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Characterization of an oligosaccharide-pyrophosphodolichol pyrophosphatase activity in yeast

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Use of Triton X-114 allowed us to develop a new method to separate hydrophilic oligosaccharidic material from hydrophobic oligosaccharide pyrophosphodolichols (oligosaccharide-*PP*-Dol). Taking advantage of this procedure we characterize, in yeast microsomal membranes, an enzymic activity that hydrolyses oligosaccharide-*PP*-Dol into oligosaccharidic material. H.p.l.c. analysis together with alkaline-phosphatase- and endo-*N*-acetyl-β-D-glucosaminidase-susceptibility demonstrate that the oligosaccharidic released material is mainly composed of oligomannosides containing a chitobiose phosphate at the reducing end. The enzymic activity requires bivalent cations and is inhibited by pyrophosphate, NAD^+ and bacitracin. As other, commercially available, pyrophosphatases have no action on lipid intermediates, the described pyrophosphatase activity appears to be the specific enzyme for oligosaccharide-*PP*-Dol. This enzymic splitting of the pyrophosphate bond might be the primary event in the catabolism of lipid intermediates.

INTRODUCTION

N-Glycosylation of proteins proceeds via the transfer of the oligosaccharide moiety from an oligosaccharide-*PP*-Dol to an asparagine residue of the nascent protein. In contrast with the voluminous literature detailing the metabolic pathway for sequential glycosylation of lipid intermediates, far less attention has been given to the degradation of oligosaccharide-*PP*-Dol. In fact, the release of soluble oligosaccharide phosphates and of neutral oligosaccharides has been observed in a number of glycoprotein-synthesizing systems (Hsu *et al.*, 1974; Oliver & Hemming, 1975; Cacan *et al.*, 1980; Anumula & Spiro, 1983). We previously demonstrated that this catabolic pathway starts with the cleavage of the pyrophosphate bond of oligosaccharide-*PP*-Dol (Cacan *et al.*, 1980, 1987), although Anumula & Spiro (1983) have proposed that part of this material could originate from a side reaction due to the oligosaccharidyltransferase using water as acceptor.

In the present paper we characterize the enzyme activity responsible for the primary event in oligosaccharide-*PP*-Dol degradation. Taking advantage of the property of Triton X-114 to segregate, after phase separation, phospholipids from more hydrophilic material (Bordier, 1981; Pryde & Phillips, 1986), we developed a procedure to extract, for further identification, the water-soluble material released from oligosaccharide-*PP*-Dol used as a substrate. Using yeast microsomal membranes as enzyme source, we describe here a novel pyrophosphatase activity acting on oligosaccharide-*PP*-Dol.

MATERIALS AND METHODS

Chemicals

GDP-[U-^{14}C]mannose (10.73 GBq/mmol) and D-[2-^{3}H]mannose (588 GBq/mmol) were from Amersham International (Amersham, Bucks., U.K.). Zymolyase-100 T from *Arthrobacter luteus* was from Kirin Brewery (Tokyo, Japan). Alkaline phosphatase from calf intestine and endo-*N*-acetyl-β-D-glucosaminidase H from *Streptomyces griseus* were from Boehringer (Mannheim, Germany). Inorganic pyrophosphatase from baker's yeast, nucleotide pyrophosphatase from *Crotalus adamanteus* venom and all other analytical-grade reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). QAE-Sephadex A-25 was obtained from Pharmacia (Uppsala, Sweden) and Bio-Gel P-2 (200–400 mesh) were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Standard oligomannosides with one *N*-acetylglucosamine residue at the reducing end were kindly provided by Dr. G. Strecker (Villeneuve d'Ascq, France). The [2-^{3}H]Man-oligosaccharide-*PP*-Dol compounds were prepared by metabolic labelling of mouse splenocytes as previously described by Cacan *et al.* (1987). The yeast strain (X 2180-1A, wild-type *Saccharomyces cerevisiae*) was kindly provided by Dr. W. Tanner (Regensburg, Germany).

Preparation of yeast microsomal membranes

Saccharomyces cerevisiae was grown at 30 °C in a medium containing 0.5% yeast extract, 1% (w/v) bactopeptone and 2% (w/v) glucose. The cells were harvested in mid-exponential phase, washed twice with 0.1 M-NH_4HCO_3, resuspended in 0.1 M-NH_4HCO_3 containing 50 mM-2-mercaptoethanol and incubated at 30 °C for 10 min under a slow stirring at 35 rev./min. The cells were collected by centrifugation at 1000 *g* for 10 min at 20 °C, washed twice with 0.1 M-sodium citrate buffer, pH 5.8, containing 0.8 M-sorbitol, then resuspended in the same buffer containing a fresh solution of Zymolyase-100 T (20 μg/ml final concn.) for spheroplast formation. The mixture was incubated at 30 °C for 1 h with continuous gentle mixing. The spheroplasts were collected by low-speed centrifugation (1000 *g* for

Abbreviation used: oligosaccharide-*PP*-Dol, oligosaccharide pyrophosphodolichols.

