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STRUCTURE AND BIODEGRADATION OF THE POLYSACCHARIDE COMPONENTS OF EGG MASSES ISOLATED FROM SNAILS OF AN *Ampullarius* SPECIES*

MARCO A. LACOMBE FEIJÓ, JOSÉ H. DUARTE,
Departamento de Bioquímica, Universidade Federal do Paraná, 80.000 Curitiba (Brasil)

SAMUEL L. MACKENZIE, AND PHILIP A. J. GORIN
National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan S7N OW9 (Canada)

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ABSTRACT

A polysaccharide preparation, isolated from egg masses deposited by snails of an *Ampullarius* species, was purified *via* precipitation with Cetavlon in the presence of sodium borate, and found to contain D-galactose and a smaller proportion of D-glucose, and to have two components with sedimentation coefficients of 10*S* and 40*S*. A polysaccharide, isolated from freshly laid egg masses, was highly branched and was shown to contain nonreducing α-D-glucopyranosyl and β-D-galactopyranosyl end-groups, and 3,6-di-*O*-substituted β-D-galactopyranosyl residues. One or more of the polysaccharide components was a D-glucopyrano-D-galactopyranan with non-reducing α-D-glucopyranosyl end-groups (1→4)-linked to β-D-galactopyranosyl residues. The polysaccharide preparations, obtained from freshly laid egg masses and from those that were left for 10 and 15 days after being laid, were structurally different from each other. With the passage of time, progressive diminution of the 10*S* component and the proportion of D-glucose in the polysaccharide took place, suggesting that each constituent was consumed preferentially by the snail embryos as an energy source.

INTRODUCTION

Since the isolation by Hammarsten[1], in 1885, of a polysaccharide from the albumen glands of *Helix pomatia*, and its characterization as a galactan by May[2] in 1931, many investigations have been carried out on the polysaccharides of the albumen glands and egg masses of snails. Such polysaccharides consist principally of highly branched structures containing nonreducing and 3,6-di-*O*-substituted[3], and smaller proportions of 3-*O*- and 6-*O*-substituted[4] β-D-galactopyranosyl residues. However, several other structures have been observed, including L-galactopyrano-

*N.R.C.C. No. 20412.

syl[5-7] in *Helix pomatia* galactan, 2-*O*-substituted β-D-galactopyranosyl, and L-fucosyl residues in the fucogalactan of egg masses of an *Ampullarius* species[8] (Morretes, Paraná, Brazil), and (1-carboxyethylidene) acetals of β-D-galactopyranosyl units in the acidic galactan from albumen glands of *Pomacea lineata* (*Ampullarius* sp.)[9] (Recife, Pernambuco, Brazil).

RESULTS AND DISCUSSION

The present study concerns (a) the structural identification of polysaccharides of the egg masses of an *Ampullarius* species, collected from streams near Pelotas, Rio Grande do Sul, Brazil, and (b) the structural changes in the polysaccharide that occur progressively after laying. Polysaccharides were isolated and purified from egg masses obtained immediately after laying (T_0), and after 10 (T_{10}) and 15 (T_{15}) further days, the purification being carried out by precipitation from aqueous solution with Cetavlon in the presence of sodium borate. Precipitation of the required polysaccharide occurred exclusively when the pH was adjusted to pH 8.5. Yields were from 56% to 60%, based on acetone powders isolated after successive extractions with chloroform–methanol and butanol saturated with water at refluxing temperature, followed by deproteination of water-soluble material with Sigma protease.

The polysaccharide preparations contained galactose and glucose in the following ratios: T_0 (76:24), T_{10} (84:16), and T_{15} (89:11). Differences in composition between the three preparations were also detected by ultracentrifugation analyses, in which at least two components were demonstrated. These had sedimentation coefficients of 10*S* and 40*S*, and the 10*S* component decreased with increasing age of the egg masses (Fig. 1). Only single, excluded fractions were obtained in chromatography using Sephadex G-200 and Sepharose 6B-100, and a single band was observed in electrophoresis using Cellogel. D-Glucose and D-galactose were present, since, after hydrolysis of the polysaccharide, they were respectively oxidized by D-glucose

