Carbohydrate Research, 98 (1981) 75–83
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

STRUCTURAL FEATURES OF THE ACIDIC POLYSACCHARIDE OF *Spondias pinnata* GUM EXUDATE

PRADYOT K. GHOSAL AND SWAPNADIP THAKUR
Department of Chemistry, The University of Burdwan, Burdwan 713104 (India)
(Received May 29th, 1981; accepted for publication, June 23rd, 1981)

ABSTRACT

The purified, homogeneous, acidic polysaccharide isolated from the gum exudate of *Spondias pinnata*, and its degraded product prepared by controlled autohydrolysis, were found to contain D-galactose, L-arabinose, and D-galacturonic acid. Complete methylation followed by hydrolysis, both before and after reduction with lithium aluminum hydride, revealed the probability of a (1→3)-linked, galactan backbone. The linkages of the interior parts were confirmed by methylation studies, and by the results of periodate oxidation and Smith degradation.

INTRODUCTION

The medicinally important, deciduous plant *Spondias pinnata*[1] is available all over India and in the tropics of Asia. Structural investigations on two sister species of *Spondias, viz., S. mangifera*[2] for the polysaccharide in its fruits, and *S. dulcis*[3] for the polysaccharide in its gum exudate, have been described. The present investigation was undertaken with a view to ascertaining the structural features of the gum exudate of *S. pinnata*. The original polysaccharide obtained from the purified gum was found to be composed of D-galactose, L-arabinose, and D-galacturonic acid in the molar ratios of 15:7:9. Autohydrolysis under controlled conditions gave a degraded polysaccharide, consisting of these three sugars in the ratios of 14:4:7. We now report the results of complete hydrolysis with acid, autohydrolysis, mild hydrolysis with acid, methylation analysis, periodate oxidation, and Smith degradation on the original and the degraded polysaccharide.

RESULTS AND DISCUSSION

The polysaccharide was isolated as a spongy mass by repeated precipitation with 9:1 (v/v) ethanol–acetone from an aqueous solution of the gum exudate. The polysaccharide thus obtained was decationized with Amberlite IR-120 (H^+) ion-exchange resin, and the acidic solution was further purified by fractionation on DEAE-cellulose. The homogeneity of the major polysaccharide fraction, $[\alpha]_D^{24}$ −38°, was checked by gel filtration on Sephadex G-100 in pyridine acetate buffer, and high-

0008-6215/81/0000–0000/$ 02.75,

voltage electrophoresis in borate buffer. Estimation by the phenol–sulfuric acid method[4] revealed the presence of 99.4% of carbohydrate in the purified, gum polysaccharide.

This original polysaccharide had a uronic acid content of 30% as estimated by the carbazole method[5]. The high percentage of uronic acid was utilized to degrade the original polysaccharide by autohydrolysis. Guided by the results of pilot, autohydrolytic experiments, degradation was conducted on the original polysaccharide by autohydrolyzing it for 120 h at 100°. The hydrolyzate was dialyzed, and concentrated, and the degraded polysaccharide isolated by precipitation with ethanol–acetone; it had $[\alpha]_D^{24}$ −25°. The uronic acid content of the degraded polysaccharide was also very high (28%), and high-voltage electrophoresis showed it to be homogeneous.

A portion of the original and of the degraded polysaccharide was each carboxyl-reduced with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate[6] (3 times), until it responded negligibly to the carbazole test. The original and the degraded polysaccharide, as well as their carboxyl-reduced derivatives, were subjected to complete hydrolysis with 0.5M sulfuric acid for 18 h at 100°, using D-xylose as the internal standard (because this monosaccharide was not a component of the polysaccharides, as observed from preliminary, hydrolytic experiments and p.c.). After the usual treatment, the monosaccharides present in the four hydrolyzates were characterized by paper chromatography (solvent systems *A* and *B*) and then isolated by preparative p.c. on Whatman 3 MM paper with solvent *B*; the configurations were determined by measurement of the specific rotation. The monosaccharides were D-galactose, L-arabinose, and D-galacturonic acid, and their relative proportions were estimated by g.l.c. as their alditol acetates (see Table I).