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10 min), gently resuspended and washed twice with citrate buffer as above. Cell lysis of spheroplasts was obtained by homogenization (ten strokes in a Potter apparatus) in 20 mM-Tris/HCl buffer, pH 7.4, containing 3 mM-$MgCl_2$ and 160 mM-NaCl. Nuclei, non-disrupted cells and mitochondria were removed by 10000 *g* centrifugation for 10 min at 4° C, and the supernatant was centrifuged at 48000 *g* for 30 min at 4 °C. The final pellet containing yeast microsomal membranes was resuspended in 100 mM-Tris/HCl buffer, pH 7.4 (30 mg of protein/ml), or was kept frozen at −80 °C until use.

Phase separation of neutral oligosaccharides and oligosaccharide phosphates from oligosaccharide-*PP*-Dol by using Triton X-114 solution

Classical sequential lipid extraction based on phase separation with chloroform/methanol/water (3:2:1, by vol.) is not suitable when exogenous oligosaccharide-*PP*-Dol are added in the presence of detergent, since they partition into both the aqueous and the organic phases. Thus a different procedure had to be developed. A simple method had been described for separating hydrophobic and hydrophilic proteins by using phase separation with Triton X-114 (Bordier, 1981). Briefly, the method is based upon solubilizing proteins in the non-ionic detergent Triton X-114 followed by heating the aqueous solution obtained above its cloud point (around 22 °C). The solution then separates into two clear phases, respectively depleted and enriched in detergent, i.e. an aqueous phase containing the hydrophilic proteins and a detergent phase containing the hydrophobic proteins. The method has been applied to oligosaccharide-*PP*-Dol.

Labelled oligosaccharide-*PP*-Dol were solubilized at 0 °C in 20 mM-Tris/HCl buffer, pH 7.4, containing 154 mM-NaCl and 1% (w/v) Triton X-114 in a total volume of 100 μl. This clear solution was overlaid at 0 °C on a cushion (500 μl) of 8.5% (w/v) sucrose in 20 mM-Tris/HCl buffer, pH 7.4, containing 154 mM-NaCl and 0.05% Triton X-114 in a 1.5 ml conical Eppendorf microcentrifuge tube. The tube was then incubated at 37 °C for 5 min and clouding of the solution occurred. The tube was centrifuged at 2500 *g* for 3 min at room temperature in a Beckman microcentrifuge. After centrifugation the detergent phase was found as an oily droplet at the bottom of the tube. To improve the extraction of oligosaccharide-*PP*-Dol, the upper aqueous phase was removed, Triton X-114 was added to give a 1% solution and again a clear solution was obtained by leaving the mixture at 0 °C. This solution was again overlaid on sucrose cushion identical with that described above, incubated at 37 °C and centrifuged as described above. As indicated on Fig. 1(*a*), to increase the percentage of oligosaccharide-*PP*-Dol in the lower phase (from 85% to more than 90%) the process has to be repeated at least twice. Fig. 1(*b*) indicates that the partition is independent of the amount of labelled oligosaccharide-*PP*-Dol.

Standard assay for oligosaccharide-*PP*-Dol pyrophosphatase activity

Approx. 150000 d.p.m. of [2-^{3}H]Man-oligosaccharide-*PP*-Dol was dissolved in 50 μl of 1% Triton X-114 in 20 mM-Tris/HCl buffer, pH 7.4, containing 154 mM-NaCl and mixed, in a conical Eppendorf microcentrifuge tube, with 20 μl of yeast microsomal membranes solubilized in 1% Triton X-114 in 100 mM-Tris/HCl buffer, pH 7.4 (30 mg of protein/ml). The mixture (total volume 100 μl) was incubated at 35 °C for 1 h and then reaction was stopped by dilution with 500 μl of ice-cold 1% Triton X-114 solution. The reaction products and remaining substrate were extracted according to the phase-separation technique described above.

Synthesis of lipid intermediates *in vitro* with GDP-[^{14}C]mannose and yeast membranes

Incorporation of [^{14}C]mannose from GDP-[^{14}C]-mannose (10.73 GBq/mmol) into endogenous yeast microsomal acceptors was performed in a final reaction volume of 0.1 ml containing 20 mM-Tris/HCl buffer, pH 7.4, 5 mM-$MgCl_2$, 2 mM-$MnCl_2$, 100 μM-UDP-*N*-acetylglucosamine, 10-μM-GDP-[^{14}C]mannose and yeast microsomal membranes (2.25 mg of protein). After incubation at 37 °C for specified time periods, reaction was stopped by diluting the mixture to 0.4 ml (4 mM-$MgCl_2$ with 1 mg of carrier immunoglobulin G) and the solution was mixed with 0.8 ml of methanol and 1.2 ml of chloroform. Lipid intermediates and glycoproteins were extracted by the multiple extraction procedure as previously described (Cacan *et al.*, 1980). The labelled oligosaccharide phosphates and neutral oligosaccharides released in the aqueous phase were separated from precursor and precursor-degradation products by gel filtration on a Bio-Gel P-2 column (1 cm × 50 cm) in 0.1 M-acetic acid. The glycoprotein pellet was dissolved in conc. formic acid by heating at 100 °C for 10 min. Radioactivities were counted in scintillation medium and quenching correction was made by external ratio standardization.

Analysis of anionic oligosaccharides on QAE-Sephadex

The number of negative charges on the oligosaccharides was determined by batch elution from QAE-Sephadex (Varki & Kornfeld, 1980). The material was dissolved in 10 mM-Tris base, passed through 1 ml column of QAE-Sephadex equilibrated in 10 mM-Tris base and eluted stepwise with increasing concentrations of NaCl in 10 mM-Tris base (5 ml each). A final elution with 1 M-NaCl in 0.1 M-HCl was done before re-equilibration with 100 mM-Tris base followed by 10 mM-Tris base.

