Role of a Mannosyl Lipid Intermediate in the Synthesis of *Neurospora crassa* Glycoproteins†

Michael H. Gold* and Hyo Joon Hahn

ABSTRACT: Particulate membrane preparations from *Neurospora crassa* incorporated mannose from GDP-[^{14}C]mannose into endogenous lipid and particulate protein acceptors. Synthesis of the mannosyl lipid is reversible in the presence of GDP. Chemical and chromatographic characterization of the mannosyl lipid suggest that it is a mannosylphosphorylpolyisoprenol. The other endogenous acceptor was precipitated by trichloroacetic acid. Gel filtration and electrophoresis studies before and after treatment with proteolytic enzymes indicate that the second acceptor is a glycoprotein(s). β-Elimination studies on the mannosyl protein formed from GDP-[^{14}C]mannose with Mg^{2+} in the reaction mixture or formed from mannosyl lipid indicate that a single mannose unit is transferred directly to form an *O*-glycosidic bond with the peptide chain. Several lines of evidence indicate that in *Neurospora crassa* the mannosyl lipid is an obligatory intermediate in the in vitro mannosylation of the protein. (a) At 15 °C the initial formation of the mannosyl lipid is faster than the initial formation of the mannosyl protein. (b) Exogenous partially purified mannosyl lipid can function as a mannosyl donor for the synthesis of the mannosyl protein. This reaction was also dependent on a divalent metal. The rate of this reaction was optimal at a concentration of Triton X-100 which effectively inhibited the transfer of mannose from GDP-[^{14}C]mannose to lipid and protein, indicating that GDP-mannose was not an intermediate in the transfer of mannose from lipid to protein. The mannosyl protein formed in this reaction was indistinguishable by several criteria from the mannosyl protein formed from GDP-[^{14}C]mannose and Mg^{2+}. (c) The effect of a chase with an excess of unlabeled GDP-mannose on the incorporation of mannose into endogenous acceptors was immediate cessation of the synthesis and subsequent turnover of the mannosyl lipid; in contrast, however, incorporation of mannose into protein continued and was proportional to the loss of mannose from the mannosyl lipid.

The role of lipid carriers as intermediates in complex carbohydrate biosynthesis is well established in bacteria (Rothfield and Romeo, 1971). Evidence is accumulating that similar intermediates also have a role in the biosynthesis of glycoproteins in animals (Lennarz, 1975) and fungi (Tanner, 1969; Bretthauer et al., 1973; Letoublon and Got, 1974; Sentandreu and Lampen, 1972). In order to elucidate further the structure and biosynthesis of the cell envelope of *Neurospora crassa* we have investigated the possible role of polyisoprenol phosphates as glycosyl carriers in this organism.

In this study we report that a cell-free membrane fraction from *Neurospora crassa* mycelia catalyzes the transfer of mannose from GDP-mannose to a mannosyl lipid and an endogenous particulate protein(s). In addition, we demonstrate that the mannosyl lipid is an obligatory intermediate in the transfer of the single mannosyl unit from GDP-mannose to the terminal protein acceptor(s). The mechanism of chain lengthening of the carbohydrate protein of the glycoprotein, as well as the exact intracellular localization of the glycoprotein, are now under investigation.

Experimental Procedure

Materials. Neurospora crassa wild type strain RL3-8A from the Rockefeller collection was maintained on slants of complete medium. The organism was grown for 16 h at 30 °C from a conidial inoculation on a rotary shaker in 2-l. Erlenmeyer flasks containing 1 l. of Vogel medium N (Vogel, 1964) using 2% sucrose as the carbon source. Mycelia were harvested by suction filtration and stored at −20 °C for less than 1 week before use. *Saccharomyces ceriviseae* was El Molino active dry yeast purchased locally. GDP-[U-^{14}C]mannose (221 mCi/mmol) was obtained from the New England Nuclear Corp. Unlabeled GDP-mannose, nonradioactive sugars, nucleotides and their derivatives, the proteases, and *E. coli* alkaline phosphatase were obtained from Sigma. Ammonyx was obtained from the Onyx Chemical Co. Hen oviduct dolichol-phosphoryl[^{14}C]mannose was a gift from Dr. J. J. Lucas, State University of New York. Silica gel G plates were obtained from Analtech. All other chemicals were reagent grade.

Radioactivity and Protein Measurements. Radioactivity was measured quantitatively in a Searle Isocap 300 scintillation system. Radiochromatograms were scanned on a Searle Actigraph III Scanner. Radioactive samples were counted in Aquasol purchased from New England Nuclear Corp. Protein was determined by the biuret procedure (Layne, 1957) using bovine serum albumin as a standard.

Particulate Enzyme Preparation. Frozen mycelium was ground with sand in a chilled mortar. All subsequent steps were performed at 0–4 °C. The broken cells were extracted for 5 min in ten volumes of 50 mM Tris-Cl, pH 7.4, 200 mM sucrose, 1 mM EDTA. The extract was centrifuged at 1000*g* for 10 min to remove cell walls, and the supernatant fraction was then centrifuged at 40 000*g* for 30 min. The resultant crude particulate preparation was resuspended in 5 mM Tris-Cl, pH 7.4, 200 mM sucrose, 1 mM EDTA to a concentration of 20 mg of protein/ml. The enzyme was prepared fresh daily.