The increased proportion of D-galactose in the carboxyl-reduced polysaccharide confirmed that the uronic acid was D-galacturonic acid; however, the percentage of D-arabinose was almost constant. After hydrolysis of both the original and degraded polysaccharide with 0.25M sulfuric acid for 8 h at 100°, neutralization ($BaCO_3$) of

TABLE I

COMPOSITION[a] OF MONOSACCHARIDES IN THE ORIGINAL AND THE DEGRADED POLYSACCHARIDES FROM *Spondias pinnata*, AND IN THEIR CARBOXYL-REDUCED DERIVATIVES

Monosaccharide	*Original polysaccharide (%)*	*Degraded polysaccharide (%)*	*Carboxyl-reduced*	
			Original polysaccharide (%)	*Degraded polysaccharide (%)*
D-Galactose	47	56	75	83
L-Arabinose	23	16	24	16
D-Galacturonic acid	30	28	trace	trace

[a]Percentage values are given to the nearest whole number.

the acid, and decationization of the hydrolyzates with Amberlite IR-120 (H^+), the neutral and acidic sugars were separated by use of De-Acidite-FF (formate) anion-exchange resin. The neutral sugars obtained by elution with water were characterized as usual, by paper chromatography (solvent *A*) and by g.l.c. as their alditol acetates, to be D-galactose and L-arabinose. On elution with 5% formic acid, the anion-exchanger gave acidic sugar mixtures in formic acid solution, and the latter acid was removed by repeated distillation with water *in vacuo*; paper chromatography (solvent *A*) of the concentrated eluate showed D-galacturonic acid and two slower-moving fractions (F_1, R_{Gal} 0.20, and F_2, R_{Gal} 0.25). Hydrolytic studies on F_1 and F_2 and their $NaBH_4$-reduced products indicated that D-galacturonic acid was the nonreducing end, and was linked to D-galactose in F_1 and to L-arabinose in F_2.

Permethylation by the Hakomori method[7] (once) and the Purdie method[8] (twice) of the original and the degraded polysaccharide gave completely methylated products showing no infrared absorption near 3500 cm^{-1}. One portion of both of the methylated materials was completely hydrolyzed, and, after conversion of the products into partially methylated alditol acetates, characterized by g.l.c.[9] using column *2*. Other portions of the methylated polysaccharides were reduced with lithium aluminum hydride[10] in oxolane–dichloromethane. These carboxyl-reduced methylated products were then hydrolyzed, and the products characterized as usual by g.l.c. in column *2*. The results of the methylation analyses are presented in Table II.

The results of the methylation analysis could be assessed by first considering the products characterized from methylated, degraded (B_1) and methylated, carboxyl-reduced, degraded polysaccharide (B_2). The conclusion would then be corroborated by considering those results given by methylated original (A_1) and methylated,

TABLE II

METHYLATION ANALYSIS OF THE ORIGINAL POLYSACCHARIDE (A_1) AND THE DEGRADED POLYSACCHARIDE (B_1) FROM *Spondias pinnata*, AND OF THEIR REDUCTION PRODUCTS (A_2 AND B_2)

Methylated sugars	*Mole proportions*			
	Original A_1	*Degraded* B_1	*Carboxyl-reduced*	
			Original A_2	*Degraded* B_2
2,3,4,6-Tetra-*O*-methyl-D-galactose	3	2	3	2
2,3,6-Tri-*O*-methyl-D-galactose	4	4	4	4
2,3,4-Tri-*O*-methyl-D-galactose	—	—	5	3
2,3-Di-*O*-methyl-D-galactose	—	—	4	4
2,6-Di-*O*-methyl-D-galactose	4	5	4	5
4,6-Di-*O*-methyl-D-galactose	3	3	3	3
2-*O*-Methyl-D-galactose	1	—	1	—
2,3,5-Tri-*O*-methyl-L-arabinofuranose	1	2	1	2
2,3,4-Tri-*O*-methyl-L-arabinopyranose	—	1	—	1
3,5-Di-*O*-methyl-L-arabinofuranose	4	—	4	—
3,4-Di-*O*-methyl-L-arabinopyranose	2	1	2	1

