[22] M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS AND F. SMITH, *Anal. Chem.*, 28 (1956) 350.
[23] A. J. MORRIS AND S. R. DICKMAN, *J. Biol. Chem.*, 235 (1960) 1404.
[24] E. FARBER AND H. SIDRANSKY, *J. Biol. Chem.*, 222 (1956) 237.
[25] L. E. HOKIN, *Biochim. Biophys. Acta*, 18 (1955) 379.
[26] P. SIEKEVITZ AND G. E. PALADE, *J. Biophys. Biochem. Cytol.*, 4 (1958) 557.
[27] V. G. ALLFREY, M. M. DALY AND A. E. MIRSKY, *J. Gen. Physiol.*, 37 (1953) 157.
[28] P. SIEKEVITZ AND G. E. PALADE, *J. Biophys. Biochem. Cytol.*, 4 (1958) 203.
[29] A. K. LAIRD AND A. D. BARTON, *Biochim. Biophys. Acta*, 25 (1957) 56.
[30] M. M. DALY, V. G. ALLFREY AND A. E. MIRSKY, *J. Gen. Physiol.*, 39 (1955) 207.
[31] J. L. VAN LANCKER AND R. L. HOLTZER, *J Biol. Chem.*, 234 (1959) 2359.
[32] P. SIEKEVITZ AND G. E. PALADE, *J. Biophys. Biochem. Cytol.*, 7 (1960) 619.

Biochim. Biophys. Acta, 69 (1963) 85–96

BBA 3845

STRUCTURE OF *HANSENULA CAPSULATA* NRRL Y-1842 PHOSPHOMANNAN

M. E. SLODKI
Northern Regional Research Laboratory, Peoria, Ill. (U.S.A.)*

(Received July 4th, 1962)

SUMMARY

Di- and trisaccharide phosphomonoesters have been isolated after mild acid hydrolysis of the phosphomannan produced by *Hansenula capsulata* NRRL Y-1842. Periodate oxidation studies on both the oligosaccharide phosphates and the intact polymer have served to elucidate further the polyphosphodiester structure. The disaccharide ester has been characterized as 2-*O*-(6-*O*-phosphoryl-β-D-mannopyranosyl)-D-mannose; the trisaccharide ester appears to contain an additional mannosyl residue α-1,2-linked to the phosphorylated moiety of the disaccharide. The biosynthetic implications of these findings are discussed.

INTRODUCTION

Among the phosphorylated mannose polymers[1] produced by yeasts of *Hansenula* and related genera, those produced by *H. capsulata* strains are the most highly phosphorylated[2]. The latter polymers are further distinguished by their low or negative optical rotations, indicating the presence of mannosidic linkages of the β-configuration[2].

Previous work[3] on the phosphate linkages in phosphomannans has shown that these extracellular polymers are polyphosphodiesters containing the α-1,6'-phosphate ester bridge between mannose units as a characteristic structural feature. Also, mild

* This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

acid hydrolysis (pH 3, 100°, 20 min) quantitatively cleaved the α-mannosyl phosphate linkages without concomitant splitting of mannosyl–mannose bonds.

This report describes the separation and characterization of the oligosaccharide phosphomonoesters formed by the "autohydrolysis" of decationized *H. capsulata* NRRL Y-1842 phosphomannan.

EXPERIMENTAL

Materials and methods

The production, isolation, and purification of *H. capsulata* NRRL Y-1842 phosphomannan, $[\alpha]_D^{25} = -2°$ (1.0% K^+ form in 0.1 M KCl), has been described previously[2]. Periodate consumption was measured by the Fleury–Lange arsenite procedure[4] and formic acid, after destruction of excess periodate with ethylene glycol, by titration with standard NaOH solution to the phenolphthalein end point. Formaldehyde was estimated by the chromotropic acid procedure[5] with appropriate periodate blanks. Oligosaccharide reducing end groups were determined by hypoiodite oxidation[6]. Theoretical iodine consumption was obtained with a 10:1 molar ratio of NaOH:phosphate ester (acid form); greater or lesser amounts of alkali gave lower values. Organic phosphate was estimated as described by LELOIR AND CARDINI[7] and mannose by the SOMOGYI–NELSON[8] procedure.

Isolation of oligosaccharide phosphate esters

A decationized solution of Y-1842 phosphomannan was prepared by dissolving 2.0 g in 100 ml of water and mixing in an excess of sulfonic acid-type (H^+ form) cation-exchange resin. The mixture was heated in a sealed flask for 20 min at 100° with occasional mixing. As shown previously[3], hydrolysis of the acid-labile secondary phosphoryl linkages does not require the presence of resin. Under these conditions, the resin does not alter the hydrolysis; *i.e.*, free mannose is not liberated.