Assay Procedures

Incorporation of [^{14}C]Mannose into Lipid and Protein. Mannosyl transferase activity was measured in a 0.1 ml reaction mixture containing 100 mM Tris-Cl, pH 8.0, 20 mM

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$MgCl_2$, 1 mg of protein and 5 μM GDP-[^{14}C]mannose (50 μCi/μmol). Reactions were started by the addition of the particulate preparation and were carried out at 22 °C for 20 min unless stated otherwise. Initially, in order to detect possible lipid-linked oligosaccharides, the reactions were stopped and prepared for counting by the method of Waechter et al. (1973) (method A). Subsequently, it was found more convenient to use the following method (method B).

Reactions were stopped at the indicated time by the addition of 1 ml of $CHCl_3$–CH_3OH (2:1) followed by 0.4 ml of H_2O. The mixture was allowed to stand for 15 min and was then centrifuged in a clinical centrifuge. The lower phase containing the mannosyl lipid was removed and washed first with 1 ml and then with 0.75 ml of 0.9% NaCl–CH_3OH (1:0.5). The washed lower phase was then transferred to a scintillation vial, evaporated to dryness, and counted. The upper phase from the original $CHCl_3$–CH_3OH extraction containing the mannosyl protein was mixed with 1.5 ml of cold 10% trichloroacetic acid (w/v) and 2 mg of bovine serum albumin. The precipitated protein was allowed to stand for at least 1 h at 0 °C and then was centrifuged in a clinical centrifuge. The protein precipitate obtained by centrifugation was washed two times with 5% trichloroacetic acid, dissolved in 0.5 ml of 98% formic acid, and counted in 5 ml of scintillation fluid. Essentially the same results were obtained with extraction procedures A and B.

Partial Purification of the Mannosyl Lipid. Mannosyl lipid (2×10^5 cpm) was applied in $CHCl_3$–CH_3OH (2:1) to a column (2.5 × 8 cm) of DEAE-cellulose prepared according to Rouser et al. (1969) and equilibrated in $CHCl_3$–CH_3OH (2:1). After the sample was applied, the column was eluted successively with 100-ml volumes of $CHCl_3$–CH_3OH (2:1), CH_3OH, and finally 0.1 M ammonium acetate in 99% CH_3OH. Fractions (7 ml) were collected and 0.2-ml aliquots from each fraction were removed, dried, and counted. The pooled mannosyl lipid fractions were concentrated under reduced pressure and washed twice by partitioning against 0.2 volume of H_2O. The lipid was finally dried under N_2 and stored at −20 °C.

Transfer of [^{14}C]Mannose from Mannosyl Lipid to Mannosyl Protein. Assay mixtures contained 4×10^3 cpm of mannosyl lipid transferred in $CHCl_3$–CH_3OH (2:1), dried under N_2, and dispersed by sonication in 25 μl of 0.4% Ammonyx (Waechter et al., 1973). The final reaction mixture (0.1 ml) contained 100 mM Tris-Cl, pH 8.0, 20 mM $MgCl_2$, and enzyme (10 mg/ml). Reactions were started by the addition of enzyme and were carried out at 22 °C. The reaction was stopped with 2 ml of $CHCl_3$–CH_3OH (2:1), and mannosylated product(s) and substrate were separated by method A.

Hydrolytic Methods. Mild acid hydrolysis of the mannosyl lipid is described in the legend to Figure 5. Strong acid hydrolysis of the glycoprotein was performed with 3 N HCl in degassed sealed tubes for 3 h at 110 °C. The hydrolyzate was then evaporated to dryness under reduced pressure. The products were chromatographed in solvents A and B described below.

Mild alkaline hydrolysis of the mannosyl lipid was carried out at 37 °C for 20 min in 0.2 ml of 0.1 N NaOH in 90% ethanol. The hydrolysis was stopped by the addition, with mixing, of 2 ml of $CHCl_3$–CH_3OH (2:1) and 0.5 ml of 40 mM acetic acid. The two phases were cooled and centrifuged, and the radioactivity in each phase was measured.

Strong alkaline hydrolysis was similar except that it was carried out for 45 min at 85 °C. An aliquot of the radioactivity which partitioned into the aqueous phase after strong alkaline hydrolysis was chromatographed in solvent system C. A second aliquot of this fraction was treated with alkaline phosphatase and chromatographed in solvent system A.

Protease Digestion of the Mannosyl Protein. Approximately 75×10^3 cpm of [^{14}C]mannose containing protein (15 mg) prepared from GDP-[^{14}C]mannose was suspended in 0.5 ml of 50 mM Tris-Cl, pH 8.0, 2 M urea, 10 mM $CaCl_2$ and 150 μg of pronase. A drop of toluene was added and the reaction was incubated at 35 °C. After 18 h an additional 150 μg of pronase was added and the reaction continued for an additional 24 h. After heating to 100 °C for 1 min the reaction mixture was adjusted to 10 mM EDTA; 150 μg of subtilisin was then added, and incubation continued for an additional 24 h. Finally, the reaction was stopped by heating to 100 °C for 1 min.

Gel Filtration. Sodium dodecyl sulfate was added to the proteolytic digest, the mixture was heated to 50 °C for 1.5 h, and glycerol was added to a concentration of 10% v/v. The mixture was applied to a Sephadex G-150 column (2.5 × 40 cm) equilibrated in 50 mM Tris-Cl, pH 7.5, 0.5% sodium dodecyl sulfate, and 0.5% mercaptoethanol. Fractions (2 ml) were collected and 0.2-ml aliquots from each fraction were counted.

