Characterization of Large Oligosaccharide-Lipids Synthesized in *Vitro* by Microsomes from *Saccharomyces cerevisiae*  

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Conditions are described for optimizing the synthesis of large oligosaccharide-lipids in microsomal preparations from *Saccharomyces cerevisiae*. On incubating microsomes with GDP-[3H]Man, the major product obtained was Man9GlcNAc2-P-P-dolichol, but when both GDP-[3H]Man and UDP-[3H]Glc were present in the incubation mixture about half of the Man9GlcNAc2 was elongated to Glc3Man9GlcNAc2-P-P-dolichol. Unlike particulate fractions from mammalian systems, little glucosylation of the yeast microsomal oligosaccharide-lipid was obtained when the concentration of UDP-Glc was less than 10 μM, but the synthesis of this product could be maximized by raising the concentration of UDP-Glc to 50 μM. Analysis of the yeast Man9GlcNAc2 species confirmed that 8 of the 9 mannose residues could be released with α-mannosidase, while the remaining mannose residue was in the core triasaccharide, Man81 → GlcNAc81 → GlcNAc. Treatment of Glc3Man9GlcNAc2 with α-mannosidase released 5 of 9 mannose residues and yielded Glc3Man9GlcNAc2. This product appeared to be identical with that obtained in parallel experiments with double labeled oligosaccharide-lipid synthesized in oviduct microsomes.

*Streptomyces plicatus* endo-β-N-acetylglucosaminidase H (Endo-H) treatment of yeast microsomal glycoproteins that were labeled with sugar nucleotides established that 15% of the label was associated with N-linked oligosaccharides. The remaining labeled sugars were released with alkali, indicating that they were linked to serine or threonine. Based on the size and distribution of [3H]glucose and [14C]mannose in the Endo-H-released oligosaccharides, it was concluded that Glc3Man9GlcNAc2 was the primary species transferred to proteins in the yeast system.

Investigations from several laboratories have established that the initial step in the formation of N-glycosidic linkages in animal systems is the *en bloc* transfer of a large oligosaccharide from an oligosaccharide pyrophosphoryl dolichol (OS-P-P-Dol)1 to an asparagine residue in the acceptor protein (1). The oligosaccharide portion of this lipid intermediate in animal systems is now known to be a heterosaccharide containing the common core sequence Manβ1 → 4GlcNAcβ1 → 4GlcNAc (ManβGlcNAc2) extended with 8 mannose and as many as 3 peripheral glucose residues (2–4). There is ample evidence implicating lipid-linked intermediates in yeast glycoprotein biosynthesis as well (5–8). Thus, in the presence of GDP-Man and UDP-GlcNAc, yeast microsomes synthesize Man-P-Dol (6, 9, 10), GlcNAc-P-P-Dol and GlcNAc-P-Dol (7, 8, 11), and the lipid-trisaccharide, ManGlcNAc2-P-P-Dol (7). The sugar moieties of these molecules are, to some extent, transferred *in vitro* to endogenous protein acceptors.

When GDP-[14C]Man is employed as the substrate for *in vitro* synthesis, more than 80% of the label incorporated into yeast microsomal proteins can be released with 0.1 N NaOH indicating that it is associated with O-glycosylated serine/threonine residues (6). Man-P-Dol has been identified as the glycolipid donor for the attachment of the initial mannose residue in these linkages, whereas chain extension is promoted by GDP-Man (6). In addition to the formation of O-mannosylglycoproteins, yeast also synthesize glycoproteins containing asparagine-oligosaccharides of the “high mannose” type. These glycoproteins contain the common core triasaccharide, ManβGlcNAc2, which is elongated by α-linked mannose residues ranging in number from about 12, in the case of carboxypeptidase Y (12, 13), to over 50, in the case of invertase (14, 15).

By analogy with the mammalian systems described recently (2–4), the synthesis of N-glycosidic oligosaccharides in yeast would be expected to proceed via large oligosaccharide-lipid intermediates. Small amounts of glycolipid exhibiting characteristics of OS-P-P-Dol from higher cells have been isolated from yeast microsomal preparations (16–19), and in one case this material served as an oligosaccharide donor for the *in vitro* glycosylation of endogenous microsomal proteins (18). It has been shown recently that log phase yeast incorporate a small amount of labeled glucose into acid-soluble oligosaccharides, which by paper chromatography appear similar to those obtained from the OS-P-P-Dol of mammalian cells (20). The largest of these species, tentatively identified as Glc3Man9GlcNAc2, appears to be associated with yeast glycoproteins after a brief pulse with labeled glucose (20).