H.p.l.c. of oligosaccharides

After treatment with endo-*N*-acetyl-β-D-glucosaminidase H of exogenous oligosaccharide-*PP*-Dol (Chalifour & Spino, 1984) or of oligosaccharide phosphates (Cacan *et al.*, 1980) the resulting oligosaccharides were desalted on Bio-Gel P-2, concentrated under vacuum and dissolved in acetonitrile/water (7:3, v/v). H.p.l.c. was performed on a Spherisorb-NH2 (5 μm particle size) column (250 mm × 4.6 mm). Elution was achieved with a linear gradient of acetonitrile/water from 7:3 to 1:1 (v/v) for 40 min at a flow rate of 1 ml/min at room temperature. Fractions (0.5 ml) were collected and their radioactivity was determined by liquid-scintillation counting. Oligosaccharides used as standards were co-chromatographed with each sample.

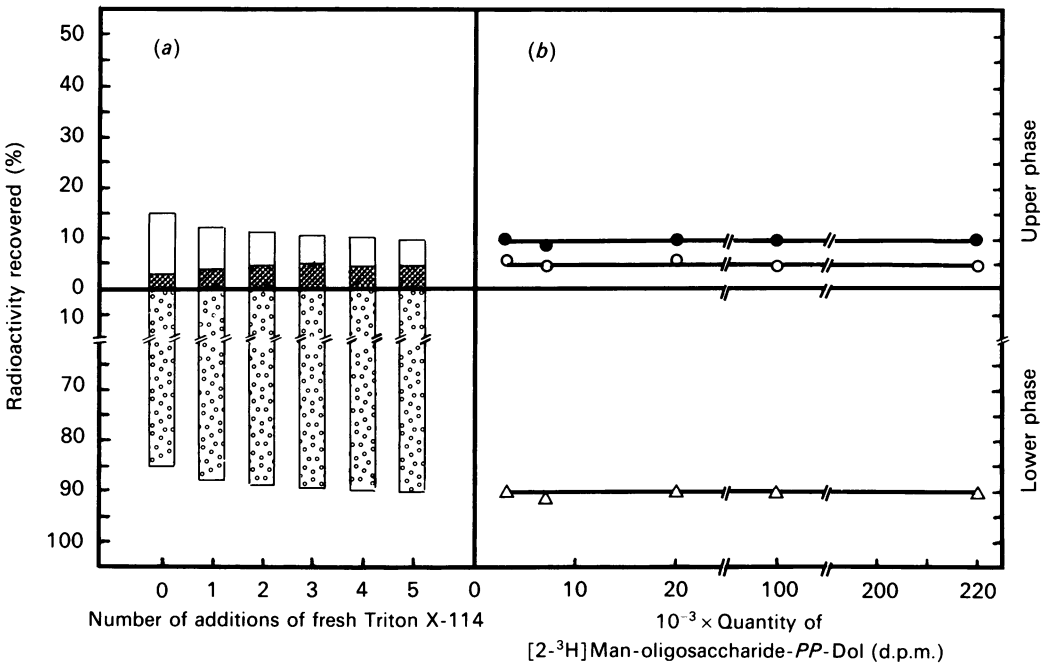

Fig. 1. Partition of oligosaccharide-*PP*-Dol after Triton X-114 phase separation

Labelled oligosaccharide-*PP*-Dol were solubilized at 0 °C in 1% Triton X-114 and the solution was overlaid on a 8.5% (w/v) sucrose cushion in buffer (see the Materials and methods section). After phase separation at 37 °C the detergent phase was separated from the aqueous phase by 2500 *g* centrifugation for 3 min. Panel (*a*) indicates the partition of radioactivity in the lower detergent phase (stippled area) and in the upper aqueous phase (hatched areas represent the radioactivity in the sucrose cushion) according to the number of repeat extractions with fresh 1% Triton X-114. Panel (*b*) represents, according to the amount of labelled oligosaccharide-*PP*-Dol added, the partition of the radioactivity in the total upper phase (●), in the sucrose cushion (○) and in the lower detergent phase (△) after two repeat extractions with 1% Triton X-114.

RESULTS

Formation of oligosaccharides during *N*-glycosylation by yeast membranes

When yeast microsomal membranes were incubated at 37 °C with GDP-[^{14}C]mannose and UDP-*N*-acetylglucosamine in the conditions described in the Materials and methods section, labelled glycosylated products were recovered as Man-*P*-Dol, oligosaccharide-*PP*-Dol and glycoproteins. In addition, the aqueous phase of the sequential lipid extraction contained, besides labelled precursors and their degradation products, a higher-molecular-mass labelled material that was eluted soon after the void volume on Bio-Gel P-2 chromatography. As shown in Fig. 2(*a*), analysis of this material on QAE-Sephadex reveals two populations: a non-retained neutral fraction (15%) and a negatively charged (2 net charges) fraction (85%). When the anionic material was treated with alkaline phosphatase (12 munits for 16 h at 37 °C) or with endo-*N*-acetyl-β-D-glucosaminidase H (10 munits for 16 h at 37 °C) it was converted into neutral compounds, indicating that its charge was due to the presence of a phosphate group linked to the *N*-acetylglucosamine residue at the reducing end of a chitobiosyl unit of an oligomannoside, as we had already described for rat spleen lymphocytes (Cacan *et al.*, 1980). At that time we had proposed that these oligosaccharide phosphates originated from the splitting of the pyrophosphate bond of oligosaccharide-*PP*-Dol, and the high yield of oligosaccharide phosphates obtained with yeast prompted us to use yeast microsomal membranes to characterize the enzymic activity responsible for the cleavage of oligosaccharide-*PP*-Dol.

