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THE USE OF LIPOSOMES IN *Aspergillus niger* VAN TIEGHEM MANNOSYLATION *IN VITRO* STUDIES

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ABSTRACT

The use of dolichyl phosphate-containing liposomes in glycoprotein biosynthesis under conditions that control the transfer of exogenous dolichyl phosphate from liposomes to microsomes is described The exogenously added dolichyl phosphate is available for mannosylation, and direct evidence for the role of dolichyl phosphate as intermediate in protein mannosylation was shown by use of liposomes containing dolichyl [^{14}C]mannosyl phosphate

INTRODUCTION

Protein mannosylation in *Aspergillus niger* van Tieghem, as well as in many other organisms, is a complex process that involves a lipid intermediate[1,2], the lipophilic moiety of which has been shown to be a poly(isoprenol)[3] These enzymic reactions occur within membranes and, *in vitro*, the main difficulty is to introduce hydrophobic compounds into the membranes To solve this problem, polar and neutral detergents have been widely used, but were shown to inhibit drastically the enzymic reaction, especially for the present system[4] Liposomes of known composition seem to be a valuable tool for the study of membrane-bound enzymes[5] In the present paper, we describe (a) the interaction between microsomes and liposomes, and (b) the influence of dolichyl phosphate and dolichyl mannosyl phosphate-containing vesicles on the transfer of mannose to lipid and endogenous proteins, respectively

EXPERIMENTAL

Material and methods — Egg yolk lecithin, phosphatidic acid, 3-*sn*-phosphatidyl ethanolamine, phosphatidylcholine, and dolichyl phosphate were obtained from Sigma Chemical Co , St Louis, MO 63178 (U S A), and radiochemicals from Amersham France, B P 563, F-78005 Versailles (France) Lipid vesicles were routinely prepared by dissolving egg yolk lecithin (10 mg), 3-*sn*-phosphatidylethanolamine (3 mg), and various concentrations of phosphatidic acid, dolichyl phosphate, or dolichyl mannosyl phosphate in 2 1 (v/v) chloroform–methanol (1 mL) After being

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dried under a nitrogen stream the lipids were swollen in 50mM Tris HCl buffer pH 8 5 (1 mL) The suspension was ultrasonically irradiated under nitrogen for 30 min at 20° at power level 4 with a Branson Sonifier Model B-12 equipped with a microtip The sonicate was then centrifuged at 100 000g for 1 h at room temperature The clear supernatant containing small, unilamellar vesicles[6] is referred to as liposomes Microsomes were prepared as previously described[7] Proteins were determined by the method of Lowry *et al* [8] with human serum albumin as standard [^{14}C]Mannose transfer to lipids and proteins was carried out according to Letoublon *et al* [7] after the extraction procedure of Folch *et al* [9] Dolichyl mannosyl phosphate [the purity of which had been controlled by t l c on silica gel-precoated plates 60F254 from Merck-France S A F-75009 Paris (France) with 60 25 4 (v/v) chloroform–methanol–water as solvent] was prepared from standard incubation mixtures[7], and kept under nitrogen at −20° as a solution in 2 1 (v/v) chloroform–methanol

Electron microscopy of liposomes — Aliquots of lipid vesicles prepared as just described were diluted 1 2 to 1 10 with 2% ammonium molybdate, pH 6 9 according to Hanover and Lennarz[10], or with 2% potassium phosphotungstate pH 7 2 Specimens were examined with a Philips EM 300 electron microscope operating at 80 kV at the Centre de Microscopie Électronique Appliquee a la Biologie Universite Claude Bernard Lyon I

Sepharose 2B chromatography — The suspension of liposomes (0 5 mL) was chromatographed on a Sepharose 2B [Pharmacia Fine Chemicals AB S-75104 Uppsala 1 (Sweden)] column (1 5 × 25 cm) equilibrated and eluted with 50mM Tris HCl buffer, pH 8 5 The absorbance was recorded at 320 nm with a Beckman 25 spectrophotometer Fractions (2 mL) were collected and aliquots (0 5 mL) used for radioactivity assay

