Molecular Organization of a Covalent
Peptido-phospho-polysaccharide Complex from the
Yeast Form of Cladosporium werneckii

Kenneth O. Lloyd

ABSTRACT: A galactomannan from the yeast form of Cladosporium werneckii contains phosphate groups (3.5% of carbohydrate) and covalently linked peptide (10%). Treatment of the complex with 0.3 m NaOH and 0.3 m NaBH₄ cleaved the carbohydrate-protein linkages (with destruction of serine and threonine) and released two types of carbohydrate chains: (a) small, reduced mannosyl-containing oligosaccharides and (b) high molecular weight, phosphated galactomannans. The latter had a molecular weight of about 60,000, their phosphate groups were diesterified and a portion of the galactose was in the furanose form. Mild acid hydrolysis (0.1 N HCl at 100°C for 15 min) cleaved the phosphate ester linkages to yield three low molecular weight fractions. One of these was phosphate free, having terminal reducing galactose residues, and an average composition of Man₃Gal₉. The other major fraction contained monoesterified phosphate groups and its molecular weight and analyses corresponded to a composition of Gal₃Man₅(PO₄)₂. A third, minor fraction had a similar carbohydrate composition but a much higher phosphate content. Three similar fractions could also be isolated after mild acid hydrolysis of the original peptido-phospho-galactomannan. A structure for the high molecular weight chain is proposed in which these fragments are linked together by phosphodiester bonds to form highly branched chains-[PO₄Gal₃Man₅(PO₄)₂Gal₉Man₅(PO₄)₂]ₙ-. The original complex these chains, as well as the more numerous mannosyl-containing oligosaccharides, would be linked to a peptide backbone through O-glycosidic linkages to serine and threonine.

Although it has been known for a number of years that the cell wall mannans of many fungi contain phosphate groups and covalently linked peptide or protein, the manner in which these three components are organized into a covalent complex is still poorly understood. Barker et al. (1967) showed that the peptide of a peptido-galactomannan from Trichophyton mentagrophytes contained high proportions of serine and threonine. Subsequently, Sentandreu and Northcote (1968, 1969) demonstrated that a peptido-mannan from that the peptide of a peptido-galactomannan from Saccharomyces cerevisiae is still poorly understood. Barker et al. (1967) showed that the peptide of a peptido-galactomannan from Trichophyton mentagrophytes contained high proportions of serine and threonine. Subsequently, Sentandreu and Northcote (1968, 1969) demonstrated that a peptido-mannan from Saccharomyces cerevisiae had mannosyl (and mannobiose and -triose) linked through O-glycosidic bonds to these amino acids. The main mannans, however, were found to be linked to the peptide through N-glycosidic bonds to asparagine. Following the demonstration (Jeanes and Watson, 1962; Slodki, 1962) that the phosphate in Hansenula mannan occurred in a diesterified form, the presence of such linkages was demonstrated in many other yeast mannans (Slodki, 1963; Stewart and Ballou, 1968) and in yeast cell walls (Mill, 1966). The structure of some of the extracellular phosphomannans of Hansenula sp. is known in some detail. Recently, the role of phosphodiester linkages in yeast cell walls was demonstrated during an investigation of a protoplast-producing enzyme (McLellan and Lampen, 1968).

Phosphodiester linkages appear to serve a variety of roles in the structures of different yeast mannans. In the Hansenula phosphomannans, they link short oligosaccharides together to form high molecular weight polymers (Jeanes et al., 1961). A galactomannan from the yeast form of Cladosporium werneckii, previously reported by this Laboratory (Lloyd, 1970a), was found to have much larger oligosaccharides joined by phosphodiester linkages (Lloyd, 1970b). More recently, Thieme and Ballou (1971) showed that the cell wall mannan from Kloeckera brevis has phosphate involved only in linking single-sugar side chains of mannose (Man) to the mannan main chain.

A more detailed analysis of the polymer from C. werneckii is now reported in which it is shown that phosphodiester linkages occur not only in the main chain but are also involved in linking side chains to the galactomannan main chain. The resulting structures are linked through alkali-labile bonds to a peptide backbone which also has numerous mannosyl-containing oligosaccharides linked to it by similar bonds. The complex is characterized by the presence of both acid- and alkali-labile bonds; this property was utilized in an analysis of its structure.