It is of note that, although the oligosaccharidic moiety of lipid intermediate was composed of Man_9- and Man_8-species (mean proportions 57% and 43% respectively), the oligosaccharidic moiety of oligosaccharide phosphates was mainly (95%) of Man_8- species, indicating the action of a specific mannosidase, as already described by Jelinek-Kelly *et al.* (1985).

Use of the Triton X-114 phase-separation method to assay the cleavage of exogenous oligosaccharide-*PP*-Dol by yeast microsomal membranes

Oligosaccharide-*PP*-Dol were prepared from mouse splenocytes after metabolic labelling with [2-^{3}H]mannose and incubated with yeast microsomal membranes in the presence of 1% Triton X-114 in the condition of the standard assay at 35 °C. After the phase-separation procedure as described in the Materials and methods section, it was observed that the radioactivity of the detergent phase containing labelled oligosaccharide-*PP*-Dol decreased with time, although the radioactivity increased in the aqueous phase (Fig. 3*a*). It has to be noted that this phenomenon was dependent on the amount of membranes added (Fig. 3*b*), being linear up to 20 μl of membrane suspension, i.e. 600 μg of protein, and did not occur when membranes were previously heat-denatured (10 min at 70 °C). The small percentage of hydrophilic material released at zero time was due to the fact that the phase-separation technique requires three

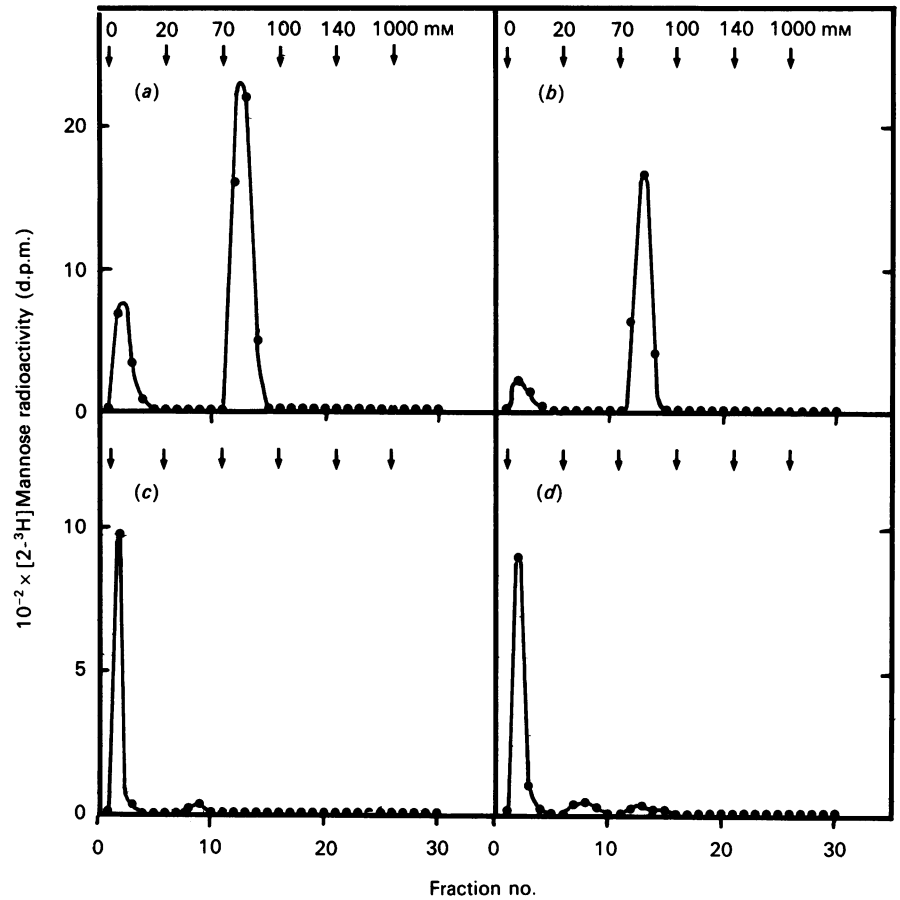

Fig. 2. QAE-Sephadex analysis of the soluble oligosaccharidic material released during oligosaccharide-*PP*-Dol degradation

Oligosaccharidic material released in the aqueous phase after synthesis of oligosaccharide-*PP*-Dol *in vitro* with labelled GDP-mannose and yeast membranes (panel *a*), or recovered in the aqueous phase obtained by Triton X-114 phase separation after incubation of exogenous labelled oligosaccharide-*PP*-Dol with Triton X-114-solubilized yeast membranes (panel *b*), was applied to QAE-Sephadex columns, and eluted stepwise with increasing concentrations of NaCl in 10 mM-Tris base (0, 20, 70, 100 and 140 mM) corresponding to a net negative charge of 0, −1, −2, −3 and −4. The charged oligosaccharidic material obtained in panel (*b*) was re-analysed on QAE-Sephadex after treatment with endo-*N*-acetyl-β-D-glucosaminidase H (panel *c*) or after treatment with alkaline phosphatase (panel *d*).

repeated heatings at 37 °C, during which reaction can occur.

