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STRUCTURAL AND IMMUNOCHEMICAL STUDIES ON *Pterospermum suberifolium* GUM

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ABSTRACT

The water-soluble polysaccharide from *Pterospermum suberifolium* gum is composed of L-rhamnose (24.0%), D-glucose (5.6%), D-galacturonic acid (32.4%), and D-glucuronic acid (19.7%), and it precipitated 77% of the antibody nitrogen from anti-Pneumococcal Type XXIII serum. From the results of methylation, periodate oxidation, and partial hydrolysis studies on the gum and its carboxyl-reduced product, a structure was assigned to its repeating unit. Inhibition of the cross-precipitation using the monosaccharides and the oligosaccharides obtained from the polysaccharide indicated that L-rhamnose and D-glucose were immunospecific, the former to the greater extent.

INTRODUCTION

The immunochemical cross-reactions of the polysaccharide from *Pterospermum suberifolium* gum with anti-Pn Type XXIII serum showed a massive reaction, precipitating about three-quarters of the antibody nitrogen. To determine the sugar groupings responsible for these cross-reactions, detailed structural and immunochemical studies on the gum PS were undertaken. We now report these results.

RESULTS AND DISCUSSION

The water-soluble portion of the gum was precipitated with ethanol, dried, and extracted with benzene to remove resinous and coloring matter. The material was dissolved in water, and the polysaccharide was precipitated by adding ethanol. The polysaccharide was slightly colored. It was redissolved in water, treated with Dowex-50W X-8 (H^+) resin, and reprecipitated. On gel chromatography using Sephadex G-100, the material was eluted as a single fraction, and, on ultracentrifugal analysis, a single peak was obtained, indicating its homogeneity. On hydrolysis, the purified polysaccharide (PS) gave L-rhamnose (24.4%), D-glucose (5.6%), and D-glucuronic acid plus D-galacturonic acid; the total uronic acid was

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estimated[1] to be 60.0%, as D-galacturonic acid.

The carboxyl groups in the PS were reduced by the method of Taylor and Conrad[2], and the product (R-PS) gave, on hydrolysis, L-rhamnose (24.0%), D-glucose (25.3%), D-galactose (32.4%), and unreduced uronic acid (6.0%). Paper chromatography (p.c.) on[3] Whatman DE-81 indicated the presence of a major amount of galacturonic acid and a trace of glucuronic acid. Further reduction did not decrease the uronic acid content. These results showed that the PS contains 19.7% of D-glucuronic acid and 32.4% of D-galacturonic acid residues.

To identify the structural features of the gum, the PS and R-PS were subjected to methylation analysis; the results are summarized in Table I. As the PS contains a major proportion of uronic acid units, better structural information could be obtained from the results of methylation studies of the R-PS. The repeating unit of R-PS contains 7.2 mol and 1.0 mol of nonreducing end D-glucose and D-galactose units, respectively. There are two (1→4)-linked D-glucopyranose and three (1→4)-linked D-galactopyranose units in the repeating unit. All of the L-rhamnose residues (8.7 mol) are (1→2)-linked. The polysaccharide is highly branched, and the D-galactose residues at the branch points are 1,2,3- (1.3 mol) and 1,2,4- (7.3 mol) linked. The hydrolyzate of the methylated PS was found to contain 3,4-di-*O*-methyl-L-rhamnose (8.7 mol) and 2,3,6-tri-*O*-methyl-D-glucose (1.7 mol), and similar values were obtained for these two methylated sugars in the corresponding product from the R-PS.

To obtain the oligosaccharides, the PS was heated with 40% formic acid for 2 h at 100°. The mixture was separated into neutral and acidic fractions on ion-exchange columns. The neutral fraction was found to contain only glucose, and three oligomers were in the acid fraction. The mixture was separated into its homogeneous constituents, and these were characterized.

