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The Cross-Reactivity of Ketha Gum and Pneumococcal Type I — Short Cut to a Constituent of a Polysaccharide*

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Summary. Although ketha gum has been reported to contain arabinose, galactose, xylose, an unidentified neutral sugar and glucuronic acid, its marked cross-reaction with Type I antipneumococcal horse sera led to the conclusion that D-galacturonic acid must be an additional component. The capsular polysaccharide of Type I pneumococcus, the determinant of immunological specificity, consists of more than 50 per cent of D-galacturonic acid. Chromatography of hydrolysates of ketha gum confirmed the presence of arabinose, galactose and xylose, and furnished spots with the mobilities of galacturonic and 4-O-methylglucuronic acids. Traces of glucuronic acid appeared only under conditions such that demethylation of the 4-O-methyl acid might have occurred. Hydrolysis of larger amounts furnished D-galacturonic acid, as yet syrupy, $[\alpha]_D+32°$ instead of $+51°$, and 4-O-methyl-D-glucuronic acid, $[\alpha]_D+20°$ instead of $+35°$. The latter, especially if present as non-reducing end-groups, would account for the strong precipitation of ketha gum in Type II antipneumococcal horse sera. When ketha gum was esterified, reduced and hydrolysed, the two acids disappeared almost entirely, a component corresponding to 4-O-methylglucose was observed, and the hexose content was increased. Oxidation of the gum with periodate, followed by reduction and hydrolysis, caused the disappearance of the 4-O-methylglucuronic acid and most of the galacturonic acid. Comparison of analyses and chromatograms of the original gum with those of samples recovered from the specific precipitates with Types I and II antisera showed little or no fractionation.

INTRODUCTION

When the composition of ketha gum, the exudate of *Feronia elephantum*, family *Rutaceae*, was originally described (Mathur and Mukherjee, 1952, 1954) it seemed possible that it might precipitate with Type II antipneumococcal sera owing to the stated presence of glucuronic acid. Tests with a number of antipneumococcal (anti-Pn) sera (Heidelberger, 1960a) not only showed that it reacted as expected (also, Heidelberger, 1960b), but that

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it precipitated anti-Pn I even more heavily and regularly. Since the type-specific capsular determinant of Type I pneumococcus contains more than 50 per cent of D-galacturonic acid (Heidelberger, Kendall and Scherp, 1936; Smith, Galloway and Mills, 1960), this pointed to the occurrence of the acid as a major constituent of ketha gum, particularly as other gums, such as those of *Khaya grandifolia* (Aspinall, Hirst and Matheson, 1956) and *K. senegalensis* (Aspinall, Johnston and Stephen, 1960), which contain much D-galacturonic acid, had been shown to precipitate anti-Pn I strongly (Heidelberger, 1960a). The chemical investigation of ketha gum was accordingly taken up once more, with results which not only confirmed the immunochemical prediction of the presence of D-galacturonic acid, but which also led to an alternative explanation of the reactivity of the gum with anti-Pn II.

MATERIALS AND METHODS

The anti-Pn I sera used were furnished by the New York City Department of Health Laboratories through the kindness of Miss Annabel W. Walter and of Dr. M. Solotorovsky (Rutgers) and by Dr. R. Wahl, of the Institut Pasteur, Paris. Quantitative microestimations of antibody nitrogen precipitated by ketha gum from the various antisera were carried out in duplicate at 0° as in previous papers (Heidelberger and Rebers, 1958; Kabat and Mayer, 1961) with the use of a cold-box for drainage of the tubes after centrifugation; determination of N in the washed precipitates was by the Markham (1942) method. The data are collected in Table 1 and experiments on the inhibition of precipitation

TABLE 1

PRECIPITATION OF KETHA GUM IN C-ABSORBED TYPE I ANTIPNEUMOCOCCAL HORSE SERA

Pneumococcal polysaccharide	μg. *Antibody N precipitated at 0° from serum no.*			
	592	*884*	*1057*	*Pasteur Inst. 173*
C	86	70	288	21
Type I (SI)	650	965	1024	1900
Ketha gum				
0.1 mg.	213	107		
0.3	265	203		
0.4				185
1.0	246*	324	417, 404	398
2.0		338	442	398
3.0		346	463	
4.0		334		373
5.0			440	
% type-specific antibody precipitated	41	36	45	21

* Supernatants at this level gave 420 μg. N with SI; total, 666 μg. N.

by sodium galacturonate and sodium glucuronate are shown in Table 2. These also confirm the importance of D-galacturonic acid in the cross-reaction.