Characterization of the products released in the aqueous phase

After incubation of labelled oligosaccharide-*PP*-Dol with yeast membranes followed by phase separation as described in the Materials and methods section, the aqueous phase was washed three times with 4 ml of theoretical lower phase [chloroform/methanol/4 mM-$MgCl_2$ (85:14:1, by vol.)] in order to eliminate residual detergent and to denature hydrophilic proteins. After being desalted on Bio-Gel P-2, the material was submitted to ion-exchange analysis on a QAE-Sephadex column. Fig. 2(*b*) reveals two populations, as had been earlier observed in the incubation with endogenously formed oligosaccharide-*PP*-Dol (Fig. 2*a*). When the anionic population was treated with endo-*N*-acetyl-β-D-glucosaminidase H (Fig. 2*c*) or with alkaline phosphatase (Fig. 2*d*), it was converted into neutral components, indicating that the major compound released in the aqueous phase was an oligomannoside terminated by a chitobiose phosphate unit. The size of this oligosaccharidic moiety was determined by h.p.l.c. analysis of the oligosaccharide released by endo-*N*-acetyl-β-D-glucosaminidase. Fig. 4(*b*) indicates that oligosaccharide phosphates are mainly composed of three populations containing nine, eight and five mannose units in the respective proportions 43 %, 23 % and 31 % on a molar basis. Since the exogenous oligosaccharide-*PP*-Dol used as substrate was composed of three species only (with nine, eight and seven mannose units in the respective proportions 78 %, 11 % and 11 %), these results indicate that during incubation some of the oligosaccharidic moieties are degraded into smaller species.

Thus the use of the Triton X-114 phase-separation procedure allowed us to demonstrate the release of oligosaccharide phosphates when oligosaccharide-*PP*-Dol are incubated with yeast microsomal membranes, indicating the action of an oligosaccharide-*PP*-Dol pyrophosphatase, which was further characterized.

To test whether this release of hydrophilic material was due to a specific enzyme, other pyrophosphate-hydrolysing enzymes were assayed on oligosaccharide-

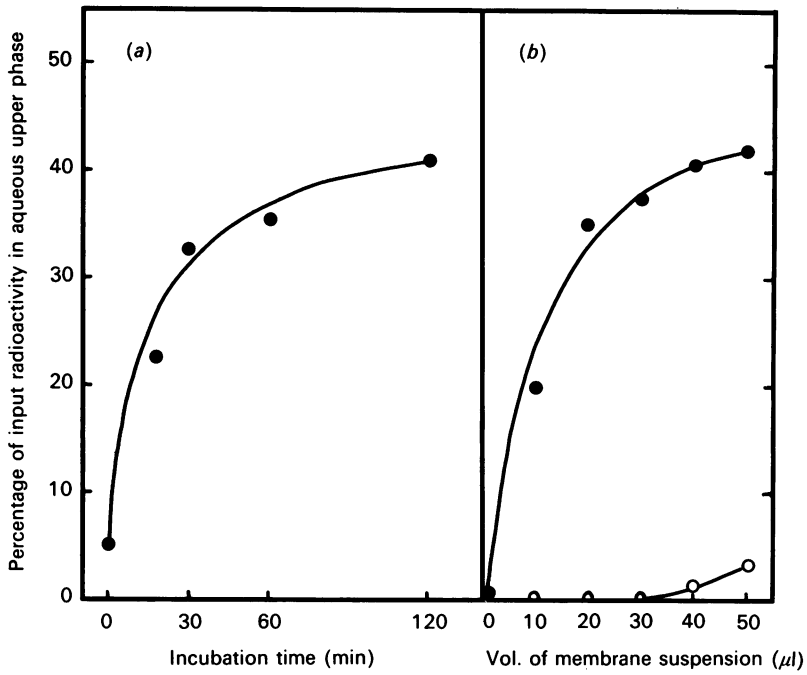

Fig. 3. Time- and membrane-concentration-dependence of oligosaccharide-*PP*-Dol hydrolysis

Labelled oligosaccharide-*PP*-Dol were incubated in the conditions described in the Materials and methods section. Extent of hydrolysis was expressed as a percentage of the input radioactivity as a function of time with 50 μl of membrane suspension (panel *a*). The membrane-concentration-dependence (panel *b*) was tested with 1 h incubations with native (●) or heat-denatured (10 min at 70 °C) yeast membranes (○).

Table 1. Bivalent-cation-dependence of oligosaccharide-*PP*-Dol pyrophosphatase activity

The assays were performed as described in the Materials and methods section in the standard incubation conditions without bivalent cations (control). Assays were then performed in the presence of chelators (EDTA, EGTA) or bivalent cations. The data represent means for three different experiments, and are expressed as percentages of the control.

Incubation conditions	Relative activity (% of control)
Complete (minus bivalent cations)	100
Plus EDTA (10 mM)	50.7
Plus EGTA (10 mM)	35.7
Plus $MgCl_2$ (10 mM)	113.2
Plus $MnCl_2$ (10 mM)	149.5
Plus $CaCl_2$ (10 mM)	150.8

PP-Dol. After a 1 h incubation, no hydrolysis was observed with alkaline phosphatase from calf intestine or with pyrophosphatase from baker's yeast, and only a little hydrolysis (1.8%) was obtained with nucleotide pyrophosphatase from *Crotalus adamanteus* venom. Control experiments indicated that the three enzymes were active on their appropriate substrates (results not shown).