TABLE I

METHYLATION ANALYSIS OF THE POLYSACCHARIDE FROM *Pterospermum suberifolium* GUM AND OF ITS CARBOXYL-REDUCED PRODUCT

Sugars	T[a]		*Mole proportion*	
	1	*2*	*Original PS*	*Carboxyl-reduced PS*
3,4-Di-*O*-methylrhamnose	0.92	0.87	8.7	8.7
2,3,4,6-Tetra-*O*-methylglucose	**1.0**	**1.0**	—	7.2
2,3,6-Tri-*O*-methylglucose	2.5	2.32	1.7	1.8
2,3,4,6-Tetra-*O*-methylgalactose	1.24	1.18	—	1.0
2,3,6-Tri-*O*-methylgalactose	2.4	2.2	—	3.2
4,6-Di-*O*-methylgalactose	3.65	2.99	—	1.3
3,6-Di-*O*-methylgalactose	4.38	3.28	—	7.3

[a]Retention times of the corresponding alditol acetates, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol as unity, on a column of (*1*) 3% of ECNSS-M (170°), and (*2*) 3% of OV-225 (170°). These were compared with authentic samples.

Oligosaccharide I: 5.6 mg; R_{GalA} 0.27, solvent *B*; $[\alpha]_D^{24}$ +24° (*c* 0.56, water). On hydrolysis, it gave L-rhamnose and D-glucuronic acid in the mol ratio of 1.0:1.1. On reduction ($NaBH_4$) of the oligosaccharide followed by hydrolysis and p.c., a spot corresponding to glucuronic acid was found. When reduced[4] with $LiAlH_4$ and the product hydrolyzed, the methylated oligosaccharide gave 3,4-di-*O*-methylrhamnose (1.0 mol) and 2,3,4-tri-*O*-methylglucose (1.1 mol). Hence, its structure is Glc*p*A-(1→2)-Rha*p*.

Oligosaccharide II: 5.0 mg; R_{GalA} 0.19, solvent *B*; $[\alpha]_D^{24}$ +13° (*c* 0.5, water). On hydrolysis, it gave L-rhamnose and D-glucuronic acid in the mol ratio of 2.3:1.0. The reducing end was occupied by a rhamnose residue. Methylation of the oligosaccharide, followed by $LiAlH_4$ reduction[4] and hydrolysis, gave 3,4-di-*O*-methylrhamnose (2.3 mol) and 2,3,4-tri-*O*-methylglucose (1.0 mol). This oligosaccharide was assigned the structure Glc*p*A-(1→2)-Rha*p*-(1→2)-Rha*p*.

Oligosaccharide III: 6.1 mg; R_{GalA} 0.096; solvent *B*; $[\alpha]_D^{24}$ +27° (*c* 0.6, water). On hydrolysis, it gave L-rhamnose, D-glucuronic acid, and D-galacturonic acid in the mol ratios of 1.1:1.0:0.9. On reduction ($NaBH_4$) of the oligomer, hydrolysis, and p.c. examination, spots corresponding to glucuronic acid and rhamnose were found, showing that the D-galacturonic acid residue occupied the reducing end. Methylation of the oligomer, followed by $LiAlH_4$ reduction[4] and hydrolysis, yielded 3,4-di-*O*-methylrhamnose (1.1 mol), 2,3,4-tri-*O*-methylglucose (1.0 mol), and 3,4-di-*O*-methylgalactose (0.9 mol). This oligosaccharide was assigned the structure Glc*p*A-(1→2)-Rha*p*-(1→2)-Gal*p*A.

The fully methylated polysaccharide was subjected to uronic acid degradation[5], using 2M methylsulfinyl sodium, and the degraded material was hydrolyzed. Examination of the resulting methylated sugars by g.l.c. showed the presence of 3,4-di-*O*-methylrhamnose and 2,3,6-tri-*O*-methylglucose in the mol ratio of 1.8:1.0. In the intact polysaccharide, the ratio of these methylated sugars was 4.8:1.0, indicating that two (1→4)-linked D-glucosyl residues and some of the (1→2)-linked L-rhamnosyl residues are present in the PS in sequence. The latter conclusion was further supported by the isolation and identification of an aldotriouronic acid (oligosaccharide II) from the PS. The structures of the oligosaccharides further showed that all of the L-rhamnose-containing branch chains have D-glucosyluronic acid groups at the nonreducing ends, and are linked to O-2 of (1→4)-linked galactosyluronic acid units in the main chain. One D-galactosyluronic

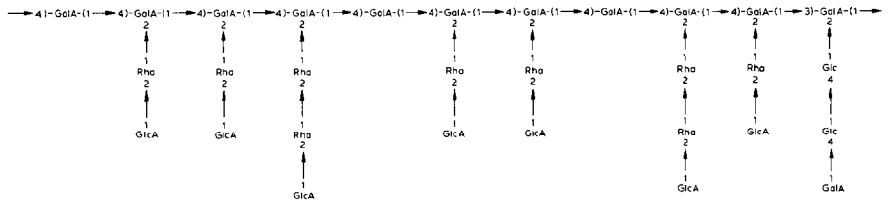

Fig. 1. The structure of the *Pterospermum suberifolium* gum.