Methods

Analytical Methods. Procedures for the determination of carbohydrate, protein, nitrogen, and total phosphate have been published previously (Lloyd, 1970a, c). Individual sugars were determined by gas chromatography of their hexitol acetates (Sawardeker et al., 1965) on a neopentyl glycol succinate column at 200°C (Lloyd, 1970a). "Acid-labile" phosphate was estimated under the conditions described by Leloir and Cardini (1957), i.e., after hydrolysis in 1 N acid at 100°C for 7 min. Monoesterified phosphate was determined with Escherichia coli alkaline phosphatase (Sigma) at pH 8.0 in 0.2 M Tris-HCl buffer. Amino acids were identified and estimated on an amino acid analyzer after hydrolysis for 15 and 30 hr at 110°C in vacuo. Specific optical rotations were determined on a Perkin-Elmer 141 polarimeter.

Paper Electrophoresis. This procedure was carried out

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Abbreviations used are: Man, D-mannose; Gal, D-galactose.
on a Savant high-voltage apparatus in acetic acid–pyridine buffer (pH 5.3) at 1500 V for 1.5 hr using Whatman No. 3MM paper. For preparative isolation, the mixture was streaked across the paper. After electrophoresis, strips were cut from the edges and sprayed with alkaline silver nitrate reagent (Trevelyan et al., 1950) to detect the components. Prior spraying of the paper with periodic acid before treating with alkaline silver nitrate reagent was necessary to detect the high molecular weight oligosaccharides.

**Get Filtration.** Agarose gel filtration for the estimation of molecular weights was performed as follows (cf. Bathgate, 1970). A column of Sepharose 4B (Pharmacia Fine Chemicals) was prepared in 1 M NaCl. The sample (ca. 2 mg) was applied to the column together with either glucose (2 mg) or blue dextran (ca. 4, methyl α- and β-D-mannopyranoside tetraacetates; 3, mannitol hexaacetate; 4, methyl α- and β-D-galactoside tetraacetates; 5, galactitol hexaacetate.

**Identification and Estimation of Reducing End Groups in Oligosaccharides.** Samples (ca. 200 μg) were reduced with NaBH₄ (700 μg) in the presence of 30 μg of L-rhamnitol (internal standard) in a total volume of 0.4 ml. After 4–6 hr the solutions were neutralized with dilute acetic acid and Dowex 50 (H⁺) (ca. 0.1 ml) was added to the tubes. The supernatant was removed and the resin was washed with water. The combined solutions were evaporated to dryness and borate was removed by evaporation with methanol (four times). The samples were methanolized in 3% HCl in methanol (0.6 ml) in a sealed tube by heating for 18 hr at 100°. After neutralization with Ag₂CO₃, the samples were evaporated to dryness and completely acetylated with 250 μl of acetic anhydride–pyridine (1:1) for 3 hr at 100°. Water (ca. 2 ml) was added to the cooled tubes and after 6–24 hr, the mixture was extracted with an equal volume of chloroform. The CHCl₃ layer was evaporated to dryness and dissolved in 50 μl of solvent for gas chromatography. Separation of methyl O-acetylglycosides from the heptitol acetate (the original terminal reducing sugar of the oligosaccharide) was achieved on a neopentyl glycol succinate column at 200°. The elution pattern of the standard compounds is shown in Figure 1. Average chain lengths were determined by estimating the ratio of heptitol to methyl glycosides in the samples. Quantitative estimations were made using a Disc integrator after determining the responses given by standard compounds.