carboxyl-reduced, original polysaccharide (A_2). The presence of 2,6-di-*O*-methyl-D-galactose (5 mol) and 4,6-di-*O*-methyl-D-galactose (3 mol) could be explained by considering a (1→3)-linked galactan (total 8 mol) chain in which 5 mol carried branching at C-4, and the other 3 mol, at C-2. The occurrence of two D-galactosyl residues as nonreducing termini was indicated by the characterization of 2,3,4,6-tetra-*O*-methyl-D-galactose (2 mol) from each of B_1 and B_2. Some nonreducing termini must also be occupied by L-arabinosyl units, in both furanose and pyranose forms, as shown by the formation of 2,3,5-tri-*O*-methyl-L-arabinofuranose (2 mol) and 2,3,4-tri-*O*-methyl-L-arabinopyranose (1 mol). The unbranched, interior parts making the branch chains were composed of (1→4)-linked galactose (4 mol) and (1→2)-linked arabinose (1 mol), as is evident from the respective products, namely, 2,3,6-tri-*O*-methyl-D-galactose (4 mol) and 3,4-di-*O*-methyl-L-arabinopyranose (1 mol). The formation of 2,3,4-tri-*O*-methyl-D-galactose (3 mol) and 2,3-di-*O*-methyl-D-galactose (4 mol) from B_2 gave evidence for the involvement of D-galacturonic acid both as nonreducing termini (3 mol) and as (1→4)-linked units (4 mol).

The results of the methylation studies on A_1 and A_2 agreed very closely with the mode of linkages proposed for B_1 and B_2. The additional formation of 2-*O*-methyl-D-galactose (1 mol) from both A_1 and A_2 indicated the presence of one highly branched galactose unit having linkages at O-1, O-3, O-4, and O-6. The characterization of 3,5-di-*O*-methyl-L-arabinofuranose (4 mol) and 3,4-di-*O*-methyl-L-arabinopyranose (2 mol) dictated the presence of (1→2)-linked arabinose residues in both the furanose (4 mol) and pyranose (2 mol) forms. Whereas 2 mol of L-arabinofuranosyl nonreducing ends were present in B_1 and B_2, there was 1 molar proportion of such a residue in A_1 and A_2, as is apparent from the formation of 2,3,5-tri-*O*-methyl-L-arabinofuranose (1 mol). The formation of 2,3,4,6-tetra-*O*-methyl-D-galactose (3 mol) from A_1 and A_2 dictated 3 molar proportions of D-galactosyl nonreducing ends in A_1 and A_2, compared to two such units in B_1 and B_2. One more D-galacturonic acid group must occur in A_1 as a nonreducing terminus than in B_1, as is evident from the formation of 5 mol of 2,3,4-tri-*O*-methyl-D-galactose in A_2, as against 4 mol of it in B_2.

The results of periodate-oxidation studies supported the aforementioned mode of linkages in the original and the degraded polysaccharide. Periodate kinetic studies[13,14] showed that the consumption of periodate became constant in 21 and 18 h for the original and the degraded polysaccharide, respectively. The amount of periodate uptake was 0.85 and 0.90 mol per mol of hexose residue for the original and the degraded polysaccharide. The observed values of periodate uptake were in accord with the theoretical amounts required for the mode of linkages proposed for them. The original polysaccharide was subjected to periodate oxidation followed by borohydride reduction, and complete hydrolysis of part of the periodate-oxidized–borohydride-reduced material revealed[15,16] the presence of 66% of D-galactose and 33% of L-arabinose. When the remaining portion was subjected to a second Smith degradation, 85% of D-galactose and 10% of L-arabinose survived. These Smith-degradation studies gave a positive implication of a (1→3)-galactan backbone in

the original polysaccharide. The first Smith-degradation of the degraded polysaccharide gave a galactan core that was immune to an attempted, second Smith-degradation.