Mild Base Treatment of the [^{14}C]Mannose-Containing Protein. The water-washed glycoprotein, prepared from either GDP-[^{14}C]mannose or lipid-[^{14}C]mannose, was incubated with 0.1 M NaOH for 24 h at 21 °C. The mixture was then dialyzed exhaustively against 200 volumes of water. The external solution was concentrated and neutralized with Dowex 50-H^+, and aliquots of this fraction and of the nondialyzable fraction were counted. Another aliquot of the external solution was chromatographed in solvent systems A and B.

Alkaline Borohydride Treatment of the [^{14}C]Mannose-Containing Protein. The mannosyl protein prepared from GDP-[^{14}C]mannose was dissolved in 0.5 ml of 0.5 M NaOH–1.0 M $NaBH_4$, incubated, and prepared for chromatography essentially as described by Bretthauer and Wu (1975). The product was chromatographed in solvent systems A and B.

Chromatography and Electrophoresis. Descending paper chromatography was conducted in the following solvent systems: (A) ethyl acetate–pyridine–H_2O (12:5:4), (B) isobutyric acid–NH_4OH–H_2O (59:4:39), (C) 1 M ammonium acetate (pH 7.5)–ethanol (3:7.5). Thin-layer chromatography of the mannosyl lipid was carried out on silica gel G in: (D) $CHCl_3$–CH_3OH–H_2O (65:25:4), (E) $CHCl_3$–CH_3OH–HOAc–H_2O (30:15:4:2), and (F) $CHCl_3$–CH_3OH–15 M NH_4OH–H_2O (80:30:0.5:3). The radioactive zones on the thin-layer plates were detected by autoradiography using Du Pont Cronex 2DC X-ray film

Results

Time Dependence of Mannosyl Transferase Activity. Incubation of *N. crassa* particulate preparations with GDP-[^{14}C]mannose at 15 °C resulted in the incorporation of radioactivity into two endogenous acceptors (Figure 1) as assayed either by method A or by method B. The mannosyl lipid is soluble in $CHCl_3$–CH_3OH (2:1). Unlike the results obtained in animal systems (Waechter et al., 1973; Behrens et al., 1973; Hsu et al., 1974) no additional radioactivity is extractable in $CHCl_3$–CH_3OH–H_2O (1:1:0.3). The radioactivity found in the insoluble residue after the two extraction procedures described above is mannosyl protein.

At 15 °C the initial rate of formation of the mannosyl lipid is faster than the initial rate of formation of mannosyl protein. The formation of mannosyl lipid reaches a steady-state level after approximately 15 min. If the concentration of GDP-mannose in the reaction mixture is reduced to 1 μM, a similar curve is generated except that the mannosyl lipid appears to

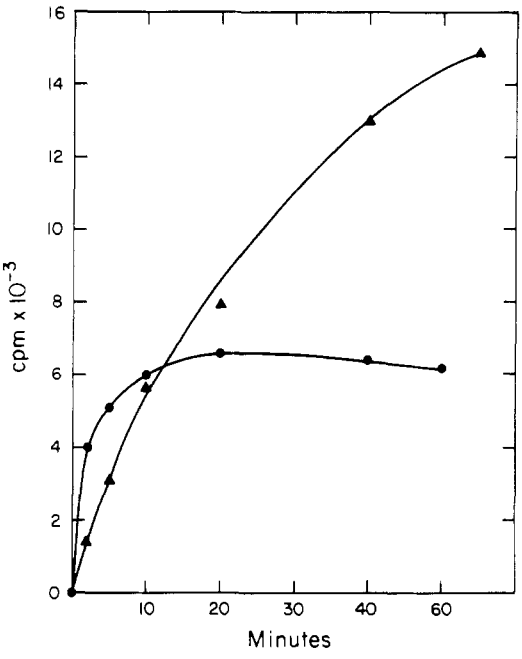


FIGURE 1: Time dependence of the transfer of mannose from GDP-[^{14}C]mannose to endogenous lipid and protein. The standard incubation conditions were used except that the incubation temperature was 15 °C, and method A was used to isolate the mannosyl lipid (●) and mannosyl protein (▲).

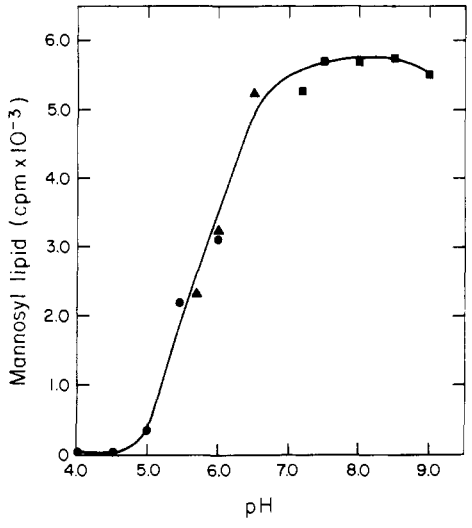


FIGURE 2: Effect of pH on mannosyl lipid synthesis. The standard incubation conditions with the following buffers were used: succinate (●); Mes (▲); Tris (■).