Temperature-, pH- and cation-dependence of the enzyme activity

No activity was detected at 0 °C and the enzymic activity showed a rather broad optimal temperature between 30° and 35 °C. Preincubation of the enzyme at different temperatures during 10 min followed by incubation for 1 h in the standard conditions showed that the enzymic activity remained stable up to 50 °C. The half-life was determined to be 1 h at 62 °C, and the activity was totally destroyed by preincubation at 70 °C.

The effect of pH was studied in the range 6–9.5 with Mes/Tris buffers. Optimal activity was observed between pH 7 and 7.5, and no activity could be detected at pH above 9. The optimal pH corresponded to a maximum of stability when the enzyme activity was assayed after preincubation at different pH values for 1 hr.

Table 1 shows that the enzyme activity was stimulated by the presence of 10 mM-Ca^{2+} and -Mn^{2+} and, to a lesser extent, of 10 mM-Mg^{2+}. The cation-dependence of the reaction was confirmed by the inhibitory effect of chelators such as EDTA and EGTA (10 mM final concn.).

Effects of phosphate, pyrophosphate, NAD^+ and bacitracin on the enzyme activity

Since the reaction involves the splitting of a pyrophosphate bond, we investigated the effect of phosphate- or pyrophosphate-containing substrates and

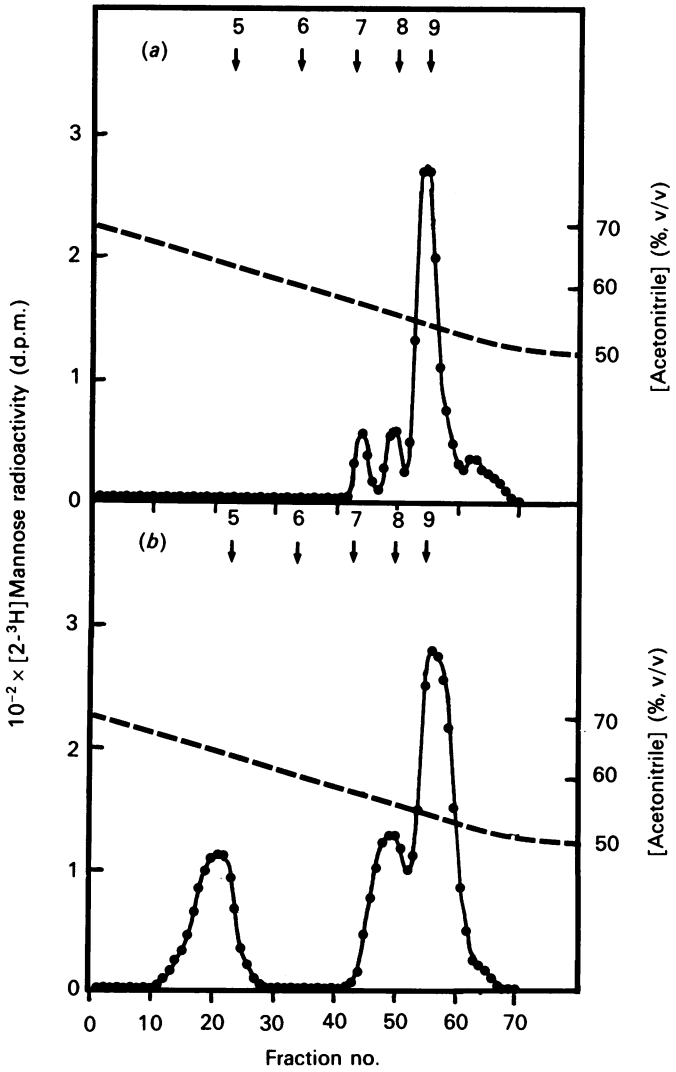

Fig. 4. H.p.l.c. analysis of labelled oligosaccharides

By endo-*N*-acetyl-β-D-glucosaminidase H treatment labelled oligosaccharides were released either from oligosaccharide-*PP*-Dol substrate (panel *a*), or from oligosaccharide phosphates (panel *b*). H.p.l.c. analyses were performed on a Spherisorb-NH2 column eluted with a linear gradient of acetonitrile/water from 7:3 to 1:1 (v/v). Arrows indicate the position of standard oligosaccharides: 9, $Man_9GlcNAc$; 8, $Man_8GlcNAc$; 7, $Man_7GlcNAc$; 6, $Man_6GlcNAc$; 5, $Man_5GlcNAc$.

of bacitracin, which is known to inhibit the cleavage of the pyrophosphate bond of dolichol pyrophosphate (Wedgwood & Strominger, 1980). Fig. 5 clearly shows that pyrophosphate and NAD^+ are potent inhibitors (total inhibition at 10 mM), although 10 mM-phosphate led to 35% inhibition only. Bacitracin was also an effective inhibitor, since an 80% inhibition was obtained when it was added at the concentration of 10 mM. We were unable to assay at a higher concentration of bacitracin since at concentrations above 10 mM the detergent effect of bacitracin rendered oligosaccharide-*PP*-Dol themselves soluble in the aqueous phase (Fig. 5*d*).