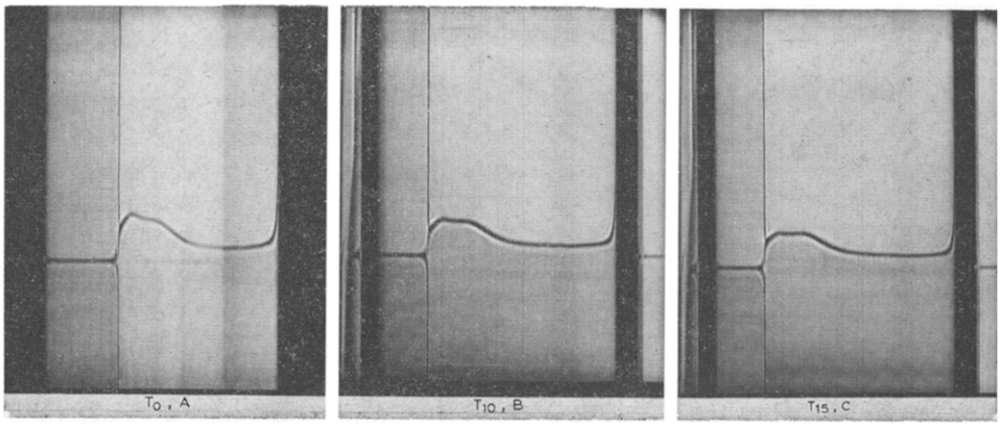

Fig. 1. Ultracentrifugation patterns of T_0(A), T_{10}(B), and T_{15}(C) obtained after 16 min at 30 000 r.p.m.

TABLE I

G.L.C. ANALYSIS OF PARTIALLY *O*-METHYLATED ALDITOL ACETATES OBTAINED FROM METHYLATED POLYSACCHARIDES[a]

Alditol acetates of[b]	T[c]	*Percentage of total peaks obtained from methylated polysaccharide*		
		T_0	T_{10}	T_{15}
2,3,4,6-Me_4-Glc	1.00	29.0	15.0	12.0
2,3,4,6-Me_4-Gal	1.25	20.0	23.0	30.0
2,4,6-Me_3-Gal	2.03		4.5	2.0
2,3,6-Me_3-Gal	2.16	7.0	11.0	8.0
2,3,4-Me_3-Gal	2.71		4.5	2.0
2,6-Me_2-Gal	3.09	5.0	7.0	6.0
2,4-Me_2-Gal	4.91	40.0	35.0	40.0

[a]As partially methylated alditol acetates. [b]Analyzed in column (b). [c]Column (a). Retention time (T) relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

TABLE II

COMPOSITION OF ALDITOL ACETATES OBTAINED BY SMITH DEGRADATION OF T_0, T_{10}, AND T_{15}, AS DETERMINED BY G.L.C.

Alditol acetate of[a]	R_{Gal}[b]	*Percentage of total peaks obtained from polysaccharide*		
		T_0	T_{10}	T_{15}
Glycerol	1.54	48.5	49.8	44.4
Threitol	1.30	9.6	11.0	10.2
Galactitol	1.00	37.4	35.3	40.9
Glucitol	1.13	4.5	3.9	4.5

[a]Column of 3% OV-225 on Chromosorb Q (80–100 mesh) (130–190°, 4°/min); arabinitol pentacetate as internal standard. [b]Paper chromatography (1:5:3:3, v/v, benzene–1-butanol–pyridine–water).

oxidase and D-galactose oxidase. The galactose was resistant to L-fucose dehydrogenase, which is specific for L-galactose and L-fucose.

Methylation analyses were carried out on T_0, T_{10} and T_{15} samples by the method of Jansson *et al.*[10], which includes g.l.c.–m.s. of the derived, partially methylated alditol acetates. As may be seen from Table I, the principal fragments obtained were acetates of 2,3,4,6-tetra-*O*-methyl-D-glucitol and -D-galactitol, and 2,3,6-tri-, 2,6-di-, and 2,4-di-*O*-methyl-D-galactitol. The most significant variation occurred with the proportions of 2,3,4,6-tetra-*O*-methyl-D-glucitol and -D-galactitol, that of the former decreasing and that of the latter increasing, respectively, on going progressively from T_0 through T_{10} to T_{15}. This suggests that the D-glucose component is con-