TABLE II

SURVIVAL OF SUGARS IN THE OXIDATION OF ACETYLATED, CARBOXYL-REDUCED POLYSACCHARIDE WITH CHROMIUM TRIOXIDE

Time (h)	myo-*Inositol*	*Rhamnose*	*Glucose*	*Galactose*
0.0	10	7.1	6.4	10.0
0.5	10	0.60	0.87	0.52
1.0	10	0.46	0.69	0.50

TABLE III

QUANTITATIVE PRECIPITIN REACTION[a] OF THE PS WITH ANTI-PNEUMOCOCCAL TYPE XXIII SERUM

Polysaccharide	*PS added (μg)*	*Antibody N precipitated (μg)*
Gum PS	500	136
	1000	204[b]
	2000	129
S-XXIII	200	265

[a]Results calculated to 1.0 mL of undiluted serum. [b]Supernatant + 50 μg of S-XXIII precipitated 64 μg of antibody N.

acid unit in eleven is 1,2,3-linked. One of the possible structures that could explain all of these facts is shown in Fig. 1.

When subjected to periodate oxidation[6], the PS consumed 1.02 mol of periodate per mol of hexosyl residue; the calculated value for the proposed structure is 1.0 mol. On hydrolysis, the periodate-oxidized and then $NaBH_4$-reduced material gave only galacturonic acid. On methylation and hydrolysis, the corresponding product R-PS gave 3,6-di-*O*-methylgalactose and 4,6-di-*O*-methylgalactose in the mol ratio of 6.7:1.0. These results supported the structure assigned to the repeating unit of the PS.

On oxidation[7] of the per-*O*-acetylated derivative of the R-PS with Cr_2O_3, it was observed (see Table II) that rhamnose, glucose, and galactose disappeared rapidly during the reaction, indicating that most of these units are β-linked.

Heidelberger[8] reported cross-reactions between anti-Pneumococcal Type XXIII serum and various plant gums. The cross-reactions quantitatively studied were those given by hualtaco and arabic gums[9] and by mangle gum[10]. The structures of these substances were also established (arabic[11], hualtaco[12], and mangle[13]). From these results, it was pointed out that the immunochemical specificities are due to the presence of similarly linked L-rhamnose or D-galactose residues, or both, in these gum molecules and in S-XXIII. The structure of S-XXIII has recently been elucidated in this laboratory[14].

S XXIII and *Pterospermum suberifolium* gum polysaccharides have L-rhamnose and D-glucose units in common. The PS precipitated 77% of the antibody nitrogen that was precipitated by S-XXIII from anti-Pn Type XXIII serum (see Table

TABLE IV

INHIBITION, BY VARIOUS SUGARS AND OLIGOSACCHARIDES, OF THE PRECIPITATION[a] OF ANTI-PNEUMOCOCCAL TYPE XXIII SERUM BY THE PS

Inhibitor	*Micromoles added*	*Antibody N precipitated (μg)*	*Inhibition (%)*
None	—	204	0
L-Rha	0.55	152	25
	1.09	135	34
	1.65	119	42
D-Glc	0.56	180	12
	1.11	165	19
	1.67	159	22
D-GlcA	1.55	199	2
Oligomer I	0.59	147	25
	1.18	135	34
	1.76	120	41
Oligomer II	0.62	129	37
	1.03	113	45
	1.65	90	56
	2.06	77	62
Oligomer III	0.58	159	22
	1.16	142	28
	1.55	129	37

[a]Results calculated to 1.0 mL of undiluted serum.

III), and, as indicated in footnote *a* thereof, precipitations by these substances involved the same fraction of antibody.