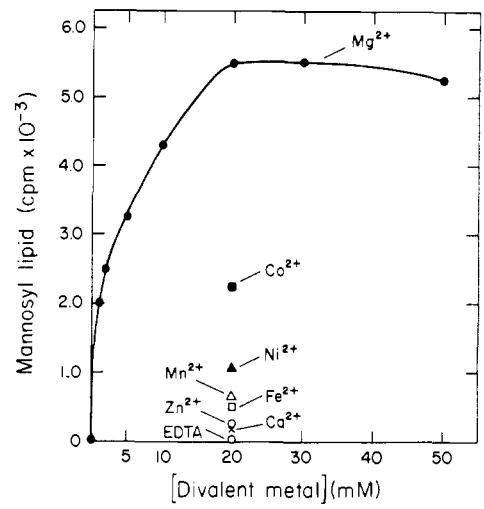


FIGURE 3: Divalent metal ion requirement for mannosyl lipid synthesis. The standard incubation and assay conditions were used.

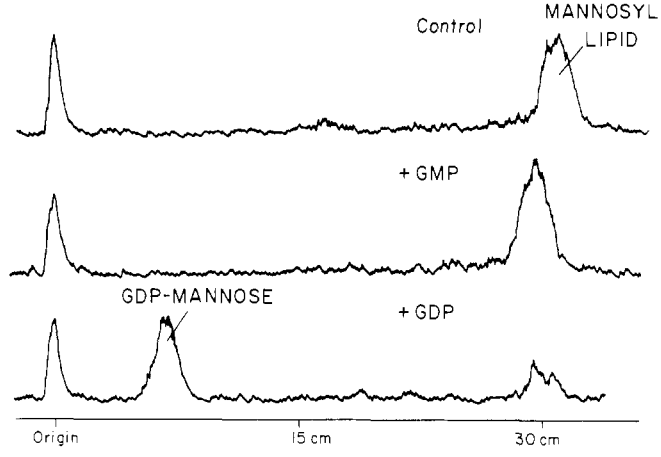


FIGURE 4: Reversibility of the mannosyl lipid synthesis. The method used was similar to that of Waechter et al. (1973). A standard reaction mixture containing GDP-[^{14}C]mannose was incubated for 5 min and centrifuged at 4 °C. The pellet was washed once with 50 mM sodium phosphate, pH 6.5, 1 mM EDTA to remove residual substrate. The washed pellet was then resuspended in the same buffer containing Mg^{2+} and the relevant guanine nucleotide addition, and incubated for 20 min at 22 °C. At the end of the incubation the reaction mixtures were spotted on Whatman 3MM paper and chromatographed in solvent system B.

turn over after 5 min. Mannosyl protein is formed at a slower rate and is metabolically stable. Each of the kinetic experiments was performed at least twice and the data presented are typical.

Optimal Conditions for the Synthesis of Mannosyl Lipid from GDP-[^{14}C]Mannose. As illustrated in Figure 2 the enzyme displayed maximum activity from pH 7.0 to pH 9.0, with activity decreasing sharply below pH 6.5. At pH 8.0 the synthetase showed absolute dependency on a divalent cation. The velocity of the reaction with increasing Mg^{2+} ion concentration is shown in Figure 3. Maximal stimulation occurs at 20 mM Mg^{2+}. Other divalent metals can substitute only partially for Mg^{2+} at pH 8.0. The saturation curve generated with increasing concentrations of GDP-mannose is hyperbolic. The apparent K_m calculated from a reciprocal plot of the data is approximately 2.2 μM. Less than 10% of the radioactivity was recovered in the product under the conditions of the assay. Maximal rate of mannosyl lipid synthesis occurred at 22 °C.

Reversibility of Mannosyl Lipid Synthesis. Particulate enzyme preparations with presynthesized lipid-[^{14}C]mannose were incubated with GDP, GMP, or with no additions, and the amount of GDP-[^{14}C]mannose formed was measured. The results in Figure 4 show that at pH 6.5, in the presence of Mg^{2+} and GDP, there is significant transfer of mannose from the mannosyl lipid to GDP-mannose. At pH 8.0 a reproducible, but significantly smaller, amount of mannose is transferred. AT EITHER PH condition, when no nucleotide is added or when GDP is replaced by GMP, no detectable amount of GDP-mannose is formed. These results indicate that the synthesis of mannosyl lipid is reversible and that GDP is the other product formed.

Properties of the Mannosyl Lipid. Mild alkaline treatment of the lipid-[^{14}C]mannose, under conditions which deacylate

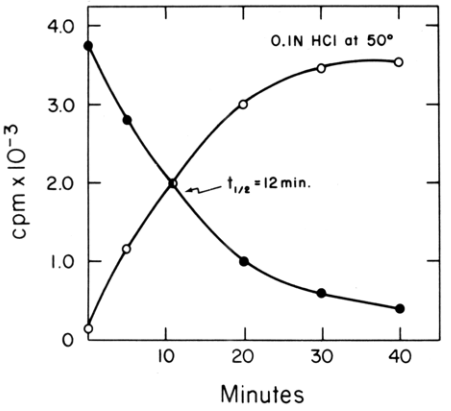


FIGURE 5: Mild acid hydrolysis of the mannosyl lipid. Lipid-[^{14}C]-mannose was dissolved in 1.0 ml of 0.1 N HCl in 50% 1-propanol and the reaction was carried out at 50 °C. At the indicated times 0.1-ml aliquots were mixed with 2 ml of $CHCl_3$–CH_3OH (2:1) and 0.5 ml of 20 mM NaOH. The two phases were separated by centrifugation, and the radioactivity in the organic (●) and aqueous phase (○) was measured.

glycosyl diglycerides (Dawson, 1967), did not release radioactivity into the aqueous phase. However, treatment of the mannosyl lipid with 0.1 M NaOH in 90% ethanol at 85 °C for 45 min resulted in the release of 80% of the radioactivity into the aqueous phase. The water soluble product cochromatographed with mannose 1-phosphate in solvent system C. After treatment with alkaline phosphatase, the water-soluble product was converted to a compound which cochromatographed with mannose in solvent systems A and B, confirming that mannosyl phosphate was the product of alkaline hydrolysis. Treatment of the mannosyl lipid with 0.1 N HCl in 50% 1-propanol at 50 °C resulted in rapid release of the radioactivity into the aqueous phase as shown in Figure 5. The mannosyl lipid has a half-life of 12 min under these hydrolytic conditions, which is similar to that of mannosylphosphorylpolyisoprenol found in other systems (Waechter et al., 1973; Baynes et al., 1973). The water-soluble product was identified as mannose after chromatography in solvent system A.