Despite these advances, a thorough characterization of the oligosaccharide moiety of yeast OS-P-P-Dol, and of the N-glycosylation reaction itself, have been hampered by the extremely low levels of OS-P-P-Dol obtained from yeast. In this communication we describe conditions which yield sufficient quantities of yeast OS-P-P-Dol from *in vitro* microsomal...
incubations to facilitate the characterization of the attendant oligosaccharide chains. Unlike the previously described animal systems (2-4), very high levels of UDP-Glc are required in vitro to obtain a significant proportion of the yeast OS-P-P-Dol in the glucosylated state. Therefore, as in the case of the animal systems, those sugars glucosylated species are transferred preferentially to endogenous proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**

UDP-N-acetylglucosamine (2-14C) (20 Ci/mole), UDP-Glc (1.3 Ci/mole), and medium-bovine serum albumin (BSA) were purchased from Amersham, and [*d*]-[4-14C]mannose (1 Ci/mole) from New England Nuclear. Radioactive labels were calibrated for the determination of radioactivity. Both osmolites were characterized for use in analytical depletion. Bovine serum albumin (BSA) and lipids were purchased as follows: BSA from Calbiochem (542864), and lipids (245.7% C) from Sigma (L-7878).

**Methods**

**Size of the Oligosaccharides Present in the OS-P-P-Dol Fraction**

To establish the size of the oligosaccharides in yeast OS-P-P-Dol synthesized in vitro, 2,500 counts/min of each of the four samples in Table I were subjected to mild acid hydrolysis, which was performed as described previously. The water-soluble products were chromatographed on a Bio-Gel P-4 column which
had been calibrated with heterosaccharides of known composition (Fig. 1, inset). As indicated in Fig. 1, the radioactivity liberated from yeast OS-P-P-Dol chromatographed as a mixture of oligosaccharides, the relative proportions of which were a function of the growth phase of the yeast and the presence of UDP-Glc. Some species were as large as Glc₂Man₆GlcNAc₂ obtained from oviduct OS-P-P-Dol, while others were as small as Man₃GlcNAc isolated from ovalbumin. In the absence of UDP-Glc, the OS-P-P-Dol obtained from both microsomal preparations contained a major oligosaccharide which eluted with a size consistent with Man₃GlcNAc₂ (Fig. 1A and B; tube 55). The mid-log phase OS-P-P-Dol always contained more of the smaller species migrating in the Man₂-GlcNAc₂ region of the profile and, even in the absence of UDP-Glc, often revealed shoulders of radioactivity in tubes 50 and 53 where Glc₂Man₆GlcNAc₂ and Glc₁Man₆GlcNAc₂ eluted, respectively. Preincubation of the microsomes at 27°C for 15 min prior to addition of the nucleotide sugars substantially reduced the species larger than Man₃GlcNAc₂ without otherwise altering the elution profile (data not shown).

On addition of 100 μM UDP-Glc to the yeast particulate system, marked changes in the Bio-Gel P-4 oligosaccharide elution profiles were noted (Fig. 1). Thus, with mid-log microsomes (Fig. 1A), nearly 45% of the oligosaccharides chromatographed the same as oviduct Glc₃Man₆GlcNAc₂. In contrast, when UDP-Glc was incubated with late-log preparations, less than 20% of the oligosaccharides migrated with the fully glucosylated oviduct marker (Fig. 1B), and in some cases, high levels of UDP-Glc failed to stimulate any synthesis of Glc₃Man₆GlcNAc₂-P-P-Dol (data not shown).