For chromatography, Whatman No. 1 or 3 mm. paper was used with the solvent systems *A*, pyridine-ethyl acetate-acetic acid-water, 5:5:1:3 (Fischer and Dörfel, 1955), *B*, n-butanol-pyridine-water, 6:4:3 (Jeanes, Wise and Dimler, 1951) and *C*, n-butanol-benzene-pyridine-water, 5:3:3:1 (upper phase) (Laidlaw and Reid, 1952). Reducing

sugars were detected with aqueous saturated aniline oxalate to which acetic acid was added for ionophoretograms. Ionophoresis on 3 mm. paper (Foster, 1952) was carried out with 0.05 M borate at pH 10 at 500 V for 5 hours. Hexose (as galactose) was determined by the modified primary cysteine reaction (Dische, 1955) and by a modified secondary cysteine reaction (Dische and Danilchenko, 1962), pentose (as arabinose) by the modified basic cysteine reaction (Dische, 1949; Heidelberger, Adams and Dische, 1956) and uronic acid (as galacturonic acid) by the carbazole method (Dische, 1947). Specific rotations were measured in water at 23°.

TABLE 2

INHIBITION OF PRECIPITATION OF KETHA GUM IN TYPE I ANTIPNEUMOCOCCAL SERA BY GALACTURONATE AND GLUCURONATE

Inhibitor	μ *mol. added*	*Total vol.* (*ml.*)	*Antibody N precipitated from* I 592C 0.2 ml. (μg.)	I 1057F,* 0.1 ml. (μg.)	*Inhibition* (%)
None		0.4	33		
Na galacturonate	3.1	,,	11		67
,, ,,	0.3	,,	30		9
,, pectate	(200 μg.)	,,	31		6
None		,,		40	
Na galacturonate	6.2	,,		18	55
,, ,,	0.6	,,		(35)†	(13)
None			30		
Na glucuronate	32.0	0.5	24		20
,, ,,	6.4	,,	28		7

* Partially purified Felton antibody solution, at same anti-SI concentration as whole serum (Table 1).

† One determination only.

There was no inhibition of the SI-1057F reaction by 6.2 μ mol. Na galacturonate, nor of the SI-592C reaction by 32 μ mol. Na glucuronate.

RESULTS

CHROMATOGRAPHIC EXAMINATION OF THE HYDROLYSATES OF KETHA GUM

The gum, 20 mg., was hydrolysed in sealed tubes at 100° with 2 ml. of 2N H_2SO_4 for 5 and 10 hours. The hydrolysates were neutralized with $BaCO_3$ and the filtrates and washings evaporated to dryness and examined for neutral sugars with solvent *B*. Galactose and arabinose were found to be major components, xylose a minor component, and glucose was absent. Rhamnose was either absent, or present only in traces. No neutral oligosaccharides were seen. When the 5 hours' hydrolysate was deionized with Dowex 50W-X2(H) and examined for free acids in solvent *A* galacturonic acid was found, also a small proportion of glucuronic acid (glucurone was not detected on a paper run for 7 hours), a component with $R_{Glu\ A}$ 1.47 and traces of three components, presumably aldobiuronic acids, with $R_{Glu\ A}$ values 0.51, 0.59 and 0.64, which had disappeared from the 10 hours' hydrolysate. All components except the traces of glucuronic acid and aldobiuronic acids were also detected when 3 mg. of gum was similarly hydrolysed for 4 hours.