Chromatography of the Mannosyl Lipid. The mannosyl lipid was retained by a DEAE-cellulose column during elution with $CHCl_3$–CH_3OH (2:1) and CH_3OH but was eluted as a sharp peak with 0.1 M ammonium acetate in 99% methanol. When chromatographed on silica gel thin-layer plates and developed in solvent systems D, E, and F, mannosyl lipids from *Neurospora* and hen oviduct were chromatographically similar but separable from *Saccharomyces cereviseae* mannosylphosphorylpolyisoprenol (Figure 6). These data together with the chemical properties of the mannosyl lipid from *Neurospora* identify it as a mannosylphosphorylpolyisoprenol. While further work is necessary to determine the exact structure of *Neurospora* mannosylphosphorylpolyisoprenol, the results of the chromatography suggest that the chain length of the lipophilic portion of the *Neurospora* lipid may be closer to the C_{90} to C_{105} found in mammalian polyisoprenols (Hemming, 1973), than the C_{75} to C_{90} found in yeast polyisoprenols (Hemming, 1973).

The Role of Mannosyl Lipid in the Glycosylation of Protein. Although the results in Figure 1 are consistent with the hypothesis that the mannosyl lipid is an intermediate in the transfer of mannose from GDP-mannose to protein, the following experiments were conducted to test this hypothesis.

Direct Transfer of Mannose from Lipid to Protein. Exogenous partially purified mannosyl lipid was tested directly as a mannosyl donor for mannosyl protein synthesis. As shown

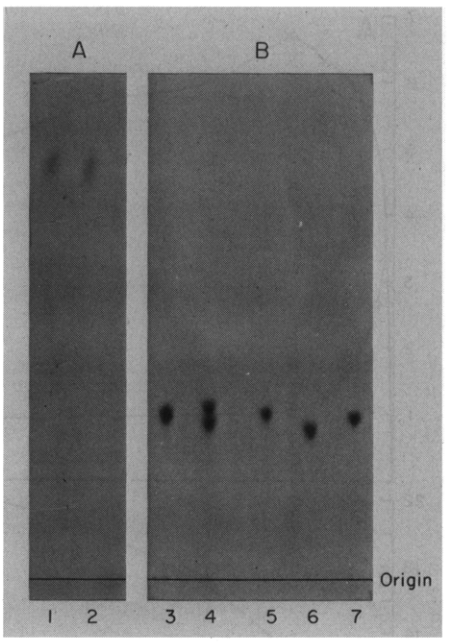


FIGURE 6: Chromatographic characterization of *Neurospora* mannosylphosphorylpolyisoprenol. (A) Chromatography of mannosyl lipids from different sources in solvent system E: mixture of hen oviduct and *Neurospora* (1), *Neurospora* (2). (B) Chromatography of mannosyl lipids from different sources in solvent system D: mixture of hen oviduct and *Neurospora* (3), mixture of yeast and *Neurospora* (4), hen oviduct (5), yeast (6), *Neurospora* (7). Crude yeast mannosylphosphorylpolyisoprenol was prepared by incubating a yeast particulate fraction with GDP-[^{14}C]mannose under the standard conditions. Before use the crude yeast mannosyl lipid was treated with mild alkali for 20 min, neutralized, and partitioned into an organic phase as described in Experimental Procedure.

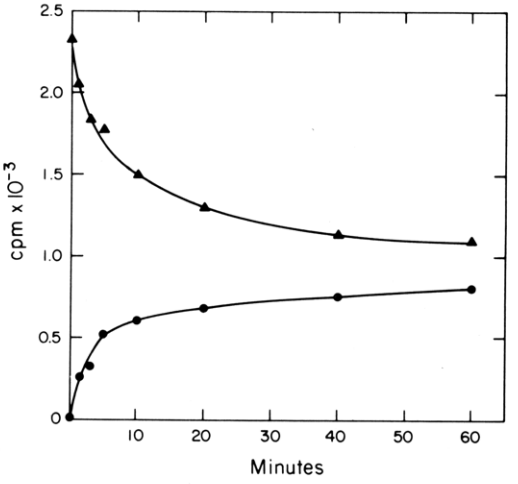


FIGURE 7: Direct transfer of mannose from lipid to protein. Incubation conditions are described in Experimental Procedure. Radioactivity in the mannosyl lipid fraction (▲) and mannosyl protein fraction (●) were separated by method A.

in Figure 7 radioactivity is released from the mannosyl lipid fraction and concomitantly is transferred to the protein fraction. No radioactive product with the solubility properties of dolichol phosphoryl oligosaccharide (Behrens et al., 1973) is formed during the reaction. The transfer of mannose from lipid to protein is dependent on a divalent metal ion. Optimal rates were obtained with 20 mM $MgCl_2$. Less than 10% of the maximal rate was obtained when no divalent metal ion was present in the reaction mixture. Crude mannosyl lipid was as good a substrate for the enzyme as partially purified mannosyl lipid.

