Biochem. J. (1987) 248, 523–531 (Printed in Great Britain)

The role of C-4-substituted mannose analogues in protein glycosylation

Effect of the guanosine diphosphate esters of 4-deoxy-4-fluoro-D-mannose and 4-deoxy-D-mannose on lipid-linked oligosaccharide assembly

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The effects of the guanosine diphosphate esters of 4-deoxy-4-fluoro-D-mannose (GDP-4FMan) and 4-deoxy-D-mannose (GDP-4dMan) on reactions of the dolichol pathway in chick-embryo cell microsomal membranes were investigated by studies with chick-embryo cell microsomal membranes *in vitro* and in baby-hamster kidney (BHK) cells *in vivo*. Each nucleotide sugar analogue inhibited lipid-linked oligosaccharide biosynthesis in a concentration-dependent manner. GDP-4FMan blocked *in vitro* the addition of mannose to Dol-*PP*-$(GlcNAc)_2Man$ from GDP-Man (where Dol represents dolichol), but did not interfere with the formation of Dol-*P*-Man, Dol-*P*-Glc and Dol-*PP*-$(GlcNAc)_2$. Although GDP-4FMan and Dol-*P*-4FMan were identified as metabolites of 4FMan in BHK cells labelled with [1-^{14}C]4FMan, GDP-4FMan was a very poor substrate for GDP-Man:Dol-*P* mannosyltransferase and Dol-*P*-4FMan could only be synthesized *in vitro* if the chick-embryo cell membranes were primed with Dol-*P*. It therefore appears that the inhibition of lipid-linked oligosaccharide formation in BHK cells treated with 4FMan [Grier & Rasmussen (1984) J. Biol. Chem. **259**, 1027–1030] is due primarily to a blockage in the formation of Dol-*PP*-$(GlcNAc)_2Man_2$ by GDP-4FMan. In contrast, GDP-4dMan was a substrate for those mannosyltransferases that catalyse the transfer of the first five mannose residues to Dol-*PP*-$(GlcNAc)_2$. In addition, GDP-4dMan was a substrate for GDP-Man:Dol-*P* mannosyltransferase, which catalysed the formation of Dol-*P*-4dMan. As a consequence of this, the formation of Dol-*P*-Man, Dol-*P*-Glc and Dol-*PP*-$(GlcNAc)_2$ may be inhibited through competition for Dol-*P*. In BHK cells treated with 10 mM-4dMan, Dol-*PP*-$(GlcNAc)_2Man_9$ was the major lipid-linked oligosaccharide detected. Nearly normal extents of protein glycosylation were observed, but very little processing to complex oligosaccharides occurred, and the high-mannose structures were smaller than in untreated cells.

INTRODUCTION

Protein glycosylation of asparagine-linked glycoproteins is a complex series of events. The first stage involves the assembly of the tetradecasaccharide precursor $Glc_3Man_9(GlcNAc)_2$ on a dolichol diphosphate carrier (Kornfeld & Kornfeld, 1985). Then, after its transfer to protein, this oligosaccharide is processed to the high-mannose and complex structures characteristic of mature glycoproteins (Kornfeld & Kornfeld, 1985). Previous studies have shown that analogues of glucose and mannose can interfere with protein glycosylation (Schwarz & Datema, 1982*a*). One class of compounds, which includes 2-deoxy-D-glucose, 2-amino-2-deoxy-D-glucose, 2-deoxy-2-fluoro-D-glucose and 2-deoxy-2-fluoro-D-mannose, inhibits the assembly of the dolichol diphosphate precursor oligosaccharide (Schwarz & Datema, 1982*a*). A second class of compounds, which includes bromoconduritol, (*N*-methyl-)1-deoxynojirimycin, castanospermine, swainsonine and 1-deoxymannojirimycin (Schwarz & Datema, 1984; Fuhrmann *et al.*, 1984), inhibits the glycosidases involved in the processing of the protein-linked oligosaccharide. With the exception of 2-amino-2-deoxy-D-glucose compounds of the first class are metabolized to their respective guanosine and uridine nucleoside diphosphate derivatives (Schwarz & Datema, 1982*a*), and it is these metabolites that are the actual inhibitory agents. These sugar analogues all contain modifications at the C-2 position. In recent years several analogues of D-mannose containing C-3 and C-4 substituents have been synthesized (Rasmussen, 1980; Rasmussen *et al.*, 1983; Scensny *et al.*, 1983). Two of these mannose analogues, 3-deoxy-3-fluoro-D-mannose (3FMan) and 4-deoxy-4-fluoro-D-mannose (4FMan), have been shown (Grier & Rasmussen, 1983) to be metabolized in yeast to their

Abbreviations used: 4FMan, 4-deoxy-4-fluoro-D-mannose; 4dMan, 4-deoxy-D-mannose; Dol-*P*, dolichyl monophosphate; Dol-*PP*, dolichyl pyrophosphate; CM, chloroform/methanol (2:1, v/v); CMW, chloroform/methanol/water (10:10:3, by vol.); BHK cells, baby-hamster kidney cells.