After cooling and removal of the resin by filtration, the hydrolyzate was neutralized to pH 8.2 with NaOH and excess 25% barium acetate added as described by LE PAGE[9]. The oligosaccharide phosphate esters were isolated as Ba^{2+} salts by the addition of 95% methanol, overnight refrigeration and centrifugation. After decantation of the methanolic supernatant liquors, the precipitates were dissolved in water and Ba^{2+} was removed by treatment with cation-exchange resin (H^+ form) before characterization.

RESULTS

Characterization and yields of phosphomonoesters

Paper chromatographic analyses of Y-1842 phosphomannan autohydrolyzates[3] revealed the presence of but a single spot with a position constant similar to that given in the literature[10] for a disaccharide monophosphate. Examination of autohydrolyzates by paper ionophoresis in either 0.1 M borax or 0.05 M barbital (600 V, 4 h) also gave single spots which migrated slower than mannose 6-phosphate. Table I shows that fractional methanolic precipitation as Ba^{2+} salts resolved the autohydrolyzate into di- and trisaccharide monophosphates. None of the fractions were distinguishable when examined individually by either paper chromatography or paper ionophoresis.

TABLE I

FRACTIONATION OF PHOSPHORYLATED OLIGOSACCHARIDE MIXTURE (AS Ba^{2+} SALTS) WITH AQUEOUS METHANOL

The mannose: phosphate molar ratio was determined after hydrolysis (4 N H_2SO_4 for 75 min at 100°, sealed tube). The $[\alpha]_D^{25°}$ value was calculated for the acid form. C = 1.02, 0.81, 1.86 and 2.47 g/100 ml, respectively. The reducing end group/P ratio was determined by iodine titration. Periodate consumption values were obtained by extrapolation to zero time (theoretical: 6.0 for trisaccharide, 5.0 for disaccharide); HCOOH liberation at 72 h (theoretical: 3.0 in all cases); CH_2O liberation measured after periodate oxidation in 0.12 N H_2SO_4 for 120 h at 4° in the dark (theoretical: 1.0 in all cases). The overall P recovery was 83%.

Methanol (%)	*Mannose: phosphate (molar ratio)*	$[\alpha]_D^{25}$	*Reducing end group/P*	*Periodate oxidation (moles/mole phosphate)*			*Per cent recovered P*	
				Periodate consumed	*HCOOH liberated*	*CH_2O liberated*		
45	3.10	+ 7°	1.01	5.8	3.1	0.76	18	Trisaccharide
60	2.13	−28°	1.05	5.1	3.2	0.83	14	Disaccharide
67	2.00	−37°	1.07	4.7	3.4	1.05	27	Disaccharide
72	1.95	−38°	0.92			1.00	41	Disaccharide

The isolated phosphomonoesters give but 12 and 20% of the expected reducing power values, respectively, with alkaline copper[8] and ferricyanide[11] reagents. This behavior is characteristic of 2-*O*-substituted sugars[12,13]. Similarly, the oligosaccharide phosphates do not react[14,15] on paper with alkaline 2,3,5-triphenyl tetrazolium chloride spray reagent; equivalent amounts of mannose 6-phosphate give a positive reaction. Under the conditions described, however, the esters are quantitatively oxidized by alkaline iodine. The reducing end group:P ratios thus obtained show that the esters are monophosphorylated oligosaccharides.

Recovery figures in Table I indicate that the di- and trisaccharides are produced in a ratio of 82:18, or approx. 4:1. Under the autohydrolysis conditions used, no free mannose or mannose 6-phosphate could be detected by paper chromatography[3]. The isolations and characterizations cited thus far have been repeated in two other instances with essentially the same results.

Periodate oxidation of phosphomonoesters

The acid forms of the phosphate esters (44–57 μmoles) were oxidized in the presence of excess sodium periodate (400 μmoles) over a period of 72 h. Although the reaction was carried out in the dark at 4° and at pH 3, considerable overoxidation was noted. The values for periodate consumption in Table I were obtained by extrapolation of the straight-line overoxidation curves back to zero time (Fig. 1). Formic acid production proceeded at a much slower rate. The rate curves for the three fractions were nearly identical and, although giving evidence of overoxidation, became asymptotic when around 3 μmoles HCOOH per μmole ester were produced.