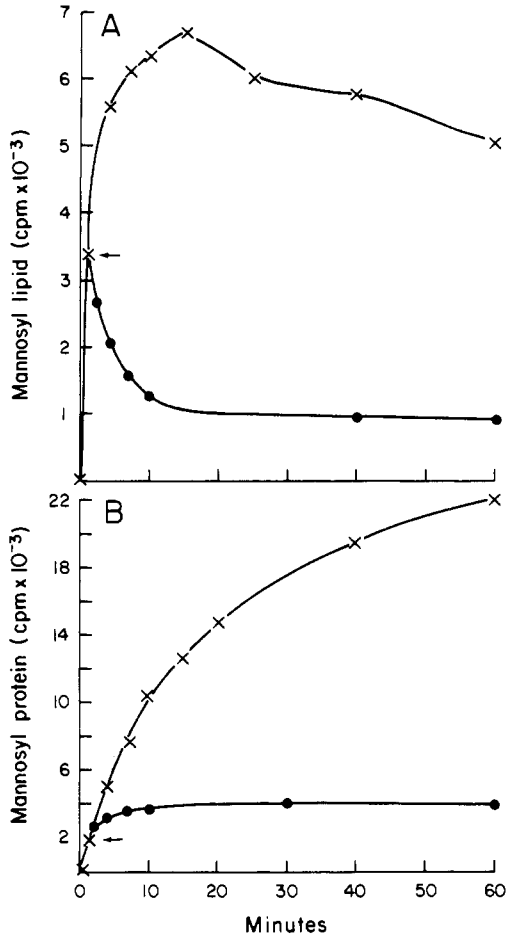


FIGURE 8: Effect of unlabeled GDP-mannose on the synthesis of mannosyl lipid and mannosyl protein from GDP-[^{14}C]mannose. Standard reaction conditions containing 20 mM Mg^{2+} were used except that at 1 min a 100-fold excess of unlabeled GDP-mannose (●) was added to the experimental tubes. An equal volume of buffer (x) was added to the control tubes. (A) Effect of unlabeled GDP-mannose on mannosyl lipid synthesis. (B) Effect of unlabeled GDP-mannose on mannosyl protein synthesis.

Table I: Effect of Triton X-100 Concentration on the Transfer of Mannose from GDP-[^{14}C]mannose to Mannosyl Lipid and Mannosyl Protein and from [^{14}C]Mannosyl Lipid to Mannosyl Protein.[a]

Triton X-100[b] %	Total Radioactivity Transferred (cpm)		
	GDP-Mannose to Mannosyl Lipid	GDP-Mannose to Mannosyl Protein	Mannosyl Lipid to Mannosyl Protein[c]
0	5987	23 659	-
0.08	4389	15 281	-
0.20	2837	10 110	-
0.40	1332	3 760	531
0.80	261	931	1987
1.60	230	635	1910

[a] Standard reaction mixtures minus GDP-mannose or mannosyl lipid were incubated with Triton X-100 on ice for 10 min. Reactions were started by the addition of either GDP-mannose or mannosyl lipid. Reactions were carried out at 22 °C. The reaction GDP-mannose to mannosyl lipid was carried out for 20 min. The other two reactions were carried out for 60 min. The reactions were stopped and prepared for counting by method B. [b] Triton X-100 was present at the final indicated concentrations expressed as percent (v/v). [c] Crude mannosyl lipid was present in the amount of 7000 cpm.

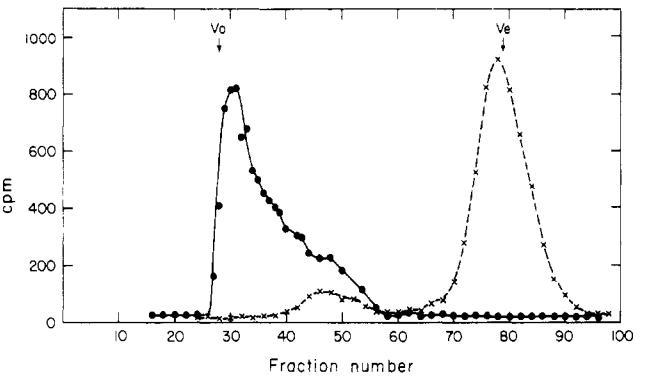


FIGURE 9: Sephadex G-150 profile of protease treated (x) or untreated (●) mannosyl protein. After incubation the proteins were solubilized in 2.0% sodium dodecyl sulfate at 50 °C for 1.5 h and then gel filtered under the conditions described in Experimental Procedure.

Effect of Triton X-100 on the Transferase Reactions. The particulate enzymes were incubated with varying concentrations of Triton X-100 and then assayed for the ability to catalyze the synthesis of mannosyl lipid and mannosyl protein from GDP-mannose, as well as for the ability to form mannosyl protein from mannosyl lipid. The results in Table I indicate that at those concentrations of Triton X-100 where mannosyl protein synthesis from mannosyl lipid is optimal, mannosyl lipid and mannosyl protein synthesis from GDP-mannose are inhibited. This indicates that GDP-mannose is not an intermediate in the transfer of the mannosyl unit from the lipid to the protein.