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respective 6-phosphates, 1,6-bisphosphates and guanosine diphosphate esters. Furthermore 4FMan was found (Grier & Rasmussen, 1984) to interfere with the glycosylation of viral G-protein in vesicular-stomatitis-virus-infected BHK cells as a consequence of depleted intracellular pools of Dol-*PP*-$(GlcNAc)_2Man_9Glc_3$. To investigate further the inhibitory effects of 4FMan on protein glycosylation we have synthesized the guanosine diphosphate ester of 4FMan and of the related 4dMan and tested their effects on reactions of the dolichol pathway *in vitro*. In addition, further studies in BHK cells on the metabolism of 4FMan and the effects of 4dMan on protein glycosylation are presented.

EXPERIMENTAL

Materials

4FMan and 4dMan were synthesized as described elsewhere (Rasmussen *et al.*, 1983). The 1-phosphates of 4FMan and 4dMan were prepared by the MacDonald (1961) procedure. GDP-4FMan and GDP-4dMan were prepared by the procedure described for the synthesis of GDP-2FMan (McDowell *et al.*, 1985). GDP-[^{14}C]-4FMan (51.1 Ci/mol) and GDP-[^{14}C]4dMan (51.1 Ci/mol) were prepared by incubation of the respective labelled sugar 1-phosphate with a yeast extract as described by Grier & Rasmussen (1982). GDP-[U-^{14}C]-Man (300 Ci/mol) and UDP-[U-^{14}C]Glc (300 Ci/mol) were from Amersham Buchler (Braunschweig, Germany). UDP-*N*-acetyl[U-^{14}C]glucosamine (230 Ci/mol), GDP-[1-^{3}H]mannose (12.6 Ci/mol), D-[2-^{3}H]mannose (24.3 Ci/mol) and D-[6-^{3}H]glucosamine (19.7 Ci/mol) were obtained from New England Nuclear. Dolichyl monophosphate and 2,3-dimercaptopropanol were bought from Sigma Chemie (Munich, Germany).

Preparation of microsomal membrane fractions and synthesis of lipid-linked oligosaccharides in cell-free systems

A crude preparation of microsomal membranes was isolated from chick embryo cells by the procedure of Krag & Robbins (1977) as described by Schwarz & Datema (1982*b*). This preparation, containing 15–20 mg of protein/ml, was either used directly or treated to complete the synthesis of partially assembled lipid-linked oligosaccharides as described previously (McDowell *et al.*, 1985).

Membranes enriched in Dol-*PP*-$(GlcNAc)_2$ were prepared by incubating the membranes with 22 μM-UDP-GlcNAc (Datema & Schwarz, 1978). Cell-free synthesis of lipid-linked oligosaccharides was performed as described by Schwarz & Datema (1982*b*). Where appropriate, ATP (0.14 mM) and 2,3-dimercaptopropanol (5 mM) (Faltynek *et al.*, 1981) were included in the cell-free assays to prevent degradation of sugar nucleotides. Dol-*P* (0.3 mg/ml final concentration) was added as a suspension in 0.2% (v/v) Triton X-100 (0.017% final concentration in the assay mixture).

Separation methods

Thin-layer plates of silica gel G-60 and paper chromatograms (Whatman 3MM paper) were developed in one of the following solvent systems (all solvent ratios are by volume): A, chloroform/methanol/conc. NH_3/water (65:35:4:4); B, ethyl acetate/pyridine/water (8:2:1); C, chloroform/methanol/water (65:25:4); D, propan-1-ol/water (7:3); E, chloroform/methanol/acetic acid/water (25:15:4:2). After development the thin-layer chromatograms were either scanned for radioactivity with a Berthold LB 2842 automatic scanner or the silica gel was scraped off the plates in 1 cm portions for subsequent measurement in a liquid-scintillation counter. Paper chromatograms were cut into 1 cm strips before radioactivity counting.

Columns (1 cm × 150 cm or 1 cm × 105 cm) of Bio-Gel P4 (–400 mesh), eluted with water containing 0.2% NaN_3 or with 0.1 M-pyridine/acetate buffer, pH 5.0, were used for the separation of oligosaccharides derived from lipid-linked oligosaccharides by mild acid hydrolysis as well as those released from Pronase-digested glycoproteins by treatment with endo-*N*-acetylglucosaminidase H.