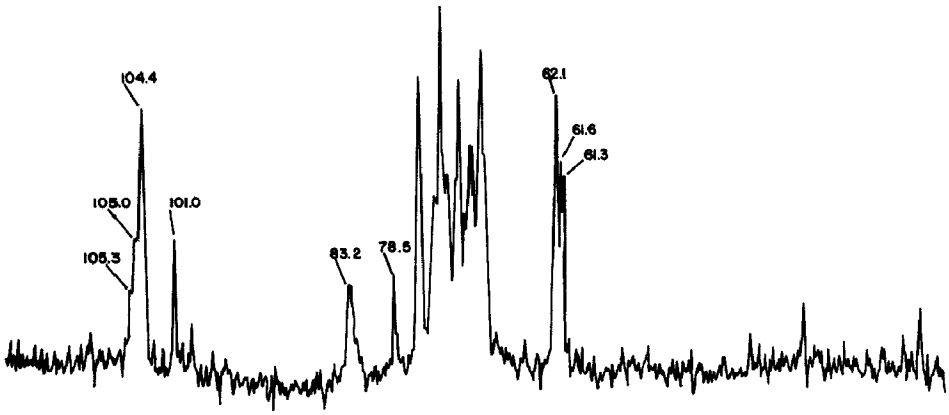

Fig. 2. ^{13}C-N.m.r. spectrum of T_0 for a solution in deuterium oxide at 70°. Depicted values are chemical shifts expressed in δ relative to an external standard of tetramethylsilane.

sumed preferentially by the snail embryos, and perhaps that D-glucose exists at a higher concentration in the 10*S* component. Also, since 2,3,4,6-tetra-*O*-methyl-D-glucitol acetate is the only D-glucose derivative detected in the course of the methylation analysis, virtually all D-glucose units are linked to a D-galactan core. The aforementioned methylation data appear to indicate the presence of 4-*O*-substituted D-galactopyranosyl, or 5-*O*-substituted D-galactofuranosyl residues, or both. However, since the acetate of 2,3,6-tri-*O*-methyl-D-galactitol could be the result of undermethylation, a Smith degradation[12] of T_0, T_{10}, and T_{15} was performed. It gave a mixture of glycerol, threitol, galactitol, and 4% of unoxidized glucitol, which were determined as the acetates by g.l.c. (Table II). The presence of D-glucose cannot be explained on the basis of the methylation data.

The quantitative periodate data for T_0 (consumption of 0.87 mol of sodium periodate/sugar unit, and production of 0.39 mol of acid/unit), and T_{10} and T_{15} (consumption of 0.79 mol of sodium periodate/sugar unit; 0.36 mol of acid/unit) agree with the quantitative methylation data (Table I).

The specific rotations of T_0, T_{10}, and T_{15} are in the range of +41 to +44°, which are higher than the value of +21° reported for a snail β-D-galactopyranan[4]. Therefore, since galactosyl and glucosyl residues are present as the D enantiomers, the presence of a small proportion of α-D-hexopyranosyl units is indicated. This was confirmed by the ^{13}C-n.m.r. spectrum of T_0 (Fig. 2), which contains a minor C-1 signal at δ 101.0, at a field higher than that of the C-1 signals at δ 105.3, 105.0, and 104.4, which correspond to β-D-galactopyranosyl or β-D-glucopyranosyl residues[13,14]. The signal at δ 101.0 corresponds to α-D-glucopyranosyl residues, since partial acid hydrolysis of the polysaccharide gave 4-*O*-α-D-glucopyranosyl-α,β-D-galactose, a structure that was indicated by methylation data and its high specific rotation of +130°. The isolation of this disaccharide, combined with the finding that degradation of the polysaccharide by mild hydrolytic conditions gave threitol (in addition to glycerol), shows the presence of a nonreducing end-group having the structure

α-D-Glc*p*-(1→4)-β-D-Gal*p*-. The β-D configuration of the galactopyranosyl residues seems likely, because the ^{13}C-n.m.r. spectrum of the β-D anomer of the disaccharide has a C-4 signal at δ 79.3 corresponding to the signal at δ 78.5 in the spectrum of the parent polysaccharide. Correspondence was also observed between the C-1 signal in the spectra of the polysaccharide (δ 101.0) and C-1 in that of the disaccharide (δ 101.8).

The absence of signals that are indicative of α- and β-D-galactofuranosyl residues, in regions typical[15–17] for C-1, C-2, C-3, and C-4 of the ^{13}C-n.m.r. spectrum, confirms the observation that all the D-galactosyl units are in the pyranose form and are 4-*O*-substituted.