The inhibitions of cross-precipitations were studied quantitatively by using the constituent monosaccharides and the oligosaccharides obtained from the PS (see Table IV). Of the three sugars used, L-rhamnose showed the maximum inhibition (42%), and D-glucose gave 22%; D-glucuronic acid had insignificant specificity. Oligosaccharide I, having the L-rhamnose residue at the reducing end, inhibited the precipitation to the extent of 41%, whereas oligomer II, having two rhamnose units, gave 62% inhibition. In the case of oligomer III, in which oligomer I is glycosidically linked to O-2 of a D-galacturonic acid residue, the inhibition was 37%. From these results, it may be concluded that L-rhamnose and D-glucose are immunospecific in the cross-reactions, the former to the greater extent. From a comparison of the structures of the repeating units of the PS and of S-XXIII, it is not possible to decide how the antibody combining-sites complementary to non-reducing or (1→4)-linked L-rhamnose units or both, show specificity for a (1→2)-linked unit. The (1→4)-linked glucose units common to the PS and S-XXIII seem to contribute to the specificity to some extent, although, in the latter, C-2 is occupied by a phosphate group.

EXPERIMENTAL

General methods. — All evaporations were conducted under diminished

pressure in a rotary evaporator at bath temperatures not exceeding 40°. Optical rotations were measured with a Perkin–Elmer 141 polarimeter. Ultraviolet and visible spectra were recorded with a Hitachi Model 100-60 spectrophotometer. Infrared spectra were recorded with a Beckman I.R.-20A instrument. Descending paper-chromatography was performed on Whatman No. 1 and 3 MM sheets and on DEAE-cellulose paper[3] (Whatman DE-81), using the following solvent systems (v/v): (*A*) 8:2:1 ethyl acetate–pyridine–water, (*B*) 9:2:2 ethyl acetate–acetic acid–water, (*C*) 3:1:1 ethyl acetate–acetic acid–water, and (*D*) 4:1:5 (upper layer) 1-butanol–acetic acid–water; chromatograms were developed with (*a*) alkaline silver nitrate, and (*b*) aniline oxalate spray-reagents. Elutions were monitored with a Waters Associates model R-403 differential refractometer. G.l.c. was performed in a Hewlett–Packard model 5730A gas chromatograph equipped with a flame-ionization detector. The glass columns (180 × 6 mm) used were packed with (*i*) 3% of ENCSS-M and (*ii*) 3% of OV-225 on Gas Chrom Q (100–120 mesh); nitrogen was the carrier gas. A Beckman Model L5-65 ultracentrifuge fitted with a Schlieren optical system was used for testing the homogeneity of the material. Periodate oxidation was conducted in the dark at 4°, and the consumption of the oxidant was monitored spectrophotometrically[6].

Isolation and purification of polysaccharide. — The reddish brown *Pterospermum suberifolium* gum (16 g) was dissolved in water (500 mL), and the insoluble material was removed by filtering the suspension through a Nylon cloth. The polysaccharide was precipitated by adding ethanol (4 vol.). The precipitate was centrifuged off, successively triturated with ethanol and acetone, and air-dried. It was then extracted with benzene several times, to remove resinous and coloring matter. This material was stirred with water overnight, cooled, and the polysaccharide precipitated by adding cold ethanol while stirring. The precipitate was centrifuged, triturated as usual, and dried over P_2O_5. An aqueous solution of the material was treated with Dowex-50W X-8 (H^+) resin, the suspension filtered, and the filtrate freeze-dried; yield, 2.0 g.

The material (65 mg) in water (3 mL) was added to a column (100 × 2.2 cm) of Sephadex G-100 which was eluted with water, 5-mL fractions being collected. It was found that the material was eluted as a single peak; yield 60 mg; $[\alpha]_D^{24}$ +55° (*c* 0.42, water). A 1% solution of the material in 0.1M phosphate buffer containing 0.1M sodium chloride (pH 7.8) gave a single peak on ultracentrifugation.