Sugar Composition of the OS-P-P-Dol Fraction—The size of the oligosaccharide chains released from yeast OS-P-P-Dol (Fig. 1) suggests that GDP-Man promotes the synthesis of Man₆GlcNAc₂-P-P-Dol primarily, and that GDP-Man plus UDP-Glc yield oligosaccharide chains with as many as 3 glucose residues. In order to ascertain the actual sugar composition of the yeast oligosaccharides eluting with sizes of Glc₃Man₆GlcNAc₂ and Man₆GlcNAc₂, OS-P-P-Dol was synthesized by incubating mid-log microsomes for 5 min at 27°C in a scaled up reaction containing 4 μM GDP-[¹⁴C]Man and 25 μM UDP-[³H]Glc. A portion of the isolated OS-P-P-Dol (80,000 ³H and 70,000 ¹⁴C cpm) was subjected to mild acid hydrolysis, and the water-soluble oligosaccharides were applied to the Bio-Gel P-4 column. As Fig. 2A depicts, nearly 80% of the ³H migrated in Peak I (centered at tube 50), the same region where the oviduct Glc₃Man₆GlcNAc₂ marker eluted. The remaining 20% of the ³H was in a peak at tube 53 consistent with a Glc₃Man₆GlcNAc₂ species. Nearly 50% of the ¹⁴C was in Peak I (centered at tube 55) where Man₆GlcNAc₂ would be expected to migrate. It should be noted that with 25 μM UDP-Glc (Fig. 2A), only 25% of the ¹⁴C eluted with Glc₃Man₆GlcNAc₂, which was about half that obtained in the previous experiment with 100 μM UDP-Glc (Fig. 1A). The relationship between UDP-Glc level and the proportion of the oligosaccharide chains glucosylated was examined more thoroughly in a subsequent section.

Fractions 49 to 51 (I) and 54 to 56 (II) in Fig. 2A were pooled separately and rechromatographed on Bio-Gel P-4 to assess the homogeneity of each. Pool I yielded a single symmetrical peak with a constant ¹⁴C/³H ratio throughout the profile (Fig. 2B). Hydrolysis of a portion of this material (Fig. 2B) to its component sugars with acid, followed by paper chromatography in Solvent D, verified that the ¹⁴C and ³H were in mannose and glucose, respectively. Correcting for the counting efficiency and specific activity of the labels present in this oligosaccharide provided a molar Man/Glc ratio of 2.93. From the estimated size of this species, Hexose₇.8GlcNAc₂ (Fig. 1), and the molar ratio of labeled sugars present, its composition was calculated to be Glc₃Glc₃Man₆GlcNAc₂.

Rechromatography of Pool II provided the profile shown in Fig. 2C. A small amount of ³H migrated in the front of the otherwise symmetrical peak, the center of which eluted as anticipated for Hexose₇GlcNAc₂. Fractions 55 to 67 were pooled and a portion of the total was subjected to acid hydrolysis followed by paper chromatography in Solvent D. All of the ¹⁴C migrated with mannose indicating that the composition of this species was Man₆GlcNAc₂.

To characterize the oligosaccharides obtained from the double labeled OS-P-P-Dol fraction further, rechromatographed Pools I and II (Fig. 2, B and C, respectively) were digested with a-mannosidase, and the products were resolved on the Bio-Gel P-4 column. The elution pattern of the Glc₃Man₆GlcNAc₂ species shown in Fig. 2D now revealed two peaks: one (tubes 95 to 97) with 57% of the ¹⁴C which migrated with mannose on paper chromatography (Solvent D) and the other (tubes 55 to 58) with a size equivalent to Hexose₇GlcNAc₂. The latter species contained 43% of the ¹⁴C and all of the ³H, and, although its profile was somewhat
broader than observed for the other oligosaccharides chromatographed on this column, the $^{14}$C/$^3$H ratio was constant in all fractions. From the corrected counting efficiencies of the radioactivity determinations, a molar Man/Glc ratio of 1.37 was obtained for the pooled oligosaccharide. Utilizing Hexose$^3$H/glcNAc$^2$ as the size of this species, its composition based on the molar ratio of sugars present was Glc2Man3GlcNAc2. Thus α-mannosidase appeared to remove 5 mannose residues from the large glucosylated heterosaccharide (Peak I).

Control experiments with uniformly labeled $[^3]$H]Glc$[^4]$C-Man$[^3]$H/GlcNAc$^2$ from oviduct OS-P-P-Dol provided results identical with those obtained with the glucosylated yeast oligosaccharide in that α-mannosidase removed 56% of the Hexose$^7$GlcNAc2 as the size of this species, its composition based on the molar ratio of sugars present was Glc2Man3GlcNAc2. Thus α-mannosidase appeared to remove 5 mannose residues from the large glucosylated heterosaccharide (Peak I).