Ion-exchange chromatography of lipid-linked oligosaccharides was carried out with columns of DEAE cellulose (acetate form) equilibrated with CMW. Monophosphate-containing material was eluted with a solution of 2 mM-ammonium acetate in CMW, and diphosphate-containing material was eluted with 20 mM- or 200 mM-ammonium acetate in CMW.

Other procedures

The mild acid hydrolysis of lipid-linked oligosaccharides and endo-*N*-acetylglucosaminidase H treatment have been described elsewhere (Schwarz & Datema, 1982*a*; Grier & Rasmussen, 1984). Protein was determined by the procedure of Lowry *et al.* (1951), with bovine serum albumin as standard. The chemical and enzymic characterization of sugar phosphate derivatives with the use of acid hydrolysis, alkaline phosphatase, and phosphodiesterase was performed as previously described (Grier & Rasmussen, 1983). BHK 21 cells were cultivated and maintained as before (Grier & Rasmussen, 1984).

RESULTS

Effect of GDP-4FMan and GDP-4dMan on lipid-linked oligosaccharide formation

Chick-embryo cell membranes saturated with Dol-*PP*-$(GlcNAc)_2$ were incubated with GDP-[^{14}C]Man in the presence of GDP-4FMan or GDP-4dMan (0–200 μM). Incorporation of label into the CM and CMW extracts and the lipid-free residue was determined. Labelled mannose incorporation into lipid-linked oligosaccharides soluble in CMW was strongly inhibited (by 90%) by either nucleotide sugar analogue (Fig. 1*b*) as was the incorporation into the residue fraction (Fig. 1*c*). However, the analogues exhibited differing effects on mannose incorporation into the CM extract (Fig. 1*a*). GDP-4dMan inhibited incorporation by 70%, whereas GDP-4FMan had little effect (Fig. 1*a*).

The reasons for these differences became apparent when the CM extracts from the incubations containing 150 μM inhibitor were examined by t.l.c. (solvent system A). In the control incubation (Fig. 2*a*) Dol-*P*-Man, Dol-*PP*-$(GlcNAc)_2Man$, Dol-*PP*-$(GlcNAc)_2Man_2$ and Dol-*PP*-$(GlcNAc)_2Man_3$ (all identified by comparison with previously characterized reference compounds) were detected. In the presence of GDP-4FMan (Fig. 2*b*) a large Dol-*P*-Man peak was present together with Dol-*PP*-$(GlcNAc)_2Man$ and decreased amounts of Dol-*PP*-$(GlcNAc)_2Man_2$ and Dol-*PP*-$(GlcNAc)_2Man_3$. The

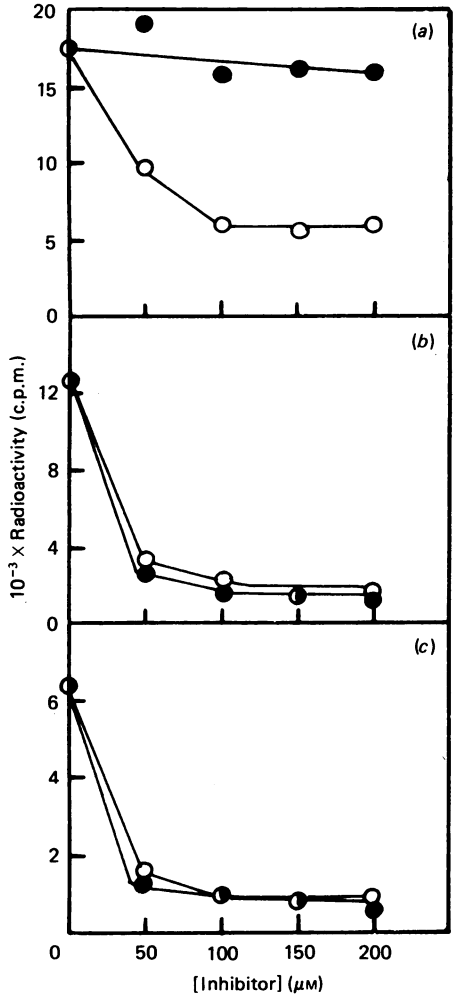

Fig. 1. Effect of GDP-4FMan and GDP-4dMan on protein glycosylation *in vitro*

Microsomal membranes enriched in Dol-*PP*-$(GlcNAc)_2$ were incubated with GDP-[^{14}C]Man (0.05 μCi) for 10 min in the presence of 0–200 μM-GDP-4FMan (●) or -GDP-4dMan (○). The assay mixtures were then extracted with CM and CMW (Schwarz & Datema, 1982*b*), and the incorporation of radioactive label into (*a*) the CM extract, (*b*) the CMW extract and (*c*) the lipid-free residue was determined.