Overoxidation by periodate, taken together with the periodate uptake and formic acid production values, implies that the 2-*O*-linked reducing end groups react in the open-chain form; the substituted malondialdehyde thus formed would be readily oxidized by periodate[16]. Reduction of the end group to the 2-*O*-mannitol derivative before periodate oxidation eliminated overoxidation without affecting the stoichiometry of periodate uptake (Fig. 1) and confirms this mechanism. That the

reducing end group does not bear the phosphoryl residue is evident from formaldehyde production (Table I). Theoretical formaldehyde liberation was obtained only when the periodate oxidation was carried out under more acidic conditions.

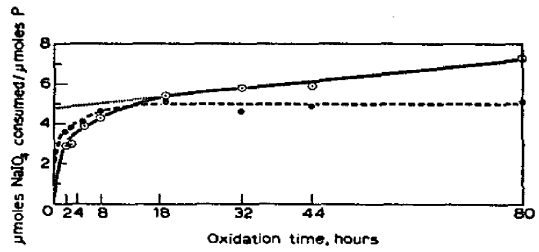

Fig. 1. Periodate oxidation of the disaccharide monophosphate (⊙—⊙) and the $NaBH_4$-reduced ester (●—●). Conditions: 4°, dark; phosphate esters, 22.6 μmoles each; $NaIO_4$, 200 μmoles; total volume 25 ml. As the unreduced ester was used in the acid form, its reaction mixture was pH 3. The reduced ester was prepared by $NaBH_4$ reduction after neutralization. Before adding $NaIO_4$, excess borohydride was decomposed by addition of 33% acetic acid to pH 4.2.

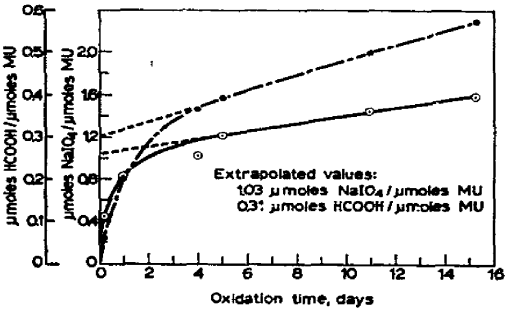

Fig. 2. Periodate oxidation of intact *Hansenula capsulata* NRRL Y-1842 phosphomannan. ⊙—⊙, Periodate consumption; ●—●, HCOOH production. MU = mannose unit.

Periodate oxidation of intact polymer

Intact Y-1842 phosphomannan (100 mg, K^+ salt) was dissolved in 100 ml water containing sufficient $NaIO_4$ to provide for the consumption of as much as 3 equiv. per mannose unit. The oxidation was allowed to proceed at 4° in the dark over a period of 15 days. Fig. 2 illustrates the results obtained by sampling the reaction mixture (pH 6) over this period. After an initially rapid oxidation, a slower linear overoxidation curve was obtained. As with the oxidation of the phosphomonoesters, it was possible to correct for the overoxidation by extrapolation.

The portion of the reaction mixture remaining after 15 days was decationized with a sulfonic acid-type resin and the filtrate neutralized with excess $BaCO_3$. After refrigeration for several days, the relatively insoluble barium salts of iodate and periodate were filtered off. The neutral filtrate was then treated with an excess of $NaBH_4$ and allowed to remain overnight at room temperature. Excess borohydride was decomposed and cations were simultaneously removed from the now alkaline solution by admixture with sulfonic acid resin. The reduced oxy-polymer was hydrolyzed by refluxing the acidic resin filtrate (congo red indicator) 1.5 h. After cooling, the hydrolyzate was lyophilized. Borate was removed from the product as trimethyl borate by dissolving the lyophilized powder in 95% methanol and distillation at 45° (reduced pressure). This step was repeated five times.

After removing methanol by a stream of N_2, the residue was taken up in water. The phosphorus in this solution (pH 3.1) was mainly in the form of organic phosphate. The solution was adjusted to pH 6.5 with 0.1 N NaOH and chromatographed on Dowex-1-Cl^- (see foot-note) as described by BYRNE AND LARDY[17]. Only a single

* Mention of this product does not constitute an endorsement by the U.S. Department of Agriculture.

organic phosphate peak was obtained by step-wise gradient elution with 0.025–1 N HCl. The fractions containing the phosphate ester were pooled, neutralized, and concentrated by lyophilization. Examination by paper chromatography[10] in two different solvent systems (ethyl acetate–acetic acid–water (3:3:1) at 4°, for 6 h, descent and *tert.*-butyl alcohol–water–picric acid (80:20:2 g) at 25° for 20 h, descent) identified the ester as glycerol phosphate. Mannose 6-phosphate was not detected. The phosphate ester was hydrolyzed in the presence of calf intestinal alkaline phosphatase and Mg^{2+} (37°, pH 9.2, 3 h). After concentration by benzene distillation under reduced pressure, the hydrolyzate was examined by paper chromatography with 5% ammoniacal $AgNO_3$ spray reagent[16] to detect polyhydric alcohols. Glycerol was identified as the sole component with two different solvent systems: *n*-butyl alcohol–pyridine–water (10:3:3, v/v) for 14 h, descent[18], and *n*-butyl alcohol–acetic acid–water (4:1:5, v/v) for 1 h, ascent[15].