RESULTS AND DISCUSSION

Aspergillus niger van Tieghem microsomes were shown to contain endogenous polyprenyl phosphate that can be enzymically mannosylated from GDP-mannose[7] the resulting compound is a lipid intermediate for glycoprotein mannosylation By modifying the amount of dolichyl phosphate available to the GDP-mannose polyprenyl phosphate mannosyltransferase (EC 2 4 1 54) an attempt was made to gain some more precise insight into the molecular mechanism of this glycosylation pathway

Table I summarizes some of the procedures used and shows that no increase of dolichyl [^{14}C]mannosyl phosphate synthesis was observed in the presence of either dolichyl phosphate alone or a suspension of dolichyl phosphate in dimethyl sulfoxide Dimethyl sulfoxide had no inhibitory effect, but Triton X-100 was inhibitory as previously observed[4 11] and the inhibition was overcome when the micelles obtained by Triton X-100 treatment contained enough dolichyl phosphate to bring the final concentration up to at least 40μM

Since liposomes have previously been used as acceptors for the assay of UDP-galactose 2-(2-hydroxyacyl)sphingosine galactosyltransferase (EC 2 4 1 45) and

TABLE I

TRANSFER OF [^{14}C]MANNOSE FROM GDP-[^{14}C]MANNOSE TO ENDOGENOUS POLYPRENYL PHOSPHATES OR ADDED DOLICHYL PHOSPHATE[a]

Addition	[^{14}C]*Mannose transferred* (*d p m mg of protein*$^{-1}$ *min*$^{-1}$)
None	550
Dolichyl phosphate (60μM)[b]	550
Dimethyl sulfoxide (0 5M)	580
Dolichyl phosphate (60μM) in dimethyl sulfoxide (0 5M)	620
Triton X-100 (0 05%)	300
Dolichyl phosphate (60–150μM) in Triton X-100 (0 05%)	950

[a]The mixtures were incubated for 5 min, the values in parentheses indicate final concentrations
[b]Dolichyl phosphate was first dissolved in 2 1 (v/v) chloroform–methanol and the solution dried under a stream of nitrogen then the incubation mixture was added to the dried residue

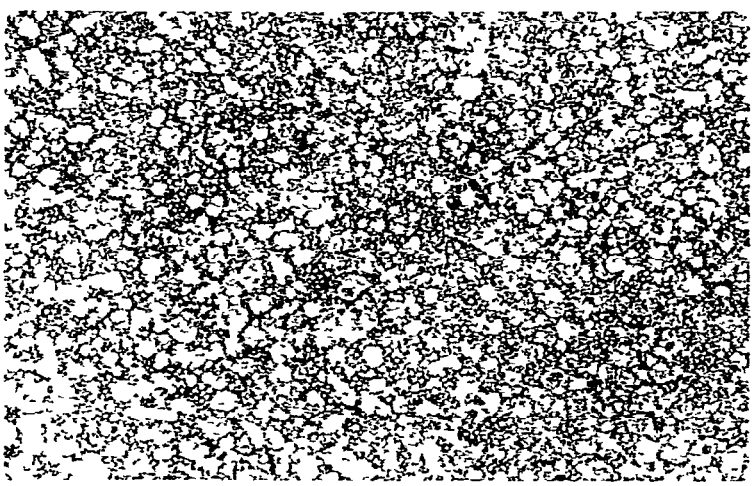

Fig 1 Electron micrograph of negatively stained preparations of small unilamellar liposomes (Magnification 34 000)

UDP-glucose ceramide glycosyltransferase[12], we attempted to adapt this technique to the present problem The shape and size of the lipid vesicles, prepared as described in the Experimental section were investigated by electron microscopy using negative staining the average size of the liposomes determined by electron microscopy was 20 nm (Fig 1), a value that is very close to the one observed by Hanover and Lennarz[10] By freeze fracture, however, a value of 40 nm was estimated[13], a value that is in good agreement with the diameters measured by Barenholz *et al*[6] In addition, gel filtration on Sepharose 2B confirmed that the lipid vesicles were homogeneous in size (see Fig 2) In order to avoid modification of the overall phospholipid composition of the microsomal membranes, the nature and ratio of the phospholipid