LARGER-SCALE HYDROLYSIS OF KETHA GUM WITH ISOLATION OF THE URONIC ACIDS

1.5 g. gum was hydrolysed for 4 hours with 2N H_2SO_4 at 95–100°. The solution was cooled, neutralized with $BaCO_3$, centrifuged and the residue washed twice with water. The combined supernatants were evaporated to dryness and treated with 25 ml. water and 25 ml. ethanol. Insoluble Ba salts were centrifuged off and the supernatant was evaporated to a syrup, taken up in 15 ml. water and passed through a column of Amberlite IR-120 (H). The eluate and washings were evaporated to small volume and applied to a column of Duolite A4(OH), to retain acidic material. The column was washed until the Molisch test was negative. When evaporated to dryness the eluate and washings gave a foam, 0.79 g., which on chromatography in solvent *C* showed galactose, arabinose and xylose, with possible traces of rhamnose. Uronic acids were eluted from the resin with 0.5N NaOH and the free acids obtained by passage through IR-120 (H) and evaporation of the eluate to dryness. Neutral sugars were absent (solvent *C*). In solvent *A*, galacturonic acid, identical with an authentic sample, and an acid with $R_{Glu\ A}$ 1.49 were the main components, together with slower-moving products. No glucuronic acid was present, and, indeed, this acid appeared only under conditions such that demethylation of 4-O-methyl-glucuronic acid might have occurred (Croon, Herrström, Kull and Lindberg, 1960).

Separation of the two main acidic components on paper with solvent *A* gave 26 mg. of Fraction 1 and 18 mg. of Fraction 2. Fraction 1 had $[\alpha]_D+26°$ (*c*, 1.29), and was chromatographically identical with D-galacturonic acid. The methyl glycoside of the methyl ester was prepared but also failed to crystallize. Fraction 2, $[\alpha]_D+20°$ (*c*, 0.89), (+35°, Bowering and Timell, 1960), $R_{Glu\ A}$ 1.49, was chromatographically identical with authentic 4-O-methyl-D-glucuronic acid (kindly supplied by Dr. E. E. Percival).

Similar hydrolysis and fractionation of a second 2 g. yielded 17 mg. of galacturonic acid with $[\alpha]_D+32°$ (*c*, 0.85), (lit., +51°) m.p. 168–171° (decomp.), which afforded *p*-bromophenyl-hydrazine *p*-bromophenylhydrazone galacturonate of m.p. and mixed m.p. 145–146° (decomp.) with an authentic sample of m.p. 145–146° (decomp.) (Niemann, Schoeffel and Link, 1933).

Material from an additional hydrolysis yielded a galacturonic acid-containing fraction which was fractionated on charcoal-Celite (1:1) (Whistler and Durso, 1950) and further purified by paper chromatography. The product, 10 mg., had $[\alpha]_D+24°$ (*c*, 0.59). Its methyl ester methyl glycoside, reduced with $NaBH_4$, hydrolysed and chromatographed in solvent *C*, gave a strong spot with the R_f of galactose and several other faint spots.

Reduction and hydrolysis (Hamilton and Smith, 1956) of periodate-oxidized ketha gum followed by chromatography showed that, of the acid components, the 4-O-methyl-glucuronic acid had been destroyed and that only minor amounts of galacturonic acid remained.

ESTERIFICATION AND REDUCTION OF KETHA GUM WITH IDENTIFICATION OF 4-O-METHYLGLUCOSE

The gum, 108 mg., was converted to the methyl ester methyl glycoside by refluxing for 5 hours with 10 ml. 1.5 per cent methanolic HCl and, after neutralization with Ag_2CO_3, the isolated product was reduced with $NaBH_4$, 30 mg. Sodium was removed by Dowex 50W-X2(H) and borate by repeated distillation with methanol. The product, 58 mg., was re-esterified and again reduced. Chromatographic examination of a 4-hour hydrolysate of an aliquot in solvent *B* showed in addition to galactose, arabinose, and xylose, R_{Rh} 0.74, a new spot with R_{Rh} 0.89, which was identical with authentic 4-O-methylglucose. After treatment

with Dowex 50(H), and examination in solvent *A* some galacturonic acid was still found. The bulk of the product was hydrolysed but only a trace of 4-O-methylglucose was recovered after fractionation on paper with solvent *B*. The sugar had M_G 0.30 (Foster, 1953) and after periodate oxidation (Lemieux and Bauer, 1953) gave the yellow spot, R_f 0.6 (approx.), characteristically produced by a 4-O-methylhexose. Chromatography of the recovered galactose in solvent *C*, using multiple development (3 × 12 hours), showed that it was free of glucose.