DISCUSSION

Consumption of one periodate equivalent for each anhydromannose unit during oxidation of intact Y-1842 phosphomannan indicates the absence of 1,3-glycosidic bonds. The complete destruction of mannose 6-phosphate, together with its conversion to a three-carbon fragment, also eliminates the possibility of 4-*O*-substituents on the phosphorylated residues. This result also carries the restriction that any structural formulation of the oligosaccharides must provide for glyceraldehyde 3-phosphate formation upon periodate oxidation. Of the permissible disaccharide monophosphate structures, only the 1,2-linked possibilities could be phosphorylated on either primary hydroxyl. The production of formaldehyde, taken together with the other periodate oxidation results, clearly establishes the structure of the disaccharide phosphate as 2-*O*-(6-*O*-phosphoryl-β-D-mannopyranosyl)-D-mannose (Fig. 3,A).

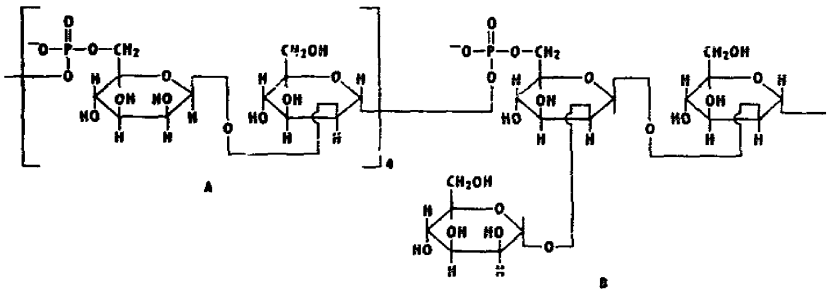

Fig. 3. Proposed structure for *Hansenula capsulata* NRRL Y-1842 phosphomannan.

The optical rotation of the trisaccharide ester indicates that it contains both α- and β-mannosyl linkages. During prolonged autohydrolysis of Y-1842 phosphomannan, free mannose can be detected before mannose 6-phosphate appears. Trisaccharide phosphate was not isolated from an autohydrolyzate heated for 30 min although total phosphate recovery (entirely in the form of the disaccharide ester) was similar to that described in Table I. Apparently the trisaccharide monophosphate contains an additional mannosyl residue α-1,2-linked to the phosphorylated moiety

of the disaccharide (Fig. 3,B). This formulation agrees with the apparent acid lability as well as with the periodate oxidation results.

Data reported here, together with those previously obtained for the phosphate linkages[3], suggest the polyphosphodiester backbone structure for Y-1842 phosphomannan illustrated in Fig. 3. If it is assumed that every fifth disaccharide unit contains an appended α-mannosyl unit, correlations can be made with the periodate oxidation analysis on the intact polymer (Table II). The positive rotation of the α-mannosyl phosphate linkage must largely cancel the negative rotation contribution of the β-linked disaccharide unit since both linkages are present in equal amounts.

TABLE II

COMPARISON BETWEEN OBSERVED PROPERTIES AND THOSE CALCULATED FOR PROPOSED STRUCTURE

Property	*Observed value*	*Theoretical value*
Mannose: P molar ratio	2.5	2.2
$[\alpha]_D^{25}$	$-2°$	approx. 0
IO_4^- uptake per mannose unit	1.03	1.45
HCOOH formed per mannose unit	0.31	0.45

Biosynthetic implications

The repeating disaccharide phosphate backbone of the structure depicted in Fig. 3 suggests that Y-1842 phosphomannan biosynthesis occurs *via* alternate mannosyl and mannose 1-phosphoryl transfers from a precursor such as guanosine diphosphate mannose (GPPM):

$$\text{GPPM} + \text{M} \longrightarrow \text{GPP} + \text{M-M}$$

$$\text{GPPM} + \text{M-M} \longrightarrow \text{GP} + \text{M-P-M-M}$$

$$\text{GPPM} + \text{M-P-M-M} \longrightarrow \text{GPP} + \text{M-M-P-M-M}$$

$$\text{GPPM} + \text{M-M-P-M-M} \longrightarrow \text{GP} + \text{M-P-M-M-P-M-M, etc.}$$