Chromatography of the α-mannosidase-digested Man$_9$GlcNAc$_2$ species also provided two peaks (Fig. 2E). In this case 91% of the $^3$H eluted in the monosaccharide region (tubes 95 to 98), and, on paper chromatography in Solvent D, this was identified as mannose. The other peak (tubes 73 to 75) contained 9% of the $^3$H and eluted in the position observed for the core trisaccharide, Man$_9$GlcNAc$_2$. The pooled material (tubes 73 to 75), when reduced with sodium borotritide (27), co-migrated with authentic Man$_9$ → 4GlcNAC$_2$ → 4GlcNAC$_2[^3]$H-ol on paper chromatography in Solvents C and D. Digestion of the reduced trisaccharide with β-mannosidase yielded a single trititated product, identified chromatographically (Solvent D) as GlcNAC$_2$ → 4GlcNAC$_2[^3]$H-ol, which on complete acid hydrolysis provided only glucosamin$[^3]$H]-itol. Thus, the Man$_9$GlcNAc$_2$ oligosaccharide isolated from the yeast glycolipid fraction contains the expected common core trisaccharide structure (25).

Effect of UDP-Glc Concentration on the Distribution of Oligosaccharide Species Present in Yeast OS-P-P-Dol—Microsomal preparations from animal cells incorporated glucose into OS-P-P-Dol when UDP-Glc was as low as 0.2 μM (41). Since the yeast system did not incorporate appreciable glucose into this glycolipid unless UDP-Glc was above 10 μM, it was of interest to determine the concentration at which UDP-Glc provided an optimal level of glucosylation. Preincubated mid-log and late-log microsomes were supplemented with 4 μM GDP-Man and varying amounts of UDP-Glc up to 100 μM. After 15 min the OS-P-P-Dol was isolated, and the oligosaccharides released by mild acid hydrolysis were applied to the Bio-Gel P-4 column. For each UDP-Glc concentration tested, the relative amount of each oligosaccharide species present in the elution profile was estimated from the amount of radioactivity in the respective peaks and shoulders. The apparent number of mannose residues in each oligosaccharide was used to normalize the various fractions. The results, shown in Fig. 3, provide the percentage of each oligosaccharide species present in the OS-P-P-Dol formed at each UDP-Glc level, and reveal that both mid-log and late-log microsomes required at least 50 μM UDP-Glc to yield the maximal amount of Glc$_3$Man$_9$GlcNAc$_2$-P-P-Dol. As indicated, there was a direct relationship between the UDP-Glc level and the maximal amount of glucosylated oligosaccharide in both systems. Thus, as the glucosylated species increased, the Man$_9$GlcNAc$_2$ species decreased indicative of a precursor-product relationship. With the exception of a decline in the putative monoglucosylated glycolipid in mid-log microsomes, the proportion of all other species was relatively unaffected by increasing the UDP-Glc concentration (Fig. 3).
Site of oligosaccharides newly transferred in endogenous microsomal systems—Since a purified microsome fraction was the invariable portion of the yeast particulate system, the preparation of newly labeled oligosaccharides from this fraction was compared with those synthesized by endogenous yeast systems and the reactions in each system were carried out at the same concentration under identical conditions. The oligosaccharides released from the yeast particulate system were compared to those released from endogenous yeast systems in which Triton X-100, a detergent, was added to the assay or not. So, in the absence of Triton X-100 (Fig. 2A), those released from microsome isolated with Triton X-100 were essentially free of the oligosaccharides found in Triton X-100-pretreated yeast microsomes and therefore presumably in the endogenous system as well. The oligosaccharides newly synthesized on the microsome fraction that were released from microsome isolated with Triton X-100 were essentially similar to those synthesized by the endogenous yeast system. The two predominant oligosaccharide-lipids synthesized by yeast microsomes from UDP-[3H]Glc and GDP-[14C]Man (Fig. 2E), the two predominant oligosaccharide-lipids synthesized by yeast microsomes from UDP-[3H]Glc and GDP-[14C]Man were shown by analysis to be Glc3ManGlcNAc2 and Man5GlcNAc2 (Fig. 2). Lesser amounts of oligosaccharides with sizes consistent with GlcManGlcNAc2, Man5GlcNAc2, and GlcManGlcNAc2 were also found in the OS-P-P-Dol fraction (Figs. 1 and 2).

The yeast microsomal system appeared to form OS-P-P-Dol in which all of the mannose residues were labeled uniformly since α-mannosidase released 91% of the label from [14C]GlcManGlcNAc2 (Fig. 2E), compared with a theoretical value of 98% expected for 8 of 9 residues. The remaining 9% of the [14C]mannose was present in a trisaccharide shown to be the common core sequence, ManβGlcNAc2, which also has been identified on the reducing end of lipid-linked oligosaccharides from oviduct (43), CHO cells (3), and NIH 51 fibroblasts (4). Treatment of the yeast Glc3ManGlcNAc2 double labeled oligosaccharide with α-mannosidase released 57% of the [14C]mannose compared with a value of 55.6% expected for 5 of 9 residues. Removing 5 mannose residues from the parent oligosaccharide should produce a HexoseGlcNAc2 species with a composition of GlcManGlcNAc2. Clearly, the [14C]-Glc/[14C]Man ratio present in the oligosaccharides before (Fig. 2B) and after (Fig. 2D) α-mannosidase digestion, coupled with the percentage of mannose removed, confirms this assumption.