Effect of detergent on the enzyme activity

When assayed with increasing concentrations of Triton X-100 (Fig. 6*a*) or Triton X-114 (Fig. 6*b*), the enzymic activity was enhanced up to the detergent concentration of about 0.5%. This increase was probably due both to the solubilization of the substrate by the detergent and to the latency of the enzyme. In fact, it is known that the oligosaccharide linked to pyrophosphodolichol is at the luminal face of the rough endoplasmic reticulum (Hanover & Lennarz, 1981), and thus it can be expected that the pyrophosphatase activity would have the same location. The enzymic activity remained stable during the 1 h incubation in the presence of up to 1% Triton X-100, but at higher concentrations denaturation occurred. It has to be noted that the phase-separation procedure with Triton X-114 was not altered by the presence of Triton X-100 (at least up to a Triton X-100/Triton X-114 ratio of 1:2), as demonstrated by control experiments without membranes (Fig. 6*a*).

DISCUSSION AND CONCLUSION

Since our previous observation (Cacan *et al.*, 1980) of the degradation of lipid intermediates during the process of glycosylation in mouse splenocytes *in vitro*, it has been also shown with other biological models (Hanover & Lennarz, 1982; Anumula & Spiro, 1983) that oligosaccharide-*PP*-Dol exhibit a dual fate, i.e. either used for the transfer of their oligosaccharidic moiety *en bloc* on to a protein acceptor or degraded into oligosaccharide phosphates and neutral oligosaccharides. In fact, the formation of oligosaccharide phosphates would be the initial step of a catabolic process leading to smaller neutral oligosaccharides (Cacan *et al.*, 1987) and presumably to monosaccharides.

In the present paper we first demonstrate that this catabolic process occurs also during *N*-glycosylation *in vitro* with yeast microsomal membranes but with a high proportion of oligosaccharide phosphates, presumably due to a slower further degradation process. Thus yeast membranes appear to be a good enzyme source for characterization of the primary event of oligosaccharide-*PP*-Dol degradation.

The characterization of this first reaction requires solubilization of the exogenous oligosaccharide-*PP*-Dol used as substrate, then separation of the hydrophilic products from the amphipathic substrate. Since the classical Folch procedure does not yield a net partition of oligosaccharide-*PP*-Dol in the organic phase when they have been previously solubilized with detergent, we devised a novel procedure based on phase separation of Triton X-114 solution at a temperature above the cloud point, as first described by Bordier (1981).

When labelled exogenous oligosaccharide-*PP*-Dol are incubated with yeast membranes, part of the radioactivity is recovered in the hydrophilic upper phase in a time-dependent and membrane-concentration-dependent manner. These labelled products have been characterized as oligosaccharide phosphates mainly with the general structures $Man_9GlcNAc_2$-*P*, $Man_8GlcNAc_2$-*P* and $Man_5GlcNAc_2$-*P* in the proportions 43%, 23% and 31% respectively. It may be noted that when oligosaccharide-*PP*-Dol are endogenously labelled with GDP-[^{14}C]mannose in the absence of detergent the