The polysaccharide (PS) (4 mg) was hydrolyzed with M sulfuric acid for 18 h at 100°, the mineral acid neutralized ($BaCO_3$), the suspension centrifuged, and the supernatant liquor decationized with Dowex-50W X-8 (H^+) resin, and evaporated. In paper chromatography (Whatman No. 1 and Whatman DE-81 papers) using solvents *A*, *B*, *C*, and *D* and spray reagent (*a*), spots corresponding to rhamnose, glucose, glucuronic acid, and galacturonic acid were detected. The neutral sugars were estimated by g.l.c. (column *i*), using *myo*-inositol as the internal standard: rhamnose, 24.4%; glucose, 5.6%. Uronic acid was estimated[1], using D-galacturonic acid as the standard, to be 60.0%.

Preparation of carboxyl-reduced PS[2]. — A solution of the PS (40 mg) in water (40 mL) was treated with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC, 1 g) and then with $NaBH_4$ following the method of Taylor and Conrad[2]. The resultant solution was dialyzed against distilled water, and then freeze-dried. The process was repeated twice, to ensure complete reduction of the carboxyl groups; yield 32 mg. The carboxyl-reduced polysaccharide (R-PS) (2 mg) was hydrolyzed with M sulfuric acid for 18 h at 100°, and, after the usual treatment, the sugars were identified and estimated by g.l.c.; rhamnose, 24.0%; glucose, 25.3%; and galactose, 32.4%. Unreduced uronic acid was estimated[1] to be 6.0%, and further reduction did not change this value.

Methylation analysis. — The PS (10 mg) and R-PS (8 mg) were permethylated by the Hakomori method[15], followed by the Purdie method[16]. The final products showed no i.r. absorptions in the region 3600–3300 cm^{-1}. The fully methylated products were hydrolyzed by heating with 90% formic acid for 2 h at 100°, and then with 0.5M sulfuric acid for 10 h at 100°. After the usual treatment, the methylated sugars were converted into alditol acetates, and these analyzed by g.l.c. The results are given in Table I.

Partial hydrolysis with acid. — A solution of the PS (150 mg) in 40% formic acid (80 mL) was heated on a boiling-water bath for 2 h. Formic acid was removed under diminished pressure, and the hydrolyzate was passed successively through columns of Dowex-50W X-8 (H^+) and Dowex-1 X-4 (HCO_3^-) resin. The eluate and washings were concentrated to a small volume. The anion-exchange resin column was eluted with 10% formic acid (200 mL), and the eluate was evaporated to dryness; yield, 100 mg. In p.c. using solvent *B*, the neutral fraction gave a single spot corresponding to glucose, whereas the acidic fraction gave three spots, having R_{GalA} (in solvent *B*) 0.27, 0.19, and 0.09, besides that of a uronic acid. The mixture was resolved into its components on 3MM papers, using solvent *B*. Each fraction was purified by passing it through a column (30 × 1 cm) of Sephadex G-25, and was found to be homogeneous in p.c.

The oligosaccharides were hydrolyzed with 0.5M sulfuric acid for 12 h at 100°, and, after the usual treatment, the neutral sugars were identified by p.c. using solvent *A*, and the acidic sugars by using Whatman DE-81 paper[3] and solvent *C*. The reducing-end unit in each oligomer was identified by treating with $NaBH_4$ solution, followed by hydrolysis, and p.c. using aniline oxalate as the spray reagent. The oligosaccharides were methylated by the Hakomori method[15], followed by reduction[4] with $LiAlH_4$. The resulting products were hydrolyzed with 0.5M sulfuric acid for 12 h at 100°, and the methylated sugars were identified, and estimated, by g.l.c. in the usual way.

Base degradation of the methylated PS[5]. — A solution of carefully dried, methylated PS (25 mg) and *p*-toluenesulfonic acid (trace) in 19:1 dimethyl sulfoxide–2,2-dimethoxypropane (4 mL) was prepared in a serum vial which was sealed with a rubber cap. After passing in nitrogen, 2M methylsulfinyl sodium (2 mL) was added, and the vial was kept for 30 min in an ultrasonic bath, and then overnight

at room temperature. The contents were poured into water (10 mL), and enough aqueous acetic acid was added to make the solution slightly acidic. The solution was extracted with chloroform (4 × 15 mL), and the extract was washed with water (5 × 15 mL), dried (Na_2SO_4), and concentrated. The product was heated with 10% aqueous acetic acid (20 mL) for 1 h at 100°, and the solution freeze-dried. It was hydrolyzed with 0.5M sulfuric acid, and the resulting methylated sugars, as their alditol acetates, were identified by g.l.c. to be 3,4-di-*O*-methylrhamnose and 2,3,6-tri-*O*-methylglucose in the mol ratio of 1.8:1.0.