An apparent discrepancy involves the elution properties of GlcManGlcNAc2 from yeast and oviduct, both of which migrated on Bio-Gel P-4 as if they were in actuality mono- or di-substituted mannose branch points (3, 4, 4, 21, 26, 44), the extended HexoseGlcNAc2 may have a greater hydrodynamic volume, and hence elute earlier, than a more compact branched oligosaccharide of the same composition. Verification of this concept should occur when the structure of the GlcManGlcNAc2 species is confirmed.

The size, Glc/Man molar ratio, and α-mannosidase sensitivity of the largest oligosaccharide in yeast OS-P-P-Dol suggests that its structure is similar to, if not identical with, that of the comparable species from mammalian and avian cells. It is not

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**FIG. 3.** Chromatography on Bio-Gel P-4 of the oligosaccharides released by α-mannosidase from newly labeled (A) and microsomal (C) systems. The yeast microsome fractions were released from the reaction mixture to prepare the double-labeled oligosaccharide-lipid described in Fig. 2.

**DISCUSSION**

Incorporation of radioactive sugars from GDP-Man and/or UDP-Glc into large oligosaccharide-lipids by yeast microsomes has been shown to rarely exceed 1% of the sugar nucleotides added (17–19). A major objective of the current work, therefore, was to improve the extent of transfer from these nucleotides to the glycolipid fraction so that the latter's attendant oligosaccharides could be more thoroughly characterized. By modifying the system of Lehle and Tanner (18) to include diithiothreitol and UDP-GlcNAc, a 5-fold increase in the yield of OS-P-P-Dol was obtained. Eliminating MnCl₂ from the reactions and optimizing the concentration of each assay component resulted in a further 3- to 4-fold increase.

Thus, the present study represents about a 20-fold improvement in the in vitro synthesis of yeast lipid-linked oligosaccharides. Relevant to previous studies (7, 17–19, 39), the following characteristics of the yeast particulate system were observed: (a) freshly isolated microsomes, or those frozen and stored in liquid N₂, were required for maximal OS-P-P-Dol synthesis; (b) microsomes stored at 0°C or −20°C lost their synthetic capacity with a half-life of 24 h and neither sucrose nor glycerol were effective in stabilizing this activity; (c) Triton X-100, even at levels below 0.1%, diminished OS-P-P-Dol synthesis by over 5-fold and doubled the Man-P-Dol levels relative to the values reported in Table 1; (d) exogenously added dolichol phosphate was without effect on the system unless added in combination with Triton X-100, in which case very large amounts of Man-P-Dol were synthesized with little appearance of OS-P-P-Dol. A major finding in these studies was that the size and sugar composition of the oligosaccharides synthesized by yeast microsomes was not only dependent upon the growth state of the yeast but also on the level of UDP-Glc added to the in vitro assays (Figs. 1 and 3). The two predominant oligosaccharide-lipids synthesized by yeast microsomes from UDP-[3H]Glc and GDP-[14C]Man were shown by analysis to be Glc3ManGlcNAc2 and Man5GlcNAc2 (Fig. 2). Lesser amounts of oligosaccharides with sizes consistent with Glc2ManGlcNAc2, Glc2ManGlcNAc2, and GlcManGlcNAc2 were also found in the OS-P-P-Dol fraction (Figs. 1 and 2).

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clear why maximal synthesis of the heterosaccharide in yeast microsomes requires a level of UDP-Glc which is 1 to 2 orders of magnitude above that needed in particulate fractions from higher cells (4, 41, 45-47). Although glycogen and glucan synthetases are very active in yeast microsomes, depletion of UDP-Glc cannot be the answer since more than half of that added remains at the end of the incubations. It is equally unclear at present why the late-log microsomes incorporate glucose poorly into OS-P-P-Dol, while synthesizing high levels of Man\textsubscript{3}GlcNAc\textsubscript{2}-P-P-Dol (Fig. 1). One explanation is that since late-log phase cells, which have a doubling time about 3 times that of mid-log phase cells, require less Glc\textsubscript{3}Man\textsubscript{3}GlcNAc\textsubscript{2}-P-P-Dol for protein glycosylation, their levels of UDP-Glc:OS-P-P-Dol glucosyltransferase enzyme(s) are correspondingly lower. Potentially related to these studies in yeast, Schmitt and Elbein (48) recently reported that by blocking protein synthesis in canine kidney cells with puromycin, a rapid inhibition of OS-P-P-Dol synthesis occurred. In this case, the decreased availability of protein acceptor sites appeared, by a feedback mechanism, to prevent accumulation of OS-P-P-Dol. Determination of the levels of yeast glycosylating enzymes at different growth phases should help clarify the case of impaired glycosylation in the late-log phase microsomes.