The absence of 3,4-di-*O*-substituted D-galactopyranosyl residues, suggested by the characterization of the acetate of 2,6-di-*O*-methylgalactitol in the methylation analysis, is indicated by the ^{13}C-n.m.r. spectrum. Undermethylation may result because snail galactans are highly branched structures and difficult to methylate[4], especially at OH-4. If such di-*O*-substituted units were present, there would be more that one signal in each region (Fig. 2) corresponding to *O*-substituted C-3 nuclei (δ 83.2) and to *O*-substituted C-4 nuclei (δ 78.5), due to β-substitution shifts[18,19]. The former signal was assigned as it was similar to the corresponding resonance in the model compound[14], methyl 3-*O*-β-D-galactopyranosyl-β-D-galactopyranoside (C-3, δ 84.0).

According to the data just presented, polysaccharide T_0 has at least two components and contains a D-gluco-D-galactan. This is highly branched, like other snail β-galactopyranans, and, as a whole, consists mainly of nonreducing β-D-galactopyranosyl and α-D-glucopyranosyl end-groups, and 3,6-di-*O*-substituted β-D-galactopyranosyl residues. The α-D-glucopyranosyl groups are mostly linked (1→4) to β-D-galactopyranosyl residues. Such a structure, having α-D-glucopyranosyl units, has not previously been recognized in galactose-containing polysaccharides from egg masses and albumen glands of snails.

EXPERIMENTAL

Isolation and purification of polysaccharides from egg masses of Ampullarius *species.* — Molluscs of an *Ampullarius* species were collected during May 1977 from small streams entering the beach of Laranjal, Lagoa dos Patos, Pelotas, RGS, Brazil, and transferred to an aquarium with artificial aeration. The molluscs, fed daily with leaves of water-cress, laid their egg masses directly on the glass walls of the aquarium. The egg masses, collected soon after laying (32 egg masses*, 6–10 h), contained a polysaccharide designated T_0. The rest of the egg masses was left on the wall for a longer time. Those that were extracted 10 (28 egg masses) and 15 (25 egg masses) days after laying gave polysaccharides designated T_{10} and T_{15}, respectively. The rest of the egg masses (17) was left for a further two days and found to be fertile, since snails were hatched.

Each of the samples of the egg masses was homogenized with acetone (200 mL)

*Each egg mass contains ~100 eggs.

in a blender, the insoluble material centrifuged off, and the resulting pellet extracted with 2:1 (v/v) chloroform–methanol (100 mL). The suspension was centrifuged, and the residue was heated under reflux with butanol saturated with water (150 mL) for 6 h. Insoluble material was filtered off, washed with acetone, and dried successively on the bench in the open air, and then *in vacuo*. Each of the acetone powders (T_0, 33 g; T_{10}, 28 g; and T_{15}, 22 g) was then suspended in 0.1M aqueous ammonium acetate (600 mL, pH 8.5), and the solution deproteinated with Sigma protease Type II (200 mg). After being kept for 72 h at 37° with the protection of toluene, the solution was centrifuged and the supernatant deproteinated by the method of Sevag[20]. After recentrifugation, the supernatant solution was treated with ethanol (3 vol.), and the resulting precipitate was filtered off, washed with acetone, dissolved in water, and lyophilized. The dry residue (T_0, 1.8 g; T_{10}, 1.5 g; and T_{15} 1.4 g) was dissolved in water (100 mL) and treated with 5% aqueous Cetavlon (100 mL, pH 7.0) to eliminate insoluble nucleic acids, and then with 0.1M aqueous sodium tetraborate (200 mL), the final pH being 8.5. The insoluble material was isolated, and the purified, nitrogen-free polysaccharide was obtained by dissolution with 2M acetic acid, followed by ethanol precipitation: T_0, 1.0 g, 55.5% of acetone powder, $[\alpha]_D^{25}$ +43.8° (*c* 1.0, water); T_{10}, 0.9 g, 60.0% of acetone powder, $[\alpha]_D^{25}$ +41.2° (*c* 1.0, water); and T_{15}, 0.8 g, 57.1% of acetone powder, $[\alpha]_D^{25}$ +40.8° (*c* 1.0, water).

Acid hydrolysis of purified polysaccharides T_0, T_{10}, and T_{15}. — A sample of each polysaccharide was hydrolyzed with 0.5M sulfuric acid for 18 h at 100°, the solution neutralized with barium carbonate, the product reduced with sodium borohydride, and the resulting alditols converted into the acetates. These were examined by g.l.c. in a conventional column (120 cm; i.d. 0.4 cm) of 3% OV-225 on Chromosorb Q (80–100 mesh) with a temperature program of 130 to 190° (4°/min, then hold). The acetates of galactitol and glucitol were respectively detected in the following ratios: T_0, 76:24; T_{10}, 84:16; and T_{15}, 89:11.