Effect of Unlabeled GDP-Mannose on Incorporation of Mannose into Lipid and Protein. When a 100-fold excess of unlabeled substrate is added to the reaction mixture prior to the cessation of mannosyl lipid synthesis (1 min), subsequent incorporation of radioactivity into lipid and protein is affected differently. As shown in Figure 8a, the addition of unlabeled GDP-mannose results in an immediate cessation of incorporation and turnover of radioactivity into lipid fraction. In contrast, incorporation of radioactivity into the protein fraction continues, although at a slower rate, following the addition. The amount of radioactivity incorporated into the protein fraction after the addition is approximately 80% of that lost from the lipid fraction. These results show that only mannosyl lipid synthesis is directly sensitive to the additions and that incorporation of mannose into protein from GDP-mannose appears to be dependent upon prior incorporation into lipid. These results, similar to those obtained by others using animal systems (Waechter et al., 1973; Baynes et al., 1973), provide additional evidence that the mannosyl lipid is an intermediate in the transfer of mannose from GDP-mannose to protein.

Characterization of the Glycoprotein(s). After hydrolysis of the labeled glycoprotein with 3 N HCl at 110 °C for 3 h and chromatography in solvent systems A and B, all of the radioactivity cochromatographed with mannose. After solubilization in sodium dodecyl sulfate, gel filtration of the mannosyl protein indicated that most of the radioactivity was eluted close to the exclusion of the column (Figure 9). When the mannosyl protein prepared from GDP-[^{14}C]mannose was digested with pronase and subtilisin prior to gel filtration, more than 90% of the radioactivity eluted in a single peak near the retention volume of the column. Identical gel filtration profiles were obtained when lipid-[^{14}C]mannose was used as a precursor of the mannosyl protein. The low-molecular-weight products of proteolytic digestion were also subjected to paper electrophoresis in formic acid and moved toward the cathode, as ex-

pected for glycopeptides. The untreated glycoprotein, in contrast, remained at the origin.

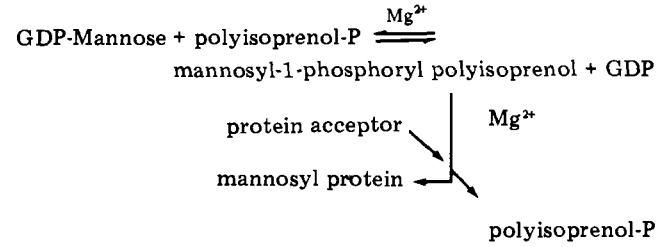
Linkage of Carbohydrate to Protein. If the labeled glycoprotein is treated with 0.1 M NaOH for 24 h at 21 °C and then dialyzed, more than 80% of the radioactivity is recovered on the outside of the dialysis bag. Chromatography of an aliquot of this fraction in solvent system A yielded a single peak which cochromatographed with mannose. β-Elimination–borohydride reduction of the glycoprotein, as described in Experimental Procedures, yielded a product which cochromatographed in solvent system A with mannitol. These results demonstrate that under the conditions described above mannosylation of the protein results in a single mannose unit linked directly to the protein through an *O*-glycosidic bond labile to mild alkaline treatment (Neuberger et al., 1972). No protein-bound mannosyl oligosaccharides appear to be synthesized when Mg^{2+} is the only divalent metal in the reaction mixtures. These results are similar to those reported by Sharma et al. (1974) for the *Saccharomyces cerviseae* mannosyl transferase system. Preliminary results indicate that as in several yeast systems (Sharma et al., 1974; Bretthauer and Tsay, 1974) inclusion of Mn^{2+} ion in the reaction mixtures results in the addition of further mannosyl residues to this simple mannosyl protein core.

Discussion

In this study we have investigated the role of mannosyl lipids in the biosynthesis of glycoprotein(s) in *Neurospora crassa*. Membrane particulate preparations catalyze the transfer of mannose from GDP-mannose to a mannosyl lipid in the presence of Mg^{2+}. The partially purified mannosyl lipid appears radiochemically pure on DEAE-cellulose and on thin-layer chromatography and is chromatographically similar to hen oviduct mannosylphosphorylpolyisoprenol. Alkaline hydrolysis of the mannosyl lipid yielded mannose 1-phosphate, whereas acid hydrolysis yielded mannose. Synthesis of the mannosyl lipid is reversible in the presence of GDP but not in the presence of GMP. All of these findings indicate that the mannosyl lipid is mannosyl-1-phosphorylpolyisoprenol. The exact structure of the polyisoprenol(s) found in *Neurospora crassa* awaits further study.

Kinetic studies of the transfer of mannose to endogenous acceptors showed that mannose was incorporated into two products. The more rapidly formed product was identified as a mannosylphosphorylpolyisoprenol. No evidence suggested the formation of oligosaccharide lipid intermediates. The second product, synthesized at a slower initial rate at low temperatures, was shown by several criteria to be a glycoprotein. After treatment with proteases almost all of the radioactivity associated with this fraction was released to low-molecular-weight glycopeptides as measured by gel filtration. Electrophoresis of these low-molecular-weight components showed they were not neutral sugars or oligosaccharides released by contaminating carbohydrases. β-Elimination of the labeled glycoprotein(s) with and without borohydride released mannitol and mannose, respectively, indicating that only one mannosyl unit was transferred directly to the peptide chain through an *O*-glycosidic bond.