Endo-H treatment of the microsomal proteins after a 5-min incubation with UDP-[\textsuperscript{3}H]Glc and GDP-[\textsuperscript{14}C]Man released a single major oligosaccharide (Fig. 5), which had the same \textsuperscript{3}H/Glc/\textsuperscript{14}C/Man ratio as the largest oligosaccharide in the OS-P-P-Dol from that reaction (Fig. 2B). The presence of the smaller oligosaccharides in the OS-P-P-Dol fraction at both 5 and 20 min (Figs. 1 and 2), but not on the proteins until 20 min, suggests that the glucosylated oligosaccharide is transferred preferentially. The absence of Glc\textsubscript{3}Man\textsubscript{3}GlcNAc\textsubscript{2} oligosaccharide in the glycolipids after a 20-min incubation without UDP-Glc (Fig. 1) and its apparent release by Endo-H from the microsomal proteins (Fig. 4) is not necessarily contradictory. In the presence of GDP-Man alone, log-phase microsomes always synthesized some Glc\textsubscript{3}Man\textsubscript{3}GlcNAc\textsubscript{2}-P-P-Dol suggesting the presence of an endogenous pool of UDP-Glc. Support for this belief was obtained on preincubating the microsomes in the absence of UDP-Glc, which resulted in a greatly reduced synthesis of Glc\textsubscript{3}Man\textsubscript{3}GlcNAc\textsubscript{2}-P-P-Dol. Consistent with this explanation was the finding that the addition of UDP-Glc promoted a substantial increase in the largest oligosaccharide in both the glycolipid (Fig. 1) and protein (Fig. 4) fractions.

A recent study by Parodi (20), performed with yeast in vivo, led to the same conclusions as those reported here with the in vitro microsomal system. Acid extraction of yeast yielded oligosaccharides which co-migrated with those isolated from the oligosaccharide-lipid synthesized by mammalian microsomes in vitro. The largest of these, most probably Glc\textsubscript{3}Man\textsubscript{3}GlcNAc\textsubscript{2}, was also found in yeast proteins after a brief pulse with \textsuperscript{14}C]glucose. On chasing with cold glucose, the labeled oligosaccharides first became shorter as a result of glucose removal and then larger due to mannose addition. Yeast OS-P-P-Dol in whole cells (20) and in the particulate fraction (Figs. 1 and 2) provide strikingly similar oligosaccharide profiles in that Glc\textsubscript{3}Man\textsubscript{3}GlcNAc\textsubscript{2} and Man\textsubscript{3}GlcNAc\textsubscript{2} are the predominant species. This pattern is somewhat different than that in higher eukaryotic cells examined to date, where the glucosylated species is present almost exclusively (49-54). Although small amounts of Man\textsubscript{3}GlcNAc\textsubscript{2} and Man\textsubscript{3}GlcNAc\textsubscript{2} have been identified as intermediates in the biosynthesis of the large glucosylated OS-P-P-Dol in CHO cells (55) and chick fibroblasts (54), respectively, Man\textsubscript{3}GlcNAc\textsubscript{2} appears to be the major precursor in yeast (Fig. 3). While smaller oligosaccharides are also found in the yeast OS-P-P-Dol fractions (Figs. 1, 2, and 3), their relationship to the glycoprotein biosynthetic pathway remains to be elucidated.