Periodate oxidation of the PS[6]. — The PS (4.0 mg) was subjected to periodate oxidation[6]. The consumption of the oxidant became constant in 51 h, and corresponded to 1.02 mol of periodate per mol of hexosyl residue. In a separate experiment, the PS (50 mg) in water (50 mL) was oxidized with sodium metaperiodate under the same conditions. The excess of periodate was decomposed with ethylene glycol, and the solution was dialyzed against distilled water, concentrated to 10 mL, and the product reduced with $NaBH_4$ (250 mg) overnight at room temperature. The mixture was made acidic (pH 7.5), dialyzed, and freeze-dried; yield, 30 mg. A part (2 mg) of the periodate-oxidized, reduced material was hydrolyzed, and the hydrolyzate obtained after the usual treatment was examined by p.c. A spot corresponding to galacturonic acid was detected. When similarly treated, the carboxyl-reduced PS gave a spot corresponding to galactose. The carboxyl-reduced and periodate oxidized material (4 mg) was methylated by the Hakomori method[15] followed by the Purdie method[16], the product hydrolyzed, and the resulting methylated sugars were identified, and estimated, by g.l.c. to be 3,6-di-*O*-methylgalactose (6.7 mol) and 4,6-di-*O*-methylgalactose (1.0 mol).

Oxidation[7] *of the R-PS with* Cr_2O_3. — A solution of the R-PS (6.0 mg) and *myo*-inositol (0.6 mg) in formamide (0.5 mL) was acetylated with acetic anhydride (2.5 mL) and pyridine (3.0 mL) at room temperature in the usual way. The per-*O*-acetylated product was dissolved in glacial acetic acid (5 mL) and the solution was kept at 50°. To it was added Cr_2O_3 (300 mg), and aliquots were removed at 0, 0.5, and 1.0 h, and immediately diluted with water. The chloroform extract of the solution was dried (Na_2SO_4), and evaporated to dryness. The product was deacetylated with 0.2M sodium methoxide for 3 h, the solution decationized with Dowex-50W X-8 (H^+) ion-exchange resin, and the product hydrolyzed with 0.5M sulfuric acid for 16 h at 100°. After the usual treatment, the hydrolyzate was analyzed by g.l.c. The results are given in Table II.

Quantitative precipitin reaction. — Anti-Pneumococcal Type XXIII serum (H-912) was kindly supplied by Prof. M. Heidelberger, New York University Medical Center, New York. Precipitin reactions[17,18] were set up, using the PS (50–200 μg) and the antiserum (0.10 mL), and the total volume was made to 0.50 mL with saline. The mixtures, and a blank containing only serum, were set up in duplicate, and kept for 7 days at 1 to 3°, these conditions being maintained throughout. The precipitates were collected by centrifugation in the cold, washed twice with chilled saline (1 mL each time), and then dissolved in 0.25M acetic acid (2.0 mL). The opti-

cal absorbance of each solution was measured at 280 nm, and the amount of nitrogen in each precipitate was calculated from a standard curve drawn by using bovine serum albumin (nitrogen, 15.03%). The results are given in Table III.

Inhibition studies. — Inhibition of the precipitin reaction was studied by using, as the inhibitors, L-rhamnose, D-glucose, and D-glucuronic acid and the oligosaccharides obtained from the graded hydrolysis of the PS. They were added, in increasing amounts, to 0.10-mL portions of antiserum, in duplicate, and diluted with appropriate quantities of saline. The contents of the tubes were kept for 1 h at 1 to 3°. To each tube was then added a solution containing 100 μg of the PS in saline, to bring the system to equivalence. The final volume of each solution was 0.50 mL. Two controls, one containing the same amounts of antigen and antiserum as in the other tubes, and the other containing the antiserum alone, were included in each set. The tubes were kept for 7 days at 1 to 3°, and the amounts of precipitated antibody nitrogen were assayed as described earlier. The results are given in Table IV.

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