EXPERIMENTAL

General methods. — The specific rotations were measured with a Perkin–Elmer Model 241 MC spectropolarimeter at 24° and 589.6 nm. Infrared spectra were recorded with an Acculab 10 Beckman instrument for KBr pellets. The homogeneity of polysaccharides was tested by high-voltage electrophoresis at 2° with a Shandon Model L-24, using borate buffer (pH 10) on Whatman No. 1 filter paper. A spectrophotometric, kinetic study of periodate consumption[13,14] at 4° was conducted with a Beckman Model 26 spectrometer at 228 nm, and all other colorimetric estimations of sugars (by the phenol–sulfuric acid and carbazole methods) in the visible region were performed with this instrument. Descending paper-chromatography was conducted on Whatman No. 1 paper for detection, and Whatman No. 3 MM paper for preparative purposes. The solvent systems (v/v) used for paper chromatography were (*A*) 8:2:1 ethyl acetate–pyridine–water and (*B*) the upper layer of 4:1:5 1-butanol–acetic acid–water. Spraying agents were (*a*) alkaline silver nitrate solution in aqueous acetone and (*b*) a saturated, aqueous solution of aniline oxalate. Gel-filtration chromatography was conducted on a column (100 × 1.4 cm) of Sephadex G-100, with pyridine acetate buffer (pH 4.5). Aqueous solutions of sugars were concentrated in a rotary evaporator at bath temperatures below 50°. Concentrated sugar solutions were always lyophilized. The gas–liquid chromatography of the monosaccharides, as their alditol acetates and partially methylated alditol acetates, was conducted in glass columns containing (*1*) 3% of OV-225 on Gas-Chrom Q (100–120 mesh) and (*2*) 3% of ECNSS-M on Gas-Chrom Q (100–120 mesh), with nitrogen as the carrier gas, in Hewlett–Packard Model 5713 A and Packard Model 419 instruments fitted with flame-ionization detectors. Non-methylated alditol acetates were characterized by matching against standard alditol acetates from mixtures of D-glucose, D-galactose, D-mannose, D-xylose, L-arabinose, and L-rhamnose. The partially methylated alditol acetates were identified by the standard retention-times[9] relative to 2,3,4,6-tetra-*O*-methylglucitol 1,5-diacetate and 2,3,6-tri-*O*-methylglucitol 1,4,5-triacetate.

Source of the gum. — The sample of the gum of *Spondias pinnata* (Anacardiaceae) was collected from the Burdwan district of West Bengal (India) during the month of August, 1977.

Isolation and purification of the gum polysaccharide. — The crude gum (30 g), brown in color (moisture content, 12%; ash content, 5%), was taken up in water (1 L) and centrifuged for 1 h at 8000 r.p.m. The polysaccharide was then precipitated from the centrifugate with 9:1 (v/v) ethanol–acetone (3 L). This purification process was repeated thrice, ultimately giving 18 g of polysaccharide. It was then decationized by passing an aqueous solution through a column (100 × 2 cm) of Amberlite IR-120 (H^+) resin, followed by fractionation in a column (75 × 2.5 cm) of DEAE-cellulose by gradient elution with alkaline (pH 10) 0.1, 0.3, and 0.5M sodium chloride solutions.

The appearance of polysaccharide in this fractionation was monitored by the phenol–sulfuric acid procedure. The eluate containing the major polysaccharide fraction was dialyzed, concentrated, and freeze-dried; yield 10 g, $[\alpha]_D^{24}$ −38° (*c* 1.2, water). Both by gel-filtration in pyridine acetate buffer through a column of Sephadex G-100, and by high-voltage electrophoresis in borate buffer, this original polysaccharide was found to be homogeneous.

Preparation of the degraded gum. — Guided by pilot experiments and the high value of the uronic acid content (30%) in the original gum polysaccharide (as estimated by the carbazole method), the best conditions for preparation of the degraded gum polysaccharide were found. Accordingly, the original polysaccharide (5 g) was dissolved in water (500 mL), and the solution heated on a boiling-water bath for 120 h, concentrated to 100 mL, and dialyzed against distilled water for 96 h at 4°. From the dialyzate, the degraded polysaccharide was precipitated with 9:1 (v/v) ethanol–acetone (1 L), and dried over P_2O_5; yield 2.5 g, $[\alpha]_D^{24}$ −25° (*c* 1, water). The degraded polysaccharide was electrophoretically homogeneous, as it migrated as a single component in high-voltage electrophoresis.

Preparation of carboxyl-reduced, original and degraded polysaccharides. — The original and the degraded polysaccharide (100 mg each) were separately dissolved in water (30 mL). 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene-sulfonate (CMC, 1 g) was added to each polysaccharide solution, with stirring, the pH being maintained at ~4.75 by dropwise addition of 0.01M hydrochloric acid during 4 h. Sodium borohydride solution (2.5M, 50 mL) was then added to each solution, the pH being maintained at ~7 by simultaneous addition of 6M hydrochloric acid. The mixtures were stirred for a further 1 h, dialyzed, and concentrated. This process of reduction was repeated twice, until the solutions responded negligibly to the carbazole test.