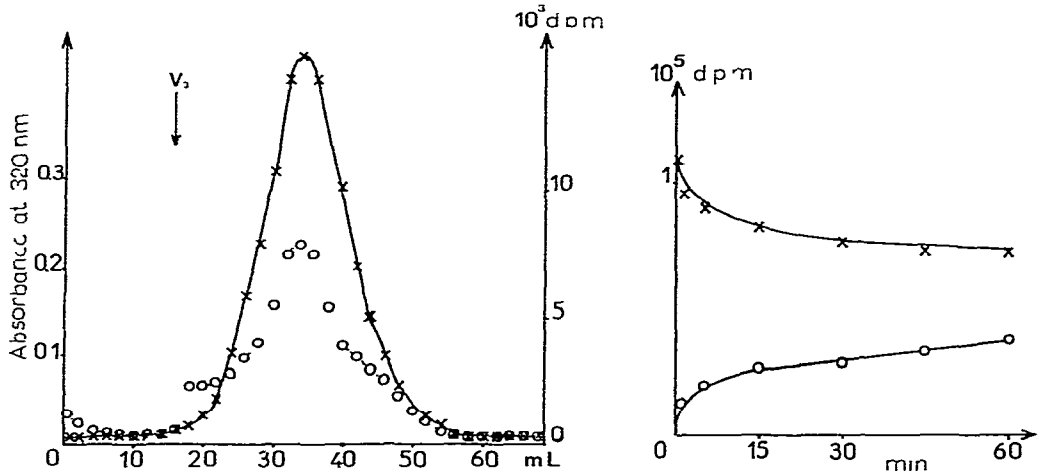

Fig 2 Sepharose 2B gel filtration The liposomes contained egg-yolk lecithin (10 mg), 3-*sn*-phosphatidylethanolamine (3 mg), dolichyl phosphate (0 5 mg), and phosphatidyl[*N*-methyl-^{14}C]choline (0 2 μCi, spec activity, 55 Ci/mol) per mL of Tris HCl buffer pH 8 5 (○——○) absorbance at 320 nm and (× ——×) radioactivity (d p m) The position of Blue Dextran is at V_0

Fig 3 Fusion between microsomes and liposomes Liposomes (20μL) containing egg-yolk lecithin (10 mg), 3-*sn*-phosphatidylethanolamine (3 mg), and dolichyl phosphate (0 5 mg) or phosphatidic acid (0 3 mg) per mL of 50mM Tris HCl buffer, pH 8 5, containing phosphatidyl[*N*-methyl-^{14}C]-choline (spec activity 55 Ci/mol) were mixed with a microsomal suspension (100 μL, 0 3 mg of protein) in the same buffer At various time-intervals the mixture was centrifuged for 3 min at 150 000*g* in an Airfuge Beckman centrifuge, and the radioactivity of the supernatant solution and the pellet measured (——) supernatant solution, (○——○) pellet

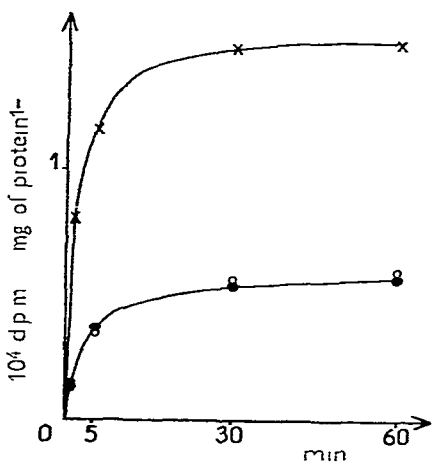

Fig 4 Synthesis of dolichyl mannosyl phosphate Liposomes (20 μL) prepared as described in the legend to Fig 3 (but without labeled phosphatidylcholine) were added to microsomal membranes (100 μL, 0 3 mg of protein) and incubated for 1 h at 32° Then GDP-[^{14}C]mannose (10 μL, 5 μCi/mL) was added to the suspension At various time-intervals, the reaction was stopped by addition of 2 1 (v/v) chloroform–methanol (20 vol) The radioactive lipids were extracted according to Folch *et al* [9] and the radioactivity was measured (●——●) microsomes without liposomes, (○——○) microsomes with phosphatidic acid-containing liposomes, and (×——×) microsomes with dolichyl phosphate-containing liposomes

components of liposomes were selected to be close to those of the naturally occurring ones