**TABLE 1: Effect of NaOH and NaBH₄ on the Amino Acid Composition of Peptido-galactomannan from C. werneckii.**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Original</th>
<th>After 0.3 M NaOH</th>
<th>After 0.3 M NaBH₄</th>
<th>After 0.1 M NaOH</th>
<th>After 0.1 M NaBH₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>16</td>
<td>21</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td>His</td>
<td>4</td>
<td>7</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td>Arg</td>
<td>7</td>
<td>12</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td>Asp</td>
<td>64</td>
<td>63</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Thr</td>
<td>248</td>
<td>48 (-81%)</td>
<td>88 (-65%)</td>
<td>88 (-65%)</td>
<td>88 (-65%)</td>
</tr>
<tr>
<td>Ser</td>
<td>153</td>
<td>66 (-57%)</td>
<td>60 (-61%)</td>
<td>60 (-61%)</td>
<td>60 (-61%)</td>
</tr>
<tr>
<td>Glu</td>
<td>81</td>
<td>81</td>
<td>87</td>
<td>87</td>
<td>87</td>
</tr>
<tr>
<td>Pro</td>
<td>51</td>
<td>43</td>
<td>48</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>Gly</td>
<td>65</td>
<td>93 (+43%)</td>
<td>66</td>
<td>66</td>
<td>66</td>
</tr>
<tr>
<td>Ala</td>
<td>120</td>
<td>127 (+6%)</td>
<td>173 (+47%)</td>
<td>173 (+47%)</td>
<td>173 (+47%)</td>
</tr>
<tr>
<td>Val</td>
<td>45</td>
<td>39</td>
<td>44</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>Ile</td>
<td>31</td>
<td>29</td>
<td>27</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>Leu</td>
<td>37</td>
<td>44</td>
<td>41</td>
<td>41</td>
<td>41</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tr</td>
<td>9</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Phe</td>
<td>20</td>
<td>24</td>
<td>28</td>
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<td>28</td>
</tr>
<tr>
<td>α-Amino-</td>
<td>0</td>
<td>0</td>
<td>23</td>
<td>23</td>
<td>23</td>
</tr>
</tbody>
</table>

*α*-Amino-butyric acid

Nmoles/mg. Average of 15- and 30-hr hydrolysis values.

Nd, not determined.

Values extrapolated to zero time.

The numbers in parentheses show the per cent change in amino acids after treatment with NaOH–NaBH₄.
had been destroyed with acetic acid, the products were fractionated on a Sephadex G-100 column (60 x 2.9 cm) by elution with water (Figure 2A). As preliminary experiments had shown that peak II was, in fact, a high molecular weight fraction, it was rechromatographed on the same column in 0.15 M NaCl (Figure 2B). In this solvent it separated to give a major peak (3a) in the excluded volume and a minor peak (3b) in the salt region. The protein component eluted at an intermediate position showing that it was no longer linked to the carbohydrate. Peak III was similarly rechromatographed to yield a minor peak of 3a and a major peak of 3b (not shown). Fractions were desalted on Bio-Gel P2 columns to yield 90 mg of 3a and 51 mg of 3b. A more accurate value (1.2:1) for the ratio of 3a:3b was obtained from the areas of the peaks in the elution diagrams (Figure 2A,B); this value also includes a portion of 3a contained in peak I. This peak also contains a small amount of peptide attached to galactomannan through alkali-stable linkages. The 3a fractions were combined and fractionated on a DEAE-Sephadex column by elution with a NaCl gradient at pH 7.2. The product (fraction 3A) was eluted with 0.3 M NaCl as a single peak completely separated from a minor protein component. It was desalted on Bio-Gel P2 column to yield 61 mg.

Properties of the High Molecular Weight Phosphogalactomannan Fraction (3A). Analyses (Table II) showed that the fraction contained galactose, mannose, and phosphate in the molar ratios of 1.0:3.7:0.55. Methylation analysis (Lloyd, 1970a, and unpublished data) showed that a portion (ca. 25%) of the galactose was present in the furanose form. Protein was found to be absent and only 0.1 N could be detected. The fraction chromatographed as a single symmetrical peak on a Sepharose 4B column at a position corresponding to a molecular weight of 50,000-60,000. The original peptido-galactomannan eluted mainly in the excluded volume but contained a minor component with a molecular weight of about 100,000.

DEAE-Sephadex chromatography had shown that the phosphate groups present were ionized; this was confirmed by paper electrophoresis which showed a single component migrating toward the anode at about half the rate of mannose 6-phosphate. Titration of a decationized sample showed only a single inflection point indicating that the phosphate was diesterified (Lloyd, 1970b; cf. Slodki, 1962). The phosphate could not be removed by alkaline phosphatase (Table III) but this does not necessarily show the absence of monoesterified phosphate since the enzyme does not always hydrolyze phosphate when it is attached to a high molecular weight polymer (McLellan and Lampen, 1968). As no inorganic phosphate was released under the conditions used by Leloir and Cardini (1957) to demonstrate acid-labile phosphate or by 0.1 N acid at 100°C for 15 min (Table III), it was concluded that pyrophosphate esters were absent from the polymer.
TABLE III: Release of Inorganic Phosphate by Acid Hydrolysis and by Alkaline Phosphatase.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Acid Hydrolysis</th>
<th>Acid Hydrolysis</th>
<th>Alkaline Phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original</td>
<td>Nd</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>3A</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>4a</td>
<td>Nd</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>4c</td>
<td>Nd</td>
<td>5</td>
<td>88</td>
</tr>
<tr>
<td>1b(a)</td>
<td>0</td>
<td>0</td>
<td>94</td>
</tr>
<tr>
<td>1b(c)</td>
<td>0</td>
<td>0</td>
<td>87</td>
</tr>
</tbody>
</table>