Estimation of monosaccharides in the original and the degraded polysaccharide, and in their carboxyl-reduced counterparts. — The polysaccharides (8 mg each) were hydrolyzed with 0.5M sulfuric acid (15 mL each) for 18 h at 100°, using D-xylose as the internal standard (because D-xylose was absent from the gum, as concluded from earlier paper-chromatographic study in pilot hydrolytic experiments). The excess of mineral acid was neutralized with $BaCO_3$, and the solution de-ionized with Amberlite IR-120 (H^+) resin. The presence of monosaccharides in these mixtures was detected by paper chromatography using solvent systems *A* and *B*, and they were quantitatively assayed as their alditol acetates by g.l.c. in column *2*. The uronic acid present in the original and in the degraded polysaccharide was galacturonic acid, and the neutral monosaccharides were galactose and arabinose. The proportion of galacturonic acid in the original and the degraded polysaccharide, as estimated by the carbazole method, was 30 and 28%, respectively (see Table I).

The configuration of each monosaccharide was ascertained by measurement of the specific rotation (for galactose, +75°; arabinose, +100°; and galacturonic acid, +20°) of the respective sugars isolated by preparative paper-chromatography in solvent system *B*.

Mild acid-hydrolysis of original and degraded polysaccharides. — The polysaccharides (50 mg each) were treated with 0.25M sulfuric acid (25 mL) for 8 h at 100°. The excess of mineral acid was neutralized with $BaCO_3$, the suspension filtered, and the filtrate decationized, and concentrated. It was then passed through a column (20 × 1 cm) of De-Acidite FF (formate) resin. The neutral monosaccharides, obtained by elution with water, were characterized as being D-galactose and L-arabinose. The acidic sugars were eluted from the column by means of 5% formic acid solution. The eluate was freed of formic acid by codistillation with water under diminished pressure. Preparative paper-chromatography of the mixture of acidic sugars in solvent system *B* revealed D-galacturonic acid and two slower-moving fractions (F_1, R_{Gal} 0.20, and F_2, R_{Gal} 0.25) from the hydrolyzate of the original polysaccharide. The degraded polysaccharide gave, however, D-galacturonic acid and F_1 only. F_1 and F_2 (2 mg each) were further hydrolyzed separately with 0.5M H_2SO_4 (5 mL). F_1 gave spots for D-galacturonic acid and D-galactose, and F_2 gave spots for D-galacturonic acid and L-arabinose. F_1 and F_2 (3 mg each) were reduced with $NaBH_4$ (20 mg); the excess of borohydride was neutralized, and, after decationization, and removal of boric acid as methyl borate, the solutions were concentrated, and the contents hydrolyzed with 0.5M H_2SO_4. Paper chromatography (solvent system *A*) revealed D-galacturonic acid as the reducing sugar.

Methylation analysis. — The original and degraded polysaccharides (30 mg each) were mixed with dry dimethyl sulfoxide (30 mL) in separate, air-tight vials, and the mixtures were ultrasonicated. Each solution was then treated with 2M methylsulfinyl sodium (30 mL) under an atmosphere of dry nitrogen. After being stirred overnight, the solutions were treated with methyl iodide (30 mL) by dropwise addition, and kept stirred for a further 4 h. The products were dialyzed, concentrated, and extracted with chloroform; yield, 20 mg each. They were then remethylated twice by the Purdie method. The infrared spectra of these permethylated products showed no absorption band near 3500 cm^{-1}.

One portion (10 mg) of each methylated product was treated with 90% formic acid (10 mL) for 4 h at 100°; then the formic acid was removed, as the water azeotrope, under diminished pressure. The materials were then completely hydrolyzed with 0.5M H_2SO_4 (10 mL) for 12 h at 100°. After the usual treatment, the partially methylated monosaccharides were converted into their alditol acetates, and characterized by g.l.c. using column *2*, as shown in Table II. Other portions (20 mg) of the methylated products were dissolved separately in dry oxolane–dichloromethane; $LiAlH_4$ was then added in portions to the mixture under reflux (45°) during 6 h. The excess of hydride was decomposed with ethyl acetate, and the solution acidified with M hydrochloric acid (to pH 3), and extracted with chloroform. The carboxyl-reduced, methylated, original and degraded polysaccharides were then hydrolyzed as before, and converted into alditol acetates. The results of g.l.c. analysis using column *2* are presented in Table II.