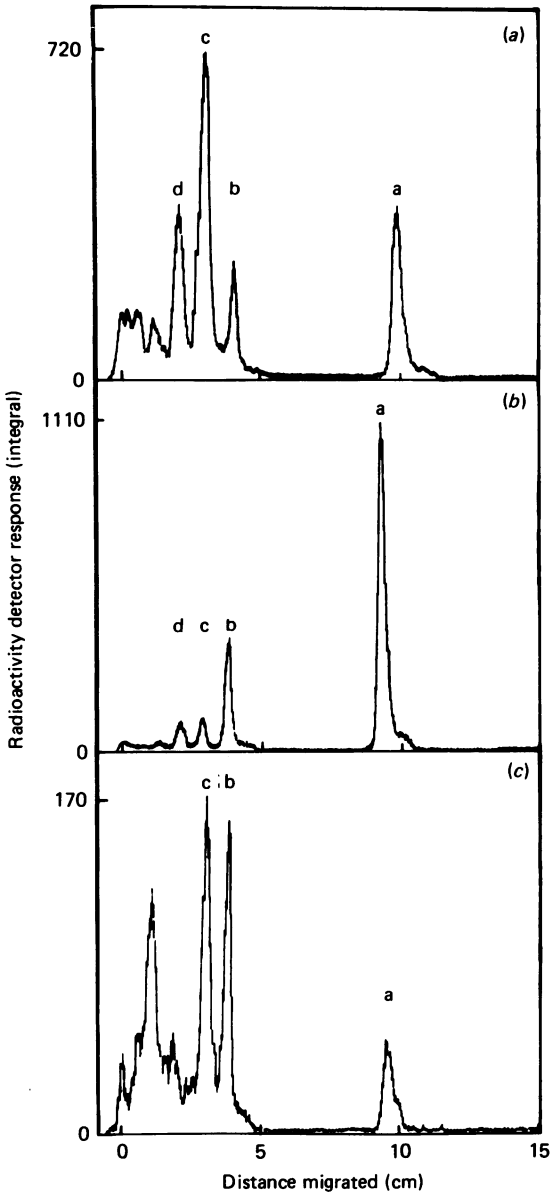

Fig. 2. T.l.c. of CM extracts of dolichol-linked saccharides synthesized in the absence (*a*) or in the presence of (*b*) GDP-4FMan or (*c*) GDP-4dMan

Microsomal membranes enriched in Dol-*PP*-$(GlcNAc)_2$ were incubated with GDP-[^{3}H]Man (0.7 μCi) for 10 min in the absence (*a*) or in the presence of (*b*) 150 μM-GDP-4FMan or (*c*) 150 μM-GDP-4dMan. The CM extracts were prepared and subjected to t.l.c. on silica gel G-60 with solvent A. The peaks marked a–d show the same mobility as reference compounds Dol-*P*-Man, Dol-*PP*-$(GlcNAc)_2$-Man, Dol-*PP*-$(GlcNAc)_2Man_2$ and Dol-*PP*-$(GlcNAc)_2$-Man_3 respectively.

chromatogram of the incubation containing GDP-4dMan (Fig. 2*c*) was quite different. The amount of Dol-*P*-Man and each of the lipid-linked oligosaccharides was decreased. Quantitative determination of the individual peaks on each chromatogram gave the values shown in Table 1. The fact that in the presence of GDP-4FMan the amounts of Dol-*P*-Man and Dol-*PP*-$(GlcNAc)_2Man$ were elevated while those of the other lipid-linked oligosaccharides were much diminished (Table 1) suggests that the transfer of the second mannose residue from GDP-Man is blocked by GDP-4FMan. Dol-*P*-Man accumulates because no Dol-*PP*-$(GlcNAc)_2Man_5$ is available to act as an acceptor of the mannose residues from Dol-*P*-Man. The observation that GDP-4dMan drastically decreases the formation of all lipid species (Table 1), especially Dol-*P*-Man, indicates that it may serve as an alternative substrate.

Effect on Dol-*P(P)*-saccharide formation

The effects of each of the two nucleotide sugar analogues on the formation of Dol-*P*-Man, Dol-*P*-Glc and Dol-*PP*-$(GlcNAc)_2$ were investigated by including GDP-4FMan or GDP-4dMan (0–200 μM) in incubation mixtures capable of synthesizing the dolichol derivatives from their respective ^{14}C-labelled sugar nucleotide donors. GDP-4dMan inhibited the synthesis of all three

Table 1. Inhibition of the formation of lipid-linked oligosaccharides soluble in CM

The areas under each peak in Fig. 2 were integrated by using software included with the Berthold LB 2842 thin-layer-chromatogram scanning system. Percentage values are expressed relative to the control.