The requirement for glucose in the initial glycosylation of proteins (41, 42) and its subsequent processing as well as that of mannose from the newly transferred oligosaccharides in animal, viral, and avian systems is now well documented (49, 51, 56-62). Since yeast do not produce the “complex” oligosaccharides found in animal cells there is no apparent reason for yeast to trim Glc\textsubscript{3}Man\textsubscript{3}GlcNAc\textsubscript{2} to Man\textsubscript{3}GlcNAc\textsubscript{2} as occurs during complex carbohydrate synthesis in higher cells (49, 51, 58, 62). Nevertheless, after a 20-min incubation in vitro, yeast microsomal proteins contained a species consistent with Man\textsubscript{3}GlcNAc which may have arisen by the processing of a larger oligosaccharide (Fig. 4). It is not known whether the yeast oligosaccharide chains are reduced in size to species smaller than Man\textsubscript{3}GlcNAc\textsubscript{2} after glucose removal (20), nor is it known how much of the original species transferred to protein is conserved in the “inner core” structure (63) of yeast mannoproteins, but processing reactions may prepare the oligosaccharide for its subsequent elongation with 50 or more mannose residues (15).

Studies currently in progress should identify the intermediates formed during OS-P-P-Dol biosynthesis as well as the processing reactions that may be required for the completion of mature yeast glycoproteins. In recent experiments (64), Glc\textsubscript{3}Man\textsubscript{3}GlcNAc\textsubscript{2}-P-P-Dol has been found to be a 20-fold more active substrate than Man\textsubscript{3}GlcNAc\textsubscript{2}-P-P-Dol for oligosaccharide transfer to endogenous protein acceptors in a solubilized yeast microsomal system. Our results, and also those of Parodi (20), thus support the thesis that the glucosylated lipid-linked oligosaccharide is the natural donor for the initial step in protein N-glycosylation in yeast, as it is in animal and avian systems (41, 42, 47, 51, 54, 58, 60).

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REFERENCES
1. Waechter, C. J., and Lennarz, W. J. (1976) Annu. Rev. Biochem. 45, 95-112
2. Spiro, R. G., Spiro, M. J., and Bhoyroo, V. D. (1976) J. Biol. Chem. 251, 6409-6419
3. Li, E., Tabas, I., and Kortfeld, S. (1978) J. Biol. Chem. 253, 7762-7770
4. Liu, T., Stetson, B., Turco, S. J., Hubbard, S. C., and Robbins, P. W. (1979) J. Biol. Chem. 254, 4554-4559
5. Babczynski, P., and Tanner, W. (1973) Biochim. Biophys. Res. Commun. 44, 1119-1124
6. Sharma, C. B., Babczynski, P., Lehle, L., and Tanner, W. (1974) Eur. J. Biochem. 46, 35-41
7. Lehle, L., and Tanner, W. (1975) Biochim. Biophys. Acta 399, 364-374
8. Reuvers, P., Habets-Willems, C., Reinking, A., and Boer, P. (1977) Biochim. Biophys. Acta 486, 541-552
9. Sentandreu, R., and Lampen, J. O. (1972) FEBS Lett. 27, 331-334
10. Jung, P., and Tanner, W. (1973) Eur. J. Biochem. 37, 1-6
11. Reuvers, F., Bser, P., and Hemming, F. W. (1978) Biochem. J. 169, 505-508
12. Trimble, R. B., and Maley, F. (1977) Biochem. Biophys. Res. Commun. 75, 935-944
13. Hasilik, A., and Tanner, W. (1978) Eur. J. Biochem. 81, 567-575
14. Tarentino, A. L., Fidjeland, T. H., Jr., and Maley, F. (1974) J. Biol. Chem. 249, 815-824
15. Lehle, L., Cohen, R. E., and Ballou, C. E. (1979) J. Biol. Chem. 254, 12209-12218
10238