* 1 N HCl at 100° for 7 min. * 0.1 N HCl at 100° for 15 min.

These conditions did, however, lead to fragmentation of the polymer as shown by chromatography of the products (fraction 4) on a Sepharose 4B column when they appeared in the salt region.

Fractionation of Fragments Formed by Partial Acid Hydrolysis of High Molecular Weight Phospho-galactomannan. A sample of 3A (30 mg) was hydrolyzed in 0.1 N HCl (3 ml) for 15 min at 100°. After neutralization with NaOH, the products were chromatographed on a Sephadex G-100 column in 0.15 M NaCl (Figure 3A). The product (fraction 4) was eluted in the salt region and none of the original high molecular weight material remained. The product was desalted on a Bio-Gel P2 column and was eluted in two peaks (Figure 3B).

These fractions, however, showed that they both contained three components. The two samples were pooled and separated into three components by preparative paper electrophoresis. The fractions were desalted on Bio-Gel P2 column. The two acidic fractions (4a and 4c) were eluted mainly in the included volume of the column. The neutral component (4b) eluted at a V/V value of 1.3 indicating a molecular weight of about 1200. Yields of the three fractions (a, b, and c) were in the ratio of 16:8:1 but this is not necessarily a reflection of their ratio in the intact polymer because of differential losses during isolation.

Purification of Corresponding Products Obtained by Partial Acid Hydrolysis of the Original Peptido-galactomannan. A low molecular weight fraction (1b) obtained by the direct hydrolysis of the peptido-polysaccharide (Lloyd, 1970b) was similarly separated into three components (1b(a), 1b(b), and 1b(c)) by paper electrophoresis and subsequent Bio-Gel P2 chromatography. Yields were in the ratio of 14:8:1.

Properties of Low Molecular Weight Galactomannan Fragments. Main Acidic Fraction (4a). Analytical data showed a galactose:mannose:phosphate ratio of 1.0:6.6:0.70 (Table II). However since the molecular weight of the fraction was about 5000 as determined by chromatography on Sephadex G-25, the composition of the fragment was approximately Gal\(_n\)Man\(_m\)(PO\(_4\))\(_n\). It moved toward the anode on paper electrophoresis at about the same rate as the unhydrolyzed, high molecular weight phospho-galactomannan. The phosphate was nearly quantitatively removed by alkaline phosphatase (Table III) showing that it was now in the monomer form. The reducing sugar of this oligosaccharide fragment was shown to be mainly mannose (Figure 1B and Table IV); the average chain length was found to be 32 by this method, in good agreement with the proposed constitution. The corresponding fraction (1b(a)) isolated directly from the original peptido-galactomannan had very similar properties (Tables II and IV) to 4a.

Minor Acidic Fraction (4c). This fraction had a similar mannose to galactose ratio as the major fraction but a much higher phosphate content (Table II). This was reflected in its higher electrophoretic mobility. Fraction 4c moved at about twice the rate of fraction 4a and had the same mobility as the original peptido-galactomannan.

Fractionation of Fragments Formed by Partial Acid Hydrolysis of High Molecular Weight Phospho-galactomannan. (A) Chromatography of products on Sephadex G-100 column (60 \(\times\) 1.8 cm) in 0.15 M NaCl. The arrow indicates the position of elution of the polymer before treatment with acid. (B) Chromatography of products (fraction 4) on Bio-Gel P2 column (60 \(\times\) 1.8 cm) in water.