	Peak area in control		Peak area with GDP-4FMan		Peak area with GDP-4dMan	
Dolichol derivative	(integral)	(%)	(integral)	(%)	(integral)	(%)
Dol-*P*-Man	6529	100	14046	215	810	12
Dol-*PP*-$(GlcNAc)_2Man$	3819	100	4999	131	2118	56
$(GlcNAc)_2Man_2$	13086	100	1363	10	2712	21
$(GlcNAc)_2Man_3$	6348	100	1627	26	919	15

Table 2. Inhibition of lipid-linked monosaccharide and disaccharide synthesis by GDP-4dMan and GDP-4FMan

Microsomal membranes were incubated with 0.05 μCi of GDP-[^{14}C]Man, UDP-[^{14}C]Glc or UDP-[^{14}C]GlcNAc and the lipid-linked sugars were extracted with CM (Schwarz & Datema, 1982*b*). IC_{50} is the concentration giving 50% inhibition of incorporation of the radiolabelled sugar into product.

	IC_{50} (μM)	
Product synthesized	GDP-4dMan	GDP-4FMan
Dol-*P*-Man	42	> 200
Dol-*P*-Glc	25	> 200
Dol-*PP*-$(GlcNAc)_2$	63	> 200

dolichol saccharides, whereas GDP-4FMan was ineffective up to 200 μM (Table 2). Addition of exogenous Dol-*P* relieved the inhibitory effects of GDP-4dMan, indicating that there is competition between inhibitor and physiological substrate for Dol-*P*. This result suggests that 4dMan can form a dolichol derivative.

Formation of Dol-*P*(*P*) derivatives of 4dMan and 4FMan

To look for the formation of dolichol derivatives containing either 4dMan or 4FMan the respective ^{14}C-labelled GDP-sugars (0.05 μCi) were incubated with chick-embryo cell membranes and the incorporation of label into the CM and CMW extracts was measured. The GDP-[^{14}C]4dMan reaction mixture gave rise to radioactive material that was soluble in CM. When examined by t.l.c. on silica gel G-60 with solvent A, this material migrated as a single peak with a mobility greater than that of Dol-*P*-Man (Fig. 3*a*). Addition of exogenous Dol-*P* stimulated incorporation 5-fold. It is characteristic of deoxy sugars to have chromatographic mobilities greater than their parent compounds in this solvent system (McDowell *et al.*, 1985), and thus it can be inferred that the peak in the chromatogram is Dol-*P*-4dMan. When the membranes were preincubated with UDP-GlcNAc to form a pool of Dol-*PP*-$(GlcNAc)_2$, a second compound, with the mobility of a lipid-linked trisaccharide, was also present on the chromatogram (Fig. 3*b*). This slower-moving compound is presumably Dol-*PP*-$(GlcNAc)_2$4dMan, indicating that 4dMan can at least replace the first mannose residue in lipid-linked oligosaccharides.

Under standard reaction conditions GDP-[^{14}C]-4FMan gave no incorporation of label into either of the two lipid extracts. Incorporation of label was only observed if the membranes were first pretreated to complete partially synthesized lipid-linked oligosaccharides and then recovered by ultracentrifugation. It was also necessary to include 2,3-dimercaptopropanol + ATP in the incubation mixture to inhibit endogenous nucleotidases. Under these conditions incorporation was very low and a single radioactive peak with a mobility greater than that of Dol-*P*-Man was just discernible on t.l.c. (Fig. 3*c*). However, addition of exogenous Dol-*P* stimulated incorporation, producing a more obvious peak on t.l.c. (Fig. 3*d*). This peak is presumably Dol-*P*-4FMan. However, it was not possible to characterize it further owing to the unavailability of amounts sufficient for analysis. Preincubation of the membranes with UDP-GlcNAc followed by reaction with GDP-[^{14}C]4FMan produced no radioactive material soluble in either extract.

The formation of lipid-linked oligosaccharides containing 4dMan was further demonstrated by first incubating membranes with UDP-[^{14}C]GlcNAc to form a pool of Dol-*PP*-([^{14}C]$GlcNAc)_2$ (Fig. 4*a*). Addition of GDP-Man resulted in the formation of large lipid-linked oligosaccharides (Fig. 4*b*), which were also observed when GDP-Man and GDP-4dMan were present together (Fig. 4*c*). When GDP-4dMan was used alone, however, an oligosaccharide with the properties of $Man_5(GlcNAc)_2$ was formed (Fig. 4*d*). Thus it appears that 4dMan residues can replace each of the first five mannose residues that are derived directly from GDP-Man. It would also appear that 4dMan cannot be transferred further from Dol-*P*-4dMan. As a result trapping of Dol-*P* may occur. GDP-4FMan did not act as a substrate for the elongation of Dol-*PP*-$(GlcNAc)_2$ (Figs. 4*e* and 4*f*). The main peak in Fig. 4(*e*) is $Man(GlcNAc)_2$, which accumulates in the presence of GDP-4FMan, as also shown in Fig. 2(*b*).