The kinetic studies discussed above are consistent with the hypothesis that the mannosylphosphorylpolyisoprenol is an intermediate in the mannosylation of the glycoprotein. Several experiments provided additional proof for this hypothesis. First, isolated, partially purified mannosylphosphorylpolyisoprenol serves as a substrate for the mannosylation of the protein under selective detergent conditions which preclude the formation of GDP-mannose from the mannosyl lipid. Second, when GDP-[^{14}C]mannose was chased with a large excess of unlabeled substrate, incorporation of radioactivity into protein continued and ultimately represented a significant portion of the radioactivity lost during turnover of mannosyl lipid. All of the results discussed above indicate the following reaction scheme:



In this report we have focused on the characterization and role of mannosyl lipid as an intermediate in the transfer of a single mannose unit to *Neurospora crassa* glycoprotein(s). Preliminary evidence indicates that the addition of Mn^{2+} ion to the reaction mixtures results in the extension of the carbohydrate chain of the glycoprotein with additional mannose units. The mechanism of that reaction in this organism is under investigation. Finally, although mannose (Mahadevan and Tatum, 1965) and protein (Wrathall and Tatum, 1973) are present in *Neurospora crassa* cell wall, it is not known whether the mannose is covalently linked to the protein as in yeast mannan (Lee and Ballou, 1965). Thus, although the mannosyl transferases and the endogenous protein acceptor(s) are particulate, strongly indicating a cell envelope location for the glycoprotein, further study will be necessary to localize the glycoprotein to the membrane and/or the wall of the organism.

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Histidine Ammonia-lyase from Rat Liver. Purification, Properties, and Inhibition by Substrate Analogues†

Larry M. Brand‡ and Alfred E. Harper*

ABSTRACT: Histidine ammonia-lyase (EC 4.3.1.3) from rat liver was purified more than 250-fold to near homogeneity. Electrophoretic determinations indicated a native molecular weight of approximately 200 000. The enzyme has a pH optimum of approximately pH 8.5. The minimum K_m for L-histidine was 0.5 mM at pH 9.0. The Michaelis constant in the physiological pH range was, however, more than 2.0 mM. D-α-hydrazinoimidazolylpropionic acid was found to be a potent competitive inhibitor of liver histidine ammonia-lyase (K_{is} = 75 μM); the L enantiomer of this compound was less effective in this regard. The enzyme was also inhibited competitively by L-histidine hydroxamate (K_{is} = 0.4 mM), and to a lesser extent by L-histidinol, D-histidine, and glycine. Failure of a wide variety of other histidine analogues to inhibit the enzyme substantially indicates high specificity of the active site for L-histidine. No alternate substrates were identified for the enzyme. DL-α-Hydrazinophenylpropionic acid, the α-hydrazino analogue of phenylalanine, was similarly shown to be a very potent competitive inhibitor of a mechanistically similar L-phenylalanine ammonia-lyase purified from *Rhodotorula glutinis*.

The properties of histidine ammonia-lyase from rat liver differ significantly from those of the enzyme from *Pseudomonas fluorescens* which has been studied most extensively to date.

Histidine ammonia-lyase (EC 4.3.1.3), the first enzyme in the major catabolic pathway of histidine metabolism, has been studied from both bacterial and mammalian sources. In mammals, this enzyme is located primarily in liver and epidermis (Zannoni and LaDu, 1963) and has been of some interest since the characterization of a human metabolic disease, histidinemia, which results from the hereditary absence of the enzyme. It is probable that the liver and epidermal enzyme species in mammals are identical in structure since studies of both mice (Kacser et al., 1973) and humans (Zannoni and LaDu, 1963) displaying genetic deficiency of liver histidine ammonia-lyase (histidinemia) have shown the skin enzyme to be lacking as well. In view of the apparent identity of the liver enzyme with the epidermal enzyme, which in humans is assayed clinically as a means of diagnosing histidinemia (LaDu, 1971), and because of the relative difficulty of extracting enzyme sufficient for characterization from epidermal tissue, the molecular and kinetic properties of the mammalian liver enzyme are of particular interest.

To date, the histidine ammonia-lyases of eukaryotes have been studied relatively little; however, the enzyme from bacterial sources has been purified and studied extensively in a number of laboratories (Magasanik et al., 1971; Rechler, 1969; Klee, 1970; Frankfater and Fridovich, 1970). Histidine ammonia-lyase from *Pseudomonas fluorescens* has been shown to exist as a tetramer with a molecular weight of 213 000, composed of apparently identical subunits (Rechler, 1969). The enzyme has been found to contain a strongly electrophilic modified amino acid side chain, referred to as "dehydroalanine" (Givot et al., 1969), but as yet not fully characterized, which participates in the deamination reaction, apparently in a manner analogous to the pyridoxal phosphate cofactor of some other amino acid deaminases, since the enzyme is irreversibly inactivated by hydrazines, borohydride, cyanide, and bisulfite (Smith et al., 1967). Cornell and Villee (1968) have partially purified the enzyme from rat liver and reported it to have an apparent requirement for divalent cations similar to that of the *Pseudomonas* enzyme. The rat liver enzyme has also been shown to have a "dehydroalanine" residue in its active site (Givot and Abeles, 1970). Okamura et al. (1974) have recently reported purification of the rat liver enzyme to homogeneity and determined its molecular weight to be 190 000. It was the purpose of the present investigation to determine some of the molecular and kinetic properties of mammalian histidine ammonia-lyase, particularly with regard to the identification and characterization of inhibitors of this enzyme.

† From the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706. *Received September 2, 1975.* This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, and by Grant No. AM 10748, National Institutes of Health, United States Public Health Service.

‡ Present address: Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154.