**TABLE IV:** Identification and Quantitation of Reducing End Groups in Fractions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Methyl Glycosides (Man + Gal) (%)</th>
<th>Mannitol (%)</th>
<th>Galactitol (%)</th>
<th>Ratio (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4b</td>
<td>91.2</td>
<td>0.86</td>
<td>8.65</td>
<td>9</td>
</tr>
<tr>
<td>1b(b)</td>
<td>89.3</td>
<td>1.05</td>
<td>9.85</td>
<td>8</td>
</tr>
<tr>
<td>4a</td>
<td>97.0</td>
<td>2.86</td>
<td>0.29</td>
<td>31</td>
</tr>
<tr>
<td>1b(a)</td>
<td>97.0</td>
<td>3.65</td>
<td>Trace</td>
<td>27</td>
</tr>
<tr>
<td>4c</td>
<td>93.5</td>
<td>5.75</td>
<td>1.05</td>
<td>14</td>
</tr>
<tr>
<td>1b(c)</td>
<td>94.7</td>
<td>2.86</td>
<td>2.34</td>
<td>18</td>
</tr>
</tbody>
</table>

*Ratio of total methyl glycosides/total sugar alcohols.  
Date of mixture of 1b(a), 1b(b), and 1b(c).
as mannose 6-phosphate. A similar fraction (1b(c)) was isolated from fraction 1b.

Neutral fraction (4b). This component had a galactose to mannose ratio of 1:7 and no phosphate (Table II). Its reducing end group was galactose (Figure 1D and Table IV) which was present in an amount corresponding to a composition of Gal,Man; for this fraction. As the fraction was shown to be heterogeneous by chromatography this is evidently only an average composition. The composition is consistent with the position of elution of the fraction from a Bio-Gel P2 column (Figure 3B). Again the corresponding fraction (1b(b)) isolated from the original had almost identical properties to the sample isolated from the high molecular weight phospho-galactomannan.

Properties of Fraction 3b. Analysis of the low molecular weight fraction formed by treatment of the peptide-galactomannan with NaOH—NaBH₄ showed that it contained only mannose and mannitol and some contaminating peptide (Table II). The mannose:mannitol ratio was 5:1 indicating that this fraction consisted of reduced oligosaccharides with an average composition Man₇mannitol. It contained only traces of PO₄⁻³ and was shown to be uncharged by paper electrophoresis. Paper chromatography showed the fraction to be heterogeneous and to consist mainly of reduced pentoses to decaoses; reduced di- and monosaccharides were not present.

Discussion

Previous studies on this peptido-phosphogalactomannan complex had shown that it consisted of phosphodiester-linked galactomannan units and more numerous mannose-containing oligosaccharides (Lloyd, 1970b) attached to a peptide backbone. This information was derived by analyzing the products obtained by treating the polymer successively by mild acid hydrolysis and then with alkali. Although it was suggested that the galactomannan units occur in long phosphodiester-linked chains, the evidence was also compatible with a number of other structures. Reversing the sequence of the degradative steps has now given definitive evidence for the structure proposed earlier (Lloyd, 1970b).

Treatment of the peptido-galactomannan with NaOH and NaBH₄ leads to the release of two types of carbohydrate chains by scission of the carbohydrate-protein linkages. Separation of the components was achieved by gel filtration on Sephadex G-100 first in water which separates the products from some residual alkali-stable material (peak I) and then in 0.1 M NaCl which separates the products into low and high molecular weight components (Figure 2). Subsequently, the high molecular weight fraction was purified on a DEAE-Sephadex column to give a peptide-free fraction (3A).

This fraction (3A) which represented about 45% by weight of the original complex, migrated as a single component on paper electrophoresis and contained almost all of the phosphate of the original complex. The phosphate is esterified such that it has only one titratable acid group and it is stable to alkali at room temperature. It is readily cleaved by mild acid hydrolysis with the appearance of monoesterified phosphate groups but no inorganic phosphate (Table III). These properties suggest the presence of diesterified phosphate in the polymer linking the C-1 of one sugar to the hydroxyl group of another sugar as in other fungal polysaccharides (Slodki, 1962; Thiem and Ballou, 1971). Its molecular weight was estimated to be in the 50,000–60,000 range.

The low molecular weight component (3b) released by treatment with NaOH—NaBH₄ and which represented about 40% of the complex, appears to be identical with the reduced mannose-containing fraction (2b) isolated previously (Lloyd, 1970b). It contains only mannose and mannitol in a 1:5 ratio and no galactose or phosphate. Paper chromatography showed the fraction to be heterogeneous and to consist of reduced oligosaccharides in the mannopentitol to decitol range. In the original polymer they would be linked to the peptide by alkali-labile bonds.