Effect of 4dMan on protein glycosylation in BHK cells

To examine the effects of 4dMan on protein glycosylation *in vivo* BHK cells were incubated for 240 min with or without 10 mM-4dMan and then labelled with [2-^{3}H]-mannose for 30 min. The cells were extracted with CM and CMW and the oligosaccharides released by mild

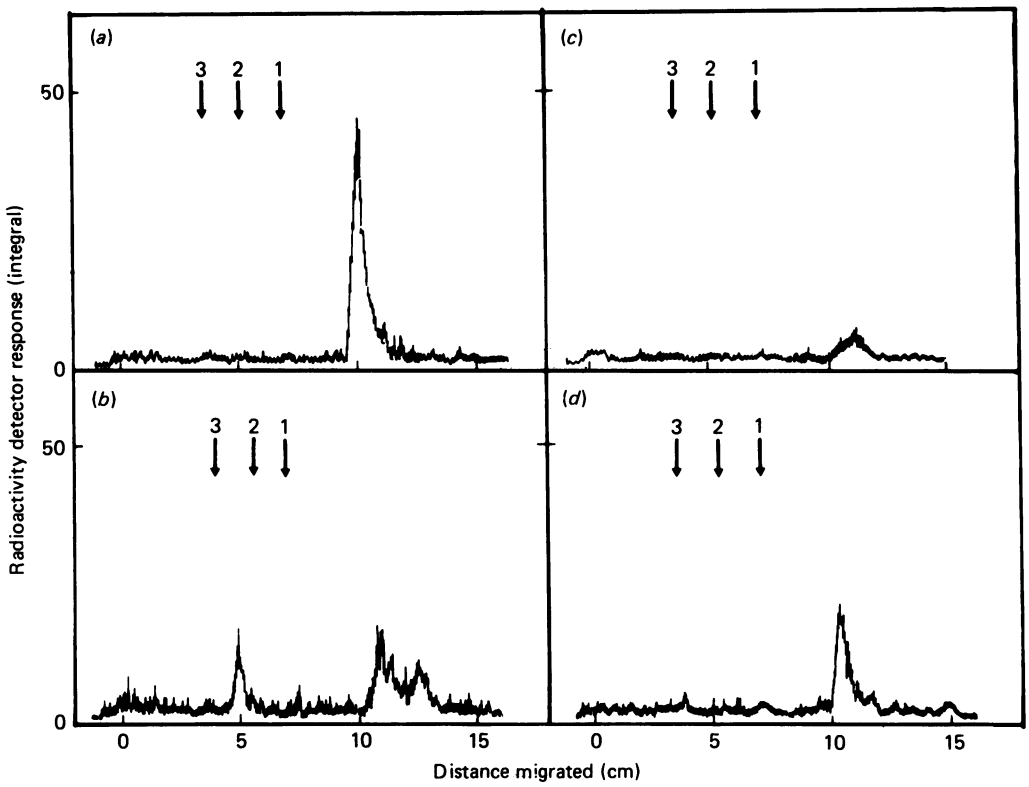

Fig. 3. Formation of dolichol derivatives of 4dMan and 4FMan

Microsomal membranes were incubated with (*a*) GDP-[^{14}C]4dMan (0.05 μCi), for 30 min at 37 °C, (*b*) UDP-GlcNAc to form a pool of Dol-*PP*-$(GlcNAc)_2$ followed by GDP-[^{14}C]4dMan for 20 min at 37 °C, (*c*) GDP-[^{14}C]4FMan (0.05 μCi) for 30 min at 37 °C, or (*d*) GDP-[^{14}C]4FMan + Dol-*P* (0.3 mg/ml) for 30 min at 37 °C. (*c*) and (*d*) also contained ATP (0.14 mM) and 2,3-dimercaptopropanol (5 mM). The CM extracts were isolated and then analysed by t.l.c. on silica gel G-60 with solvent A. The arrows indicate the positions of 1, Dol-*P*-Man, 2, Dol-*PP*-$(GlcNAc)_2$, and 3, Dol-*PP*-$(GlcNAc)_2Man$.

acid hydrolysis were analysed by Bio-Gel P4 chromatography (Figs. 5*a* and 5*b*). In the sample from the untreated cells the major oligosaccharide present was $Glc_3Man_9(GlcNAc)_2$ (Fig. 5*a*). Only a minor amount of $Glc_3Man_9(GlcNAc)_2$ oligosaccharide was observed in the 4dMan-treated sample (Fig. 5*b*). The major peak was eluted at a position corresponding to $Man_9(GlcNAc)_2$ (Fig. 5*b*).