ATTEMPT TO FRACTIONATE KETHA GUM BY PRECIPITATION WITH ANTI-PN I AND ANTI-PN II

Fifty ml. of type I C-absorbed serum 592 was diluted with 50 ml. of saline at 0° and 50 mg. of ketha gum in 2.5 ml. saline was added (cf. Table 1). After several days at 0° the precipitate was centrifuged, washed several times with chilled saline and finally with ice-cold water, and dissociated with 5 per cent trichloroacetic acid (Heidelberger, Dische, Neely and Wolfrom, 1955). The dissolved polysaccharide was precipitated in the cold with ethanol, washed with ethanol and acetone, and dried over P_2O_5 *in vacuo*. The recovered polysaccharide, 15 mg., K I, had $[\alpha]_D$ +5° (*c*, 0.41) (cf. Table 3).

TABLE 3

ANALYSIS OF KETHA GUM, ITS FRACTIONS PRECIPITATED BY ANTISERA AND ITS PRODUCT OF REDUCTION

Sample	$[\alpha]_D$ *in water*	*Hexose, as % anhydro-galactose (Dische)* *	†	*Pentose, as % anhydro-arabinose (Dische)*	*Uronic acid, as % anhydro-galacturonic acid (Dische)*	*Total anhydro-sugars, (%)*
K	+4°	24	35	36	30	101
K I	+5°		33	34	25	92
K II	+2°	29	35	34	28	97
K Red		44	52	29	9	90

*By primary cysteine-sulphuric acid colour-reaction.
†By secondary cysteine-sulphuric acid colour-reaction. These values were used in computing totals.

Ketha II, 15 mg., was similarly obtained from the specific precipitate given by 23 ml. of Type II antiserum 513 (Heidelberger, 1960b) and had $[\alpha]_D$ +2° (*c*, 0.56) (cf. Table 3).

Ketha I and ketha II were heated in 2N H_2SO_4 at 100° for 6 hours and worked up as before. Chromatograms of both in solvent *A* showed galacturonic acid, 4-O-methyl-glucuronic acid, and traces of glucuronic acid, possibly heavier in ketha II. There was thus no obvious fractionation.

DISCUSSION

The experiments described in the present paper demonstrate once more how immuno-chemical methods may point unerringly to the nature of an unknown constituent of a complex polysaccharide. In this instance it was anticipated that ketha gum, said to contain glucuronic acid, would show cross-precipitation in Type II antipneumococcal sera. Unexpected, however, was the consistent reactivity in Type I antipneumococcal

sera and, as this involved 20 to 45 per cent of the type-specific antibody, it was evident that ketha gum contained multiples, in terms of the quantitative precipitin theory of Heidelberger and Kendall (1935), of a major antigenic grouping in common with the specific capsular polysaccharide of Type I pneumococcus, the determinant of Type I specificity. Since more than one-half of this substance consists of D-galacturonic acid and sugar acids, like other organic acids (Landsteiner, 1945), have been shown to be major determinants of immunological specificity (Goebel, 1936; Goebel and Hotchkiss, 1937; Marrack and Carpenter, 1938), it was a foregone conclusion that D-galacturonic acid would be found as a component of ketha gum. Its identification among the products of hydrolysis is outlined above, as is also that of 4-O-methyl-D-glucuronic acid, which, instead of the previously reported glucuronic acid, appears to be the other principal acidic constituent and is responsible for the cross-reactivity of the gum in Type II antipneumococcal sera. An O-methyl group in the 4-position of D-glucuronic acid, as in the hemicelluloses, does not prevent cross-precipitation of such substances in Type II antipneumococcal sera (Heidelberger, 1960 a, b), although the D-glucuronic acid is unsubstituted in the specific capsular polysaccharide of Type II pneumococcus, the determinant of Type II specificity.

Studies with other gums which precipitate Type I antipneumococcal sera will appear in another communication and work is in progress on the constitution of the Type I pneumococcal polysaccharide.

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