Treatment of the peptido-galactomannan by NaOH—NaBH₄ is accompanied by releases of most of the peptide moiety (Figure 2B) and a decrease in its serine and threonine content (Table I). These results suggest that the carbohydrate chains are linked to the peptide by O-glycosidic bonds to these amino acids. In the presence of BH₄⁻ a portion of the dehydroamino acids formed by alkali elimination are usually converted to alanine (from serine) and α-amino butyric acid (from threonine). Under the conditions (0.3 M NaOH and 0.3 M NaBH₄) used for the release of the chains from the peptide described above this conversion was not noticed; there was however a 43% rise in the glycine peak. A similar increase in glycine was observed by Adams (1965) when he treated a mucin from human breast carcinoma with alkali; he suggested that the glycine was formed by degradation of threonine residues. When the peptido-polysaccharide was treated with 0.1 M NaOH and 0.3 M NaBH₄, there was less destruction of serine and threonine but it was accompanied by the expected rise in alanine and the appearance of α-amino butyric acid. Carbohydrate-protein linkages involving O-glycosidic linkages to serine and threonine were first demonstrated in mammalian mucopolysaccharides (Anderson et al., 1963) but they have since been found to be widespread in nature (Spyro, 1970). Sentandreu and Northcote (1968) were the first to demonstrate their presence in fungal glycoproteins. They showed that a glycoprotein from Saccharomyces cerevisiae had numerous mannose (and some mannobiose and triose) residues linked to serine and threonine. The C. werneckii peptido-polysaccharide differs from this complex in that it has longer oligosaccharides (and no mannose or mannobiose) linked to the hydroxymyano acids. In addition to the mannose residues, S. cerevisiae has long mannan chains attached to the peptide (through alkali-stable bonds); in C. werneckii only about 10% by weight of the complex consists of chains of this type.

Mild acid hydrolysis of fraction 3A, with scission of phosphodiester bonds, results in complete fragmentation of the chain into low molecular weight products (Figure 3A). The fraction was separated into two major and one minor components by preparative paper electrophoresis. One major fraction (4b) was found to be neutral and to have an average composition of Man₇Gal, (Table V). The other major fraction (4a) was much larger and contained monoesterified phosphate groups. Its analyses and molecular size corresponded to a composition of Gal,Man,PO₄²⁻, (Table V). The minor fraction (4c) had a similar sugar composition but a much higher phosphate content.

The fraction (1b) previously isolated after mild hydrolysis of the original complex (Lloyd, 1970b) could also be separated into three components by paper electrophoresis. These three fractions (1b(a), (b), and (c)) were almost identical in composition with the three derived from the isolated high molecular weight phospho-galactomannan chain (Table II).

These experiments confirm the overall structure already proposed for the peptido-phosphogalactomannan complex (Lloyd, 1970b) but also demonstrate that the phosphate-
linked chains are more complex than was originally thought.
The main chain consists of the larger galactomannan units
linked together by phosphodiester bonds; to this chain the
smaller galactomannan units are linked also by phospho-
diester bonds as side chains (Table V). Each of the larger
units has two PO$_4^{2-}$ groups available for substitution by side
chains; this would lead to a value of about 3.5% for the PO$_4^{2-}$
content of the intact chain. The observed value was about
5.8% PO$_4^{2-}$. This difference can be accounted for by the fact
that some of the PO$_4^{2-}$ groups may be unsubstituted and by
the presence in the chain of the minor but highly phosphated
unit (4c). The role of this fraction in the structure is uncer-
tain but it clearly occurs as a part of the main chain. The high
molecular weight phosphated chain has a higher Gal :Man
ratio than the isolated fragments (Table V); this is due to
the presence of galactofuranosyl groups in the intact structure
which are removed, concomitantly with phosphodiester
bond scission, during mild acid hydrolysis.