Periodate oxidation and Smith-degradation studies on the original and the degraded polysaccharide. — The original and the degraded polysaccharide were treated

with 0.04M sodium metaperiodate in the dark at 4°, the rates of consumption of the oxidant being monitored spectrophotometrically[13,14]. The periodate consumption became constant at 21 and 18 h, respectively, and was found to correspond to 0.85 and 0.90 mol of periodate per mol of hexose residue.

Smith degradation of the original and the degraded polysaccharide was conducted as follows. The original and the degraded polysaccharide (100 mg each) was each treated with 0.04M sodium metaperiodate (250 mL) for 36 h at 4° in the dark. The solutions were then treated with ethylene glycol (10 mL), kept overnight, dialyzed, and concentrated, and the contents reduced with sodium borohydride (1 g) at room temperature. The excess of borohydride was decomposed with acetic acid (to pH 5), the solution decationized with Amberlite IR-120 (H^+) resin, the boric acid removed by addition of methanol and evaporation, and an aqueous solution of the residue lyophilized (30 mg). Part (10 mg) of the periodate-oxidized–borohydride-reduced material was hydrolyzed with 0.5M sulfuric acid (10 mL), and the product, after conversion into alditol acetates, analyzed by g.l.c. in column *I*. The other part (20 mg) was hydrolyzed with 0.5M sulfuric acid (30 mL) for 12 h at room temperature, the acid neutralized with $BaCO_3$, and the solution decationized, and dialyzed. The dialyzate was concentrated to 20 mL, and the contents subjected to a second Smith-degradation; after the usual processing, the polysaccharide fragment that resisted periodate oxidation was isolated by precipitation with ethanol–acetone (yield, 8 mg).

These periodate-immune polysaccharide fragments were subjected to acid hydrolysis, and the monosaccharides were characterized by g.l.c. in column *I*.

ACKNOWLEDGMENTS

Sincerest thanks are accorded to Prof. C. V. N. Rao, Dr. A. K. Mukherjee, and Sri S. Basu of the Indian Association for the Cultivation of Science, Calcutta, and to Dr. S. B. Bhattacharya of I.I.E.M., Calcutta, for helpful suggestions and discussions and some instrumental facilities.

REFERENCES

1 R. N. Chopra, S. L. Nayar, and I. C. Chopra, *Glossary of Indian Medicinal Plants*, C.S.I.R., New Delhi, 1956, p. 233.
2 Q. N. Haq and N. I. Mollah, *Bangladesh J. Sci. Ind. Res.*, 8 (1973) 37–41.
3 S. Basu, *Carbohydr. Res.*, 81 (1980) 200–201.
4 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1966) 350–356.
5 Z. Dische, *J. Biol. Chem.*, 167 (1947) 189–198.
6 R. L. Taylor and H. E. Conrad, *Biochemistry*, 11 (1972) 1383–1388.
7 S.-I. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
8 T. Purdie and J. C. Irvine, *J. Chem. Soc.*, 85 (1904) 1049–1070.
9 H. Björndal, C. G. Hellerqvist, B. Lindberg, and S. Svensson, *Angew. Chem. Int. Ed. Engl.*, 9 (1970) 610–619.
10 G. O. Aspinall and A. K. Bhattacharjee, *J. Chem. Soc.*, (1970) 361–365.
11 G. W. Hay, B. A. Lewis, and F. Smith, *Methods Carbohydr. Chem.*, 5 (1965) 357–361.

12 M. ABDEL-AKHER, J. K. HAMILTON, R. MONTGOMERY, AND F. SMITH, *J. Am. Chem. Soc.*, 74 (1952) 4970–4971.
13 J. S. DIXON AND D. LIPKIN, *Anal. Chem.*, 26 (1954) 1092–1093.
14 G. O. ASPINALL AND R. J. FERRIER, *Chem. Ind. (London)*, (1957) 1216.
15 Z. DISCHE, L. B. SHETTLES, AND M. OSNOS, *Arch. Biochem.*, 22 (1949) 169–184.
16 Z. DISCHE, *J. Biol. Chem.*, 181 (1949) 379–392.