Similar phosphoryl transfers from nucleotide precursors have been demonstrated in phospholipid biosynthesis[20] and postulated for teichoic acid biogenesis[21]. As previously noted[3], this scheme accounts for the configuration of the α-mannosyl phosphate linkage since that bond would not be disturbed during mannose 1-phosphoryl transfer from GPPM to the primary hydroxyl of the acceptor. The biosynthesis study of MUNCH-PETERSEN[22] indicates that the mannosyl residue in GPPM is of the α-anomeric configuration. The hypothetical scheme may also account for our inability to find a mannose-containing nucleotide other than GPPM in extracts of *H. holstii* NRRL Y-2448 (see ref. 23). This organism produces a less highly phosphorylated phosphomannan that has a relatively high positive specific rotation[1]. Even so, the participation in phosphomannan biosynthesis of a mannose nucleotide analogous to the uridine diphosphate-*N*-acetylglucosamine-6-phospho-1-galactose from hen oviduct[24-26] cannot be ruled out. While other mannosyl phosphate donors and acceptors may be involved in alternative pathways, the scheme given serves to rationalize the polymer structure in plausible biogenic terms.

ACKNOWLEDGEMENT

The author gratefully acknowledges the helpful discussions with Drs. J. W. VAN CLEVE and J. H. SLONEKER during the course of this work.

REFERENCES

1 A. JEANES, J. E. PITTSLEY, P. R. WATSON AND R. J. DIMLER, *Arch. Biochem. Biophys.*, 92 (1961) 343.
2 M. E. SLODKI, L. J. WICKERHAM AND M. C. CADMUS, *J. Bacteriol.*, 82 (1961) 269.
3 M. E. SLODKI, *Biochim. Biophys. Acta*, 57 (1962) 525.
4 E. L. JACKSON, *Org. Reactions*, 2 (1944) 361.
5 M. LAMBERT AND A. C. NEISH, *Can. J. Research*, B28 (1950) 83.
6 A. C. NIESH, *Analytical Methods for Bacterial Fermentations*, National Research Council of Canada, Saskatoon, 1952, p. 22.
7 L. F. LELOIR AND C. E. CARDINI, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. III, Academic Press, New York, 1957, p. 840.
8 S. SOMOGYI, *J. Biol. Chem.*, 195 (1952) 19.
9 G. A. LEPAGE, in W. W. UMBREIT, R. A. BURRIS AND J. F. STAUFFER, *Manometric Techniques*, Burgess Publishing Co., Minneapolis, 1957, p. 268.
10 A. A. BENSON, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. III, Academic Press, New York, 1957, p. 119.
11 J. T. PARK AND M. J. JOHNSON, *J. Biol. Chem.*, 181 (1949) 149.
12 H. SOBOTKA, *J. Biol. Chem.*, 69 (1926) 267.
13 G. ZEMPLÉN, *Ber.*, 59 (1926) 2404.
14 K. WALLENFELS, *Naturwiss.*, 37 (1950) 491.
15 D. J. BELL AND R. DEDONDER, *J. Chem. Soc.*, (1954) 2866.
16 M. CANTLEY, L. HOUGH AND A. O. PITTET, *Chem. and Ind.*, (1959) 1126.
17 W. L. BYRNE AND H. A. LARDY, *Biochim. Biophys. Acta*, 14 (1954) 495.
18 L. HOUGH, *Nature*, 165 (1950) 400.
19 J. G. BUCHANAN, C. A. DEKKER AND A. G. LONG, *J. Chem. Soc.*, (1950) 3162.
20 E. P. KENNEDY AND S. B. WEISS, *J. Am. Chem. Soc.*, 77 (1955) 250.
21 J. BADDILEY AND J. G. BUCHANAN, *Quart. Rev.*, 12 (1958) 166.
22 A. MUNCH-PETERSEN, *Arch. Biochem. Biophys.*, 55 (1955) 592.
23 M. E. SLODKI AND H. P. MELOCHE, Jr., *Abstr. of Papers, 134th Meeting, Am. Chem. Soc.*, September, 1958, p. 56C.
24 J. L. STROMINGER, *J. Biol. Chem.*, 237 (1962) 1388.
25 S. SUZUKI, *J. Biol. Chem.*, 237 (1962) 1393.
26 O. GABRIEL AND G. ASHWELL, *J. Biol. Chem.*, 237 (1962) 1400.