The lipid-free residue of cellular proteins from the above extraction was digested with Pronase, then desalted on Bio-Gel P2, and the glycopeptides were treated with endo-*N*-acetylglucosaminidase H. The endo-*N*-acetylglucosaminidase H-treated Pronase digests were then analysed by Bio-Gel P4 chromatography (Figs. 5*c* and 5*d*). The profile for the sample from the control cells (Fig. 5*c*) was almost identical with that reported by Hubbard & Robbins (1979) for a similar experiment in chick-embryo cells with the two peaks being eluted as $GlcMan_9GlcNAc$ and $Man_9GlcNAc$ respectively. The sample from the 4dMan-treated cells contained less endo-*N*-acetylglucosaminidase H-resistant material and smaller oligosaccharide structures (Fig. 5*d*) than the control, with the two main peaks being eluted as $Man_9GlcNAc$ and $Man_7GlcNAc$ respectively. When the results obtained *in vitro* with GDP-4dMan (in Figs. 3*b* and 4*b*), showing that 4dMan residues can be incorporated into lipid-linked oligosaccharides at an early stage in the biosynthetic pathway, are taken into account, it would seem more appropriate to consider the compositions of the main peaks in Figs. 5(*b*) and 5(*d*) to be $(Man+4dMan)_9(GlcNAc)_2$ (Fig. 5*b*) and $(Man+4dMan)_9GlcNAc$ and $(Man+4dMan)_7GlcNAc$ (Fig. 5*d*).

Metabolism of 4FMan by BHK cells

To correlate the data obtained *in vitro* and *in vivo* for the mannose analogues it was desirable to investigate their metabolism in BHK cells. Such an investigation with yeast cells (Grier & Rasmussen, 1983) showed that 4FMan was metabolized to its 6-phosphate and GDP derivatives. BHK cells were labelled with [1-^{14}C]4FMan (43 μCi) for 240 min and then extracted to give aqueous, CM and CMW fractions. Analysis of the aqueous fraction (extracted with 4 mM-$MgCl_2$) by paper chromatography (Fig. 6), and chemical and enzymic reactivities (Table 3) indicated that 4FMan was metabolized to 4FMan-6-*P* and GDP-4FMan. Analysis of the CM fraction by t.l.c. (Figs. 7*a*–7*c*) indicated the presence of a radiolabelled product with the chromatographic properties expected for Dol-*P*-4FMan. This product was eluted from the silica and further characterized by DEAE-cellulose ion-exchange chromatography. Most of the label (75%) could be eluted from the column with 2 mM-ammonium acetate in CMW, indicating the presence of a monophosphate derivative. Acid hydrolysis followed by paper-chromatographic analysis confirmed that 4FMan was the sugar moiety (Fig. 7*d*). Thus Dol-*P*-[1-^{14}C]4FMan is indeed a metabolite of [1-^{14}C]4FMan. However, a similar labelling experiment performed with

