per cent*	*per cent*	*per cent*
Calcium	5.8	6.7	5.5	5.3
Reducing sugars (as glucose) (Schaffer-Hartmann)	44.8	44.3	34.4	48.0
Aldose (as glucose) (Willstätter-Schudel)	53.3	58.1	61.5	48.0

Fraction I[3] thus corresponds fairly closely to a calcium aldobionate, $(C_{12}H_{19}O_{12})_2Ca$, while the succeeding fractions show increasing contamination.

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[2] The experimental work in this section was carried out by one of us (M. H.) in the laboratories of the Mt. Sinai Hospital and the Presbyterian Hospital, New York. For the facilities offered by these institutions, and for the kindness of Dr. Forrest E. Kendall, of the Presbyterian Hospital, in carrying out the analyses, the writers wish to express their hearty thanks.

[3] A crystalline cinchonidine salt, and through this, the crystalline aldobionic acid have since been isolated, and will form the subject of a separate communication.

The analyses indicate that the chief impurity in Fraction II is possibly a salt of the type of calcium glucuronate, while Fraction III presumably contains galactose, especially as the mother-liquors from this deposited crystals of galactose on standing (melting point, 160–168°; yielded mucic acid, melting point, 206–207° on oxidation).

DISCUSSION.

That a polysaccharide with specific properties should occur among the higher plants without any apparent relation to the life processes of micro-organisms, is additional evidence of the wide-spread occurrence of carbohydrates with immunologically specific properties. Speculation as to their function and chemical and immunological relationships, while enticing, must be postponed until more information is at hand. There is evidence which has been interpreted by Beijerinck (9) as pointing to the elaboration of gum arabic as a result of the activities of molds, and by Greig Smith (10) as showing the gum to be a product of the metabolism of certain bacteria. As neither of these workers has proved, however, that contamination of the gum with the appropriate organism did not take place after its formation the hypothesis that gum arabic originates through the activities of micro-organisms, though attractive from the standpoint of bacterial specificity, must be considered as unproved.

In fact, certain chemical data obtained in the present investigation argue against this point of view. On partial hydrolysis about one-half of the original material is recovered with its specific activity increased 100 to 150-fold, showing that the specifically reacting gum does not exist as such in the original gum arabic, but is formed from it, probably by removal of a pentose grouping in glucosidic union, since the specific fraction contains less than one-half the pentose of the gum arabic itself. The low reactivity of the original gum would then be accounted for on the basis of traces of the specific material formed on exposure, in the process of refining, or by enzyme action. It is not excluded however, that the specific gum owes its presence to the synthetic activity of the strong hydrochloric acid on the hydrolytic products of the original gum (11), although this would seem less probable.

Another finding of chemical interest is the large proportion of complex sugar acids. It has recently been shown (*3a* and *3b*) that the chief product of the hydrolysis of the soluble specific substance of

Type III pneumococcus is an aldobionic acid of which one component is glucose and the other glucuronic acid. A similar acid also forms one of the hydrolytic products of the soluble specific substance of the Type A Friedländer bacillus (12). At least one of the crude acid fractions from the hydrolysis of the specific gum arabic corresponds roughly to an aldobionic acid, while in the other the agreement is not so good. While both of these acid fractions may still be mixtures, their rough correspondence to important hydrolytic products of bacterial specific polysaccharides is of interest.

While the exact significance of these more or less complex sugar acids is still to be established, the finding of at least two of these acids as hydrolysis products of the specific gum arabic renders it evident that the specific gum, if actually a single substance in its present state of purity, is a more complex product than the bacterial specific polysaccharides hitherto investigated in detail. The recovery of the specific gum from its precipitate with Type II antipneumococcus serum with its reactivity for both Type II and Type III sera augmented (see experimental part) may be taken as evidence that it is a single constituent of the gum which precipitates both sera. This is in agreement with the relative complexity of the specific gum, since a substance containing the molecular groupings necessary for reaction with antibodies to both Type II and Type III pneumococci might be expected to yield more varied products on hydrolysis than one precipitating either serum alone. Whether any of these hydrolysis products of the specific gum arabic and the specific polysaccharides of Type II and Type III pneumococci are identical cannot be stated as yet. Thus far galactose has been found only in the first of the three, and this constitutes a marked difference.

From Table II it will be seen that Type II antipneumococcus serum which has been precipitated by the specific gum arabic still retains practically unimpaired its agglutinating power for the Type II pneumococcus and its precipitating power for the Type II soluble specific substance, whereas all of the antibodies are removed on absorption of Type II antiserum with Type II pneumococcus. This lack of reciprocal antibody absorption is suggestive of the relationship between Pneumococcus Type II and Type B Friedländer bacillus, in which the writers found (13) a certain chemical similarity between the specific polysaccharides of these bacteria and a corresponding immunological

relationship between the micro-organisms themselves. The serological and antigenic similarity of the otherwise unrelated bacteria was interpreted as an example of heterogenetic specificity; it is not unlikely that the reactions of specific gum arabic with Types II and III antipneumococcus sera may be accounted for in the same way.

SUMMARY.

1. By partial acid hydrolysis a specific carbohydrate may be isolated from gum arabic (gum acacia). This carbohydrate is comparable in its precipitating activity for Type II (and Type III) antipneumococcus serum with the bacterial soluble specific substances themselves.

2. On hydrolysis this fraction yields galactose and two or more complex sugar acids, one of which appears to be a disaccharide acid comparable with those isolated from the specific polysaccharides of the Type III pneumococcus and the Type A Friedländer bacillus.

3. The significance of these findings is discussed.

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