Yeast Oligosaccharide-Lipids

16. Parodi, A. J. (1976) FEBS Lett. 71, 283–286
17. Parodi, A. J. (1978) Eur. J. Biochem. 83, 253–259
18. Lehle, L., and Tanner, W. (1978) Biochim. Biophys. Acta 539, 218–229
19. Dominguez, A., Villaneuva, J. R., and Sentandreu, R. (1978) Antonie van Leeuwenhoek J. Microbiol. Serol. 44, 183–192
20. Parodi, A. J. (1979) J. Biol. Chem. 254, 8343–8352
21. Ito, S., Yamashita, K., Spiro, R. G., and Kobata, A. (1977) J. Biochem. (Tokyo) 81, 1621–1631
22. Tarentino, A. L., Plummer, T. H., Jr., and Maley, F. (1973) J. Biol. Chem. 248, 5547–5548
23. Huang, C.-G., Mayer, H. E., Jr., and Montgomery, R. (1970) Carbohydr. Res. 13, 127–137
24. Tarentino, A. L., and Maley, F. (1969) Arch. Biochem. Biophys. 130, 295–303
25. Tarentino, A., Plummer, T. H., Jr., and Maley, F. (1975) Biochemistry 14, 5516–5523
26. Trimble, R. B., Tarentino, A. L., Plummer, T. H., Jr., and Maley, F. (1978) J. Biol. Chem. 253, 4508–4511
27. Tarentino, A. L., Plummer, T. H., Jr., and Maley, F. (1970) J. Biol. Chem. 245, 4150–4157
28. Tarentino, A. L., and Maley, F. (1974) J. Biol. Chem. 249, 811–817
29. Tarentino, A. L., Trimble, R. B., and Maley, F. (1978) Methods Enzymol. 50, 574–580
30. Tan, C. C., Muldrey, J. E., Li, S.-C., and Li, Y.-T. (1976) J. Biol. Chem. 251, 4384–4388
31. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
32. Waechter, C. J., Lucas, J. J., and Lennarz, W. J. (1973) J. Biol. Chem. 248, 7570–7579
33. Lucas, J. J., Waechter, C. J., and Lennarz, W. J. (1975) J. Biol. Chem. 250, 1992–2002
34. Behrens, N. H., and Tabora, E. (1978) Methods Enzymol. 50, 402–435
35. Trevelyan, W. E., Procter, D. P., and Harrison, J. S. (1950) Nature 166, 444–445
36. Randerath, K., and Randerath, E. (1964) J. Chromatogr. 16, 111–125
37. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956) Anal. Chem. 28, 350–356
38. Nakajima, T., and Ballou, C. E. (1974) J. Biol. Chem. 249, 7679–7684
39. Parodi, A. J. (1977) Eur. J. Biochem. 75, 171–180
40. Axelos, M., and Peaud-Lenoel, C. (1978) Biochimie 60, 35–44
41. Turco, S. J., Stetson, B., and Robbins, P. W. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 4411–4414
42. Spiro, M. J., Spiro, R. G., and Bhoyroo, V. D. (1979) J. Biol. Chem. 254, 7668–7674
43. Chen, W. W., Lennarz, W. J., Tarentino, A. L., and Maley, F. (1975) J. Biol. Chem. 250, 7006–7013
44. Tai, T., Yamashita, K., Ito, S., and Kobata, A. (1977) J. Biol. Chem. 252, 6687–6694
45. Robbins, P. W., Krag, S. S., and Liu, T. (1977) J. Biol. Chem. 252, 1780–1785
46. Hershco, A., Bugge, B., and Jeanloz, R. W. (1977) J. Biol. Chem. 252, 2271–2277
47. Chen, W. W., and Lennarz, W. J. (1978) J. Biol. Chem. 253, 5774–5779
48. Schmitt, J. W., and Elbein, A. D. (1979) J. Biol. Chem. 254, 12281–12294
49. Tabas, I., Schlesinger, S., and Kornfeld, S. (1978) J. Biol. Chem. 253, 716–722
50. Spiro, M. J., Spiro, R. G., and Bhoyroo, V. D. (1976) J. Biol. Chem. 251, 6420–6425
51. Robbins, P. W., Hubbard, S. C., Turco, S. J., and Wirth, D. F. (1977) Cell 12, 893–900
52. Speake, B. R., and White, D. A. (1978) Biochemistry 17, 993–1000
53. Staneloni, R. J., and Leloir, L. F. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 1162–1166
54. Hubbard, S. C., and Robbins, P. W. (1979) J. Biol. Chem. 254, 4505–4507
55. Li, E., and Kornfeld, S. (1979) J. Biol. Chem. 254, 2754–2758
56. Hunt, L. A., and Summers, D. F. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 754–758
57. Chen, W. W., and Lennarz, W. J. (1978) J. Biol. Chem. 253, 5780–5785
58. Kornfeld, S., Li, E., and Tabas, I. (1979) J. Biol. Chem. 253, 7771–7777
59. Scher, M. G., and Waechter, C. J. (1979) J. Biol. Chem. 254, 2630–2637
60. Turco, S. J., and Robbins, P. W. (1979) J. Biol. Chem. 254, 4560–4567
61. Spiro, R. G., Spiro, M. J., and Bhoyroo, V. D. (1979) J. Biol. Chem. 254, 7659–7667
62. Tabas, I., and Kornfeld, S. (1978) J. Biol. Chem. 253, 7779–7786
63. Nakajima, T., and Ballou, C. E. (1974) J. Biol. Chem. 249, 7688–7694
64. Trimble, R. B., Byrd, J., Tarentino, A. L., and Maley, F. (1980) Fed. Proc. 39, 1900