In the study of the rate and extent of fusion between liposomes and microsomes a plateau was reached after 1 h of incubation, and ~40% of the negatively charged lipid vesicles had fused with the microsomal membranes (see Fig 3) The results obtained with *Aspergillus niger* microsomes, which have a very high protein-to-phospholipid ratio (>15 1) as compared to that from rat liver microsomes (ratio ~4 1), show that the extent of fusion is about the same, but the initial velocity of the phenomenon is much higher in the latter[14]

The fusion of dolichyl phosphate-loaded vesicles and microsomes (see Fig 4) resulted in (a) an increase of the initial velocity of the mannosylation, and (b) a tremendous enhancement of the dolichyl mannosyl phosphate synthesis, whereas the fusion of charged liposomes that did not contain any dolichyl phosphate did not affect the mannosyltransferase activity These observations indicate that the substrate (exogenous dolichyl phosphate) had access to the GDP-mannose dolichyl phosphate mannosyltransferase The chromatographic behavior and hydrolysis properties of the mannophospholipids formed upon addition of dolichyl phosphate were identical with those of dolichyl mannosyl phosphate[15]

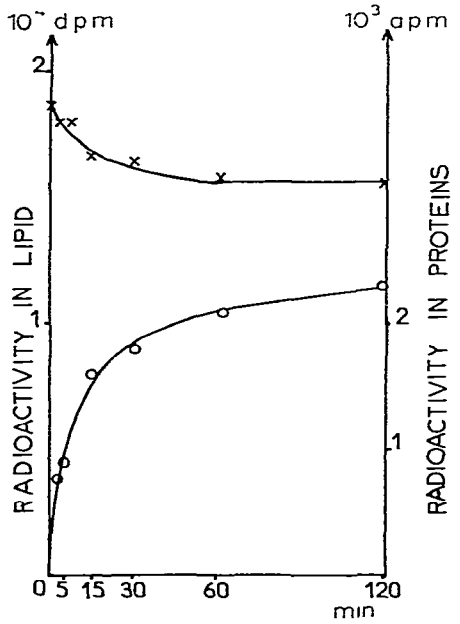

Fig 5 Influence of dolichyl [^{14}C]mannosyl-containing liposomes on glycoprotein biosynthesis Liposomes (20 μL) containing egg yolk lecithin (10 mg), phosphatidylethanolamine (3 mg), phosphatidic acid (0 3 mg), and dolichyl [^{14}C]mannosyl phosphate (10^6 d p m) per mL of 50mM Tris HCl buffer pH 8 5 were added to microsomes (100 μL 0 3 mg of protein) from *A niger* At various time intervals, the reaction was stopped by addition of 2 1 (v/v) chloroform-methanol (2 mL) and the radioactivity of lipid (×——×) and protein (○——○) measured as described in the Experimental section

The apparent K_m for dolichyl phosphate is $\sim 30 \mu M$ this value is very close to that obtained by Forsee and Elbein[16] for ficaprenyl phosphate in the presence of a detergent It is of interest that, by use of liposomes it is possible to control the amount of lipophilic substrate available to the microsomal enzyme

In previous experiments[1], some evidence that dolichyl phosphate is an intermediate in protein mannosylation had been presented and this conclusion was based on kinetic results the use of dolichyl [^{14}C]mannosyl phosphate-containing liposomes provides direct evidence for the transfer of [^{14}C]mannose from the lipid to endogenous proteins (see Fig 5) 80% of the total radioactivity was recovered as [^{14}C]mannolipid after 120 min of incubation of the remaining 20%, 11% were [^{14}C]mannose covalently bound to proteins, and the rest was recovered as [^{14}C]mannose and [^{14}C]mannosyl phosphate no oligosaccharide (or related compound) was found

The data presented herein show that liposomes can be used for the assay of two different membrane-bound mannosyltransferases thus, addition of detergents which in some cases have inhibitory effects or organic solvents to disperse the lipids is no longer required As previously mentioned[13] phospholipid vesicles may be expected to be used for a more general application than for glycosyltransferases only, namely for all the enzymes that require lipids or amphipatic molecules as activator or substrate

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