Configuration of glucose and galactose residues. — The hydrolyzate obtained from T_0 was shown to be susceptible to oxidation by D-glucose oxidase[21] and D-galactose oxidase[22]. The galactose portion was unaffected by L-fucose dehydrogenase[23].

Criteria of heterogeneity of polysaccharides T_0, T_{10}, and T_{15}. — The polysaccharides gave only single-excluded fractions when passed through columns of Sephadex G-200 (80 × 2 cm; 10 mL/h) and Sepharose 6B-100 (100 × 2 cm; 12 mL/h). The polysaccharides each gave a single band when submitted to electrophoresis on Cellogel[24].

Each polysaccharide (10 mg), dissolved in 0.5M sodium hydroxide (2 mL), was submitted to ultracentrifugation at 20° and 30 000 r.p.m. At least two components were present for 10*S* and 40*S*. As may be seen from the patterns obtained after 16 min (Fig. 1), the proportion of the 10*S* component decreased progressively in the samples T_0, T_{10}, and T_{15}, respectively.

Methylation analysis of polysaccharides. — Samples of T_0, T_{10}, and T_{15} were methylated successively by the methods of Haworth[25] and Hakomori[26]. The methyl-

ated polysaccharides were converted to partially methylated alditol acetates by successive treatment with 5% methanolic hydrogen chloride (5 h, 100°) and 0.5M hydrochloric acid (5 h, 100°), reduction with sodium borohydride, and acetylation with acetic anhydride–pyridine. The products were examined by g.l.c. in (a) a glass-capillary column (30 m), coated with OV-225, from 95 to 230° (2°/min), and (b) a conventional column (1.20 m, 0.4 cm i.d.) of 3% (w/w) ECNSS-M on Chromosorb W (80–100 mesh), from 120 to 170° (4°/min, then hold). The partially methylated fragments were characterized by the retention times on the capillary column, and by electron-impact mass spectrometry[10]. The conventional column was used for quantitative determination. The results are presented in Table I.

Isolation and methylation analysis of disaccharide formed from polysaccharide. — Polysaccharide (500 mg) was partially hydrolyzed with 0.5M sulfuric acid (50 mL) for 2 h at 70°. The solution was neutralized ($BaCO_3$), filtered, and the filtrate evaporated to a syrup. This was fractionated on a cellulose column; 7:1 (v/v) acetone–water eluted D-glucose and D-galactose, and 4:1 (v/v) acetone–water a disaccharide (15 mg) having $[\alpha]_D^{25}$ +130° (*c* 0.2, water). Acid hydrolysis of the disaccharide gave D-glucose and D-galactose.

A portion of the disaccharide was methylated by the method of Haworth using mild-alkaline conditions in the initial stage[25], and the partly methylated product extracted continuously with chloroform from the neutralized mixture. Methylation was completed by the procedure of Kuhn *et al.*[27], and the product converted into a mixture of *O*-methylhexitol acetates, as described earlier. These were shown, by g.l.c.–m.s. using column (a), to be mainly acetates of 2,3,4,6-tetra-*O*-methylglucitol and 2,3,6-tri-*O*-methylgalactitol, indicating a 4-*O*-α-D-glucopyranosyl-D-galactose structure.

Smith degradation of polysaccharides T_0, T_{10}, and T_{15}. — Each polysaccharide (50 mg) was oxidized with 0.01M aqueous sodium metaperiodate for 94 h at 0–2°, in the dark. The product was reduced with sodium borohydride, and the polyalcohol hydrolyzed with 0.5M sulfuric acid (5 h, 100°). Reduction with sodium borohydride, followed by acetylation, gave a mixture of acetates of glycerol, threitol, galactitol, and glucitol, which was analyzed by g.l.c. with the conventional OV-225 column described earlier, and with the aid of an internal standard of arabinitol pentaacetate. The quantitative results are presented in Table II. Partial hydrolysis of the polyalcohol from T_0, at pH 2 for 1 h at 100° gave mainly glycerol with less threitol.