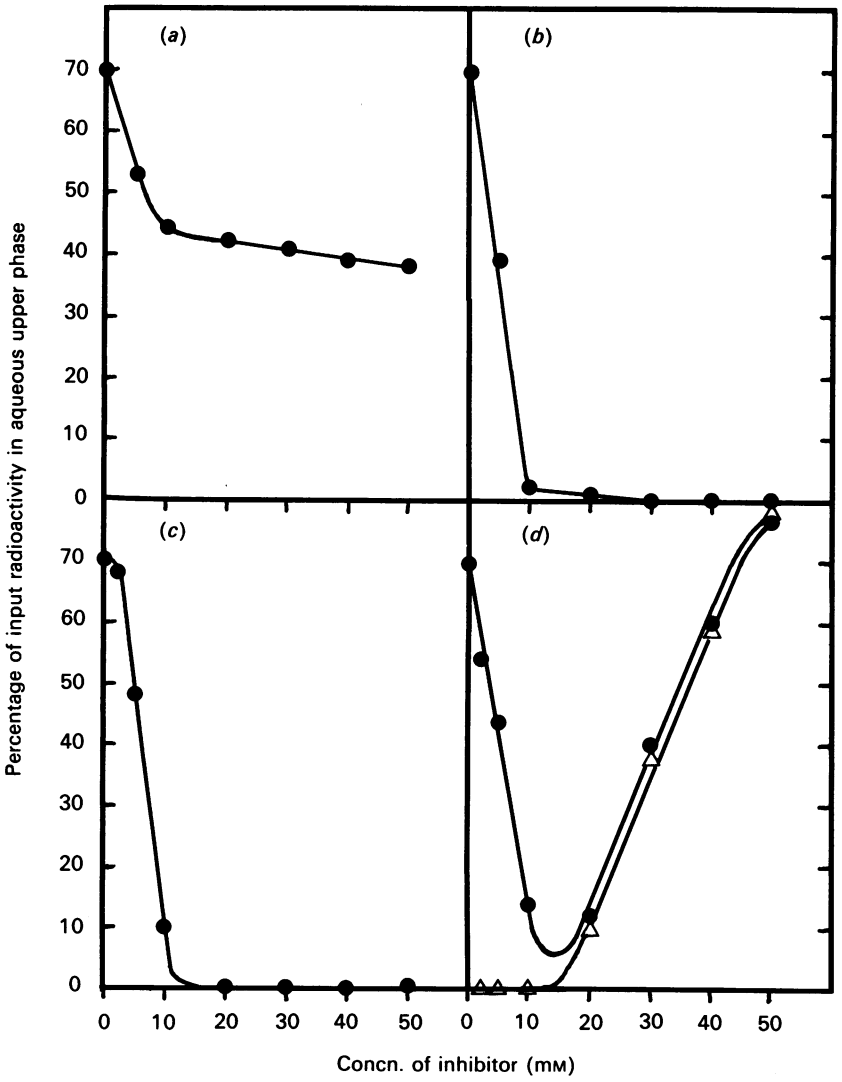

Fig. 5. Inhibition of oligosaccharide-*PP*-Dol pyrophosphatase activity

Incubations were performed in the standard conditions as described in the Materials and methods section with various concentrations (from 0 to 50 mM) of inorganic phosphate (panel *a*), pyrophosphate (panel *b*) or NAD^+ (panel *c*). Panel (*d*) indicates the effect of increasing amounts of bacitracin incubated in the presence (●) or in the absence (△) of yeast membranes.

oligosaccharide phosphates produced are mainly of $Man_8GlcNAc_2$-*P* structure although the donor lipid intermediates possess nine mannose residues. This is in agreement with the rough-endoplasmic-reticulum location of a specific mannosidase reported by Bischoff & Kornfeld (1983) and described also in yeast by Jelinek-Kelly *et al.* (1985), which reinforces the proposal that the splitting of oligosaccharide-*PP*-Dol occurs on their biosynthetic site, i.e. the lumen of rough endoplasmic reticulum. The presence of $Man_5GlcNac_2$-*P* species in the assay with exogenous oligosaccharide-*PP*-Dol is presumably due to the presence of other mannosidases released by detergent, as mentioned by Jelinek-Kelly *et al.* (1985).

The pyrophosphatase activity described appears to be the specific enzyme for oligosaccharide-*PP*-Dol, since other pyrophosphatases do not act on these lipid intermediates. This enzyme exhibits an optimal pH between 7 and 7.5 and an optimal temperature between 30° and 35 °C, and appears to be cation-dependent, mainly for Mn^{2+} and Ca^{2+}. More interesting is its inhibition by pyrophosphate-containing compounds and by bacitracin, which is known to interact with the pyrophosphate bond of lipid intermediates, as has been demonstrated by Wedgwood & Strominger (1980) and previously utilized by Cacan *et al.* (1980) to inhibit the degradation of lipid intermediates in rat splenocytes. This raises the question whether the enzyme activity described in the present paper would be supported by the pyrophosphodolichol pyrophosphatase described by Wedgwood & Strominger (1980), the function of which would be to regenerate dolichol phosphate from dolichol pyrophosphate released after the action of the oligosaccharidyltransferase.

Thus the biological significance of this enzyme activity could be a regulatory step in the *N*-glycosylation pathway: it could control the availability of oligosaccharide-*PP*-Dol used as donors, and this first degradation event would regenerate dolichol phosphate required for the so-called 'dolichol cycle'.

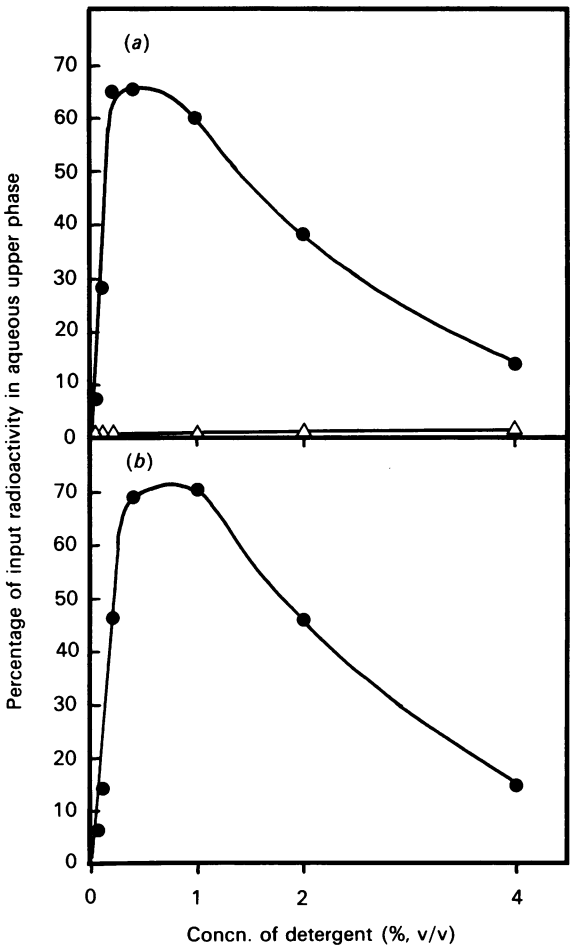

Fig. 6. Effect of detergent on the oligosaccharide-*PP*-Dol pyrophosphatase activity

Incubations were performed in the standard conditions as described in the Materials and methods with increasing concentrations of Triton X-100 (panel *a*) in the presence (●) or in the absence (△) of yeast membranes. Panel (*b*) indicates the effect of increasing concentrations of Triton X-114.

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