Figure 4 shows the structure proposed for the peptido-
galactomannan which best explains the fragmentation pattern
obtained by sequential treatment with acid and alkali and vice versa.
Treatment of the complex with NaOH–NaBH$_4$
leads to elimination of the long phosphated galactomannan
chains (3A) and the small mannose-containing oligosaccha-
rides (3b) from the peptide component. The former is frag-
mented into two major (4a and 4b) and one minor (4c) low
molecular weight components by mild acid hydrolysis. Simi-
lar fragments are formed by direct hydrolysis of the peptido-
galactomannan together with a high molecular weight prod-
uct (1a) representing the mannose-containing units still at-
ached to the peptide (Lloyd, 1970b). Since the mannose-
containing oligosaccharides represent about half the weight of the
complex (Lloyd, 1970b) and since the molecular weight of the phosphated chains is about 60,000, the ratio of small
to long chains must be about 50:1. This ratio would give a
value of 10% for the galactose content and about 3% for the
PO$_4^{2-}$ content of the original. These values are in close agree-
ment with the observed values of 13 and 3.2%, respectively.
The peptide also has a small amount of a galactomannan
chain linked to it by alkali-stable bonds but this component
does not represent more than 10% of the weight of the com-
plex.

In conclusion, it should be noted that the peptido-galacto-
mannan sample on which these experiments were performed
is heterogeneous on DEAE-Sephadex chromatography and
yields a series of fractions varying in their PO$_4^{2-}$ and galactose
contents (Lloyd, 1970a). It has been shown, however, that
each of the fractions contains all the structural features
of the original (Lloyd, 1970a).

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A New Phosphagen, 

$N'$-Phosphorylguanidinoethylphospho-O-(\(\alpha-N,N\)-dimethyl)serine (Phosphothalassemine)†

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**ABSTRACT:** A new guanidine compound, L-thalassemine, the corresponding phosphagen, phospho-L-thalassemine, and the corresponding phosphagen kinase, ATP: L-thalassemine phosphotransferase, have been found in the body wall muscle of an echiuroid worm, Thalassema neptuni. L-Thalassemine has been isolated from Thalassema muscle as the free-base monohydrate: \(\text{C}_8\text{H}_{15}\text{N}_4\text{O}_7\text{P}\cdot\text{H}_2\text{O}\), mp 184°, [\(\alpha\)]D = 11.2°. Its structure was elucidated as the guanidinoethylphospho-O-(\(\alpha-N,N\)-dimethyl)serine or \(\alpha-N,N\)-dimethylombricine and confirmed by synthesis from L-lombricine. Lombricine (guanidinoethylphospho-O-serine), which is likely to be the precursor of thalassemine, was isolated from Thalassema viscera and found to be of the same L series as thalassemine from Thalassema muscle and as lombricine isolated from another echiuroid, Urechis caupo, in contrast with lombricine present in various oligochetous and polychetous annelids, which was shown to be the D isomer. The biological origin of the methyl groups of thalassemine from L-[methyl-\(^{14}C\)]methionine in T. neptuni was established from in vivo experiments. Phosphothalassemine was identified in Thalassema muscle. The phosphagen kinase responsible for the phosphorylation of thalassemine was characterized in the same tissue and some properties of the enzyme were investigated.

The mono- and disubstituted guanidines which can be phosphorylated into muscular phosphagens are amino acid (arginine) or amino acid degradation products, guanidinoacetic acid and creatine originating from glycine, and amidino-hypotaurine (hypotaurocyamine) and amidinotaurine (taurocyamine) from cysteine. Another compound issued from serine, guanidinoethanol, is phosphorylated into guanidinoethyl phosphate which is either esterified with serine giving rise to guanidinoethylphospho-O-serine (lombricine) (I) or methylated by methionine into guanidinoethyl methyl phosphate (opheline) (II) (see review by Thoai and Robin, 1969).

Actually opheline is found only in a marine polychete, Ophelia neglecta (Thoai et al., 1963), while lombricine, isolated for the first time from Lumbricus terrestris (Thoai and Robin, 1954), is widely distributed in all oligochetes (Robin and Roche, 1965), in polychetes (Thoai et al., 1963), and in two echiuroids, Urechis caupo (Robin, 1964) and Bonellia viridis (Thoai et al., 1967).

In the muscle of the last worm, lombricine is present only in traces. The main muscular product is a \(\beta\)-aspartyllombricine, called bonellidine (III) (Thoai et al., 1967). Even though the \(N'$-phosphorylated derivative has not been found in Bonellia, it may be presumed that bonellidine, the only guanidine com-