Quantitative periodate oxidation of T_0, T_{10}, and T_{15}. — Each polysaccharide (50 mg) was oxidized in 0.01M sodium metaperiodate (100 mL) at 0–2° in the dark. The consumption of oxidant and production of formic acid was followed at time intervals of up to 95 h, the values being based on anhydrohexose units. Extrapolation of the curves to zero time gave the following results: T_0 (0.87 mol/mol uptake, 0.39 mol/mol acid), T_{10} (0.79 mol/mol uptake, 0.36 mol/mol acid), and T_{15} (0.79 mol/mol uptake, 0.36 mol/mol acid).

^{13}C-N.m.r. spectra of T_0 and T_{10}. — These spectra were obtained for solutions in deuterium oxide at 70° under the conditions previously described[28].

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REFERENCES

1 O. HAMMARSTEN, *Pfluegers Arch.*, 36 (1885) 373–456.
2 F. MAY, *Z. Biol.*, 91 (1931) 215–220.
3 E. BALDWIN AND D. J. BELL, *J. Chem. Soc.*, (1938) 1461–1465.
4 J. H. DUARTE AND J. K. N. JONES, *Carbohydr. Res.*, 16 (1971) 327–335.
5 F. MAY, *Z. Biol.*, 92 (1932) 325–350.
6 D. J. BELL AND E. BALDWIN, *J. Chem. Soc.*, (1941) 125–131.
7 K. LEONTEIN, B. LINDBERG, AND J. LÖNNGREN, *Carbohydr. Res.*, 62 (1978) 359–362.
8 M. A. LACOMBE FEIJÓ AND J. H. DUARTE, *Carbohydr. Res.*, 44 (1975) 241–249.
9 P. A. J. GORIN, M. MAZUREK, H. S. DUARTE, AND J. H. DUARTE, *Carbohydr. Res.*, 92 (1981) c1–c4.
10 P.-E. JANSSON, L. KENNE, H. LIEDGREN, B. LINDBERG, AND J. LÖNNGREN, *Chem. Commun. Univ. Stockholm*, 8 (1976) 1–75.
11 P. A. J. GORIN AND D. E. EVELEIGH, *Biochemistry*, 9 (1970) 5023–5027.
12 I. J. GOLDSTEIN, G. W. HAY, B. A. LEWIS, AND F. SMITH, *Abstr. Pap. Am. Chem. Soc. Meet., 135* (1959) 3D.
13 L. D. HALL AND L. F. JOHNSON, *J. Chem. Soc., Chem. Commun.*, (1959) 509–510.
14 P. A. J. GORIN, *Carbohydr. Res.*, 101 (1982) 13–20.
15 P. A. J. GORIN AND M. MAZUREK, *Can. J. Chem.*, 53 (1975) 1212–1223.
16 L. MENDONÇA, P. A. J. GORIN, K. O. LLOYD, AND L. R. TRAVASSOS, *Biochemistry*, 15 (1976) 2423–2431.
17 P.-E. JANNSON AND B. LINDBERG, *Carbohydr. Res.*, 82 (1980) 97–102.
18 D. E. DORMAN, S. J. ANGYAL, AND J. D. ROBERTS, *J. Am. Chem. Soc.*, 92 (1970) 1351–1354.
19 P. A. J. GORIN AND J. F. T. SPENCER, *Can. J. Microbiol.*, 18 (1972) 1709–1715.
20 M. G. SEVAG, *Biochem. Z.*, 273 (1934) 419–423.
21 A. DAHLQVIST, *Biochem. J.*, 80 (1961) 547–555.
22 D. AMARAL, F. F. KELLY, AND B. L. HORECKER, *Methods Enzymol.*, 9 (1966) 87–92.
23 M. F. GUIMARÃES, L. U. RIGO, AND L. A. VEIGA, in A. STOPANI, M. BACILA, AND B. L. HORECKER (Eds.), *Biochemistry and Genetics of Yeasts*, Academic Press, New York, 1978, pp. 161–170.
24 W. F. DUDMAN AND C. T. BISHOP, *Can. J. Chem.*, 46 (1968) 3079–3084.
25 W. N. HAWORTH, *J. Chem. Soc.*, 107 (1915) 8–16.
26 S. HAKOMORI, *J. Biochemistry (Tokyo)*, 55 (1964) 205–208.
27 P. KUHN, H. TRISCHMANN, AND I. LÖW, *Angew. Chem.*, 67 (1955) 32–37.
28 P. A. J. GORIN, R. H. HASKINS, L. R. TRAVASSOS, AND L. MENDONÇA-PREVIATO, *Carbohydr. Res.*, 55 (1977) 21–23.