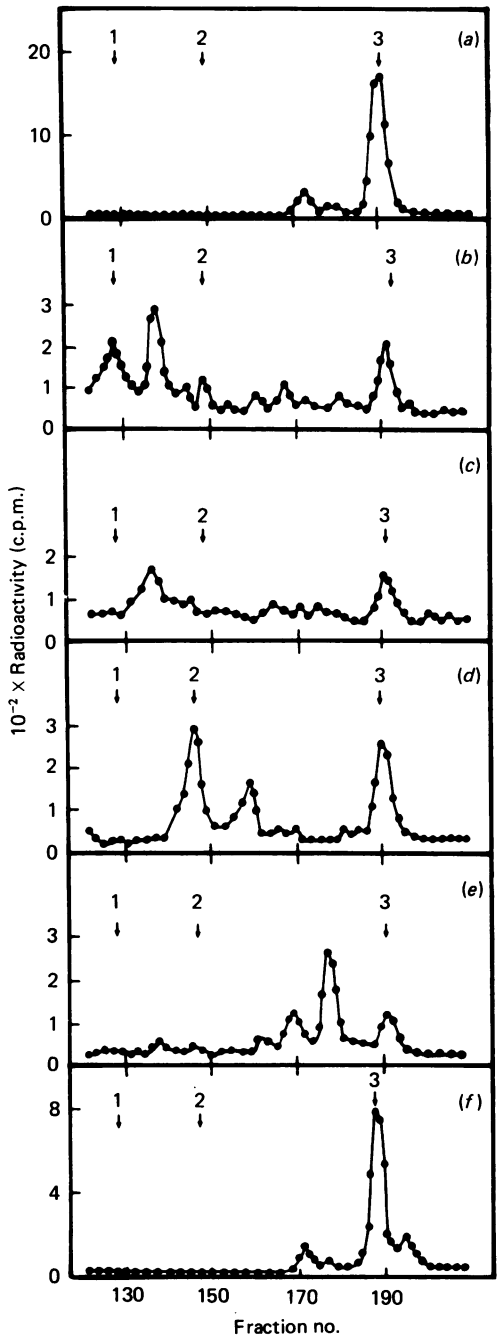

Fig. 4. Bio-Gel P4 chromatography of oligosaccharides labelled with UDP-[^{14}C]GlcNAc in a cell-free system in the absence or in the presence of GDP-Man or GDP-4dMan or GDP-4FMan

Chick-embryo cell membranes were incubated with UDP-[^{14}C]GlcNAc (0.1 μCi) for 20 min and then incubated for a further 20 min with (*a*) no additions, (*b*) GDP-Man (8.3 μM), (*c*) GDP-Man + GDP-4dMan (150 μM), (*d*) GDP-4dMan alone, (*e*) GDP-Man + GDP-4FMan (150 μM), or (*f*) GDP-4FMan alone. The reactions were terminated with CM, and the lipid-linked oligosaccharides present in the CM and CMW extracts were combined and the oligosaccharides released by mild acid hydrolysis were analysed on a column (1 cm × 150 cm) of Bio-Gel P4. Arrows 1, 2 and 3 indicate the elution positions of $Glc_3Man_9(GlcNAc)_2$, $Man_5(GlcNAc)_2$ and $(GlcNAc)_2$ respectively. The void volume of the columns was at fraction 81.

[1-^{14}C]4dMan did not result in the accumulation of measurable amounts of metabolites. This is not surprising in view of the low efficiency with which 4-deoxy sugars are phosphorylated by hexokinase (Finch & Merchant, 1979).

DISCUSSION

Previous studies (Schwarz & Datema, 1982*a*; McDowell *et al.*, 1985) have shown that fluoro sugars can inhibit protein glycosylation by interfering with the assembly of the lipid-linked tetradecasaccharide precursor intermediate. In the case of 2dGlc and 2FMan the guanosine diphosphate esters are the actual inhibitory agents (Datema & Schwarz, 1978; McDowell *et al.*, 1985). In the present study the inhibitory effects of 4FMan towards protein glycosylation (Grier & Rasmussen, 1984) can also be attributed to the guanosine diphosphate ester. Specifically, GDP-4FMan was produced by BHK cells and found *in vitro* to inhibit the transfer of mannose from GDP-Man to Dol-*PP*-$(GlcNAc)_2$Man. Interestingly, GDP-4FMan had no observable effect *in vitro* on the synthesis of Dol-*P*-Man in spite of the fact that Dol-*P*-4FMan was identified as a metabolite of 4FMan in BHK cells treated with the labelled sugar analogue. In contrast, Dol-*P*-Man formation is inhibited by GDP-2FMan owing to the formation of a Dol-*P*-sugar analogue derivative (McDowell *et al.*, 1985). One explanation for this difference is that GDP-4FMan is a poor substrate for GDP-Man:Dol-*P* mannosyltransferase. Thus it was extremely difficult to detect Dol-*P*-4FMan formation from labelled GDP-4FMan *in vitro*. The microsomal membranes had to be primed with Dol-*P* and the reaction was carried out in the presence of 2,3-dimercaptopropanol + ATP to inhibit endogenous phosphatases present in the membrane preparation (Faltynek *et al.*, 1981).

Unlike the 4-fluoro analogue, treatment of BHK cells with 10 mM-4dMan did not significantly decrease the extent of glycosylation of newly synthesized proteins. Subsequent processing of the oligosaccharides appeared to be abnormal, however. Decreased amounts of endo-*N*-acetylglucosaminidase H-resistant chains and smaller high-mannose structures were present. The major lipid-linked oligosaccharide in BHK cells treated with 10 mM-4dMan was Dol-*PP*-$(GlcNAc)_2Man_9$. Only a trace amount of Dol-*PP*-$(GlcNAc)_2Man_9Glc_3$ was detected. Examination of the effects *in vitro* of GDP-4dMan on lipid-linked oligosaccharide biosynthesis provided a partial explanation of these observations.

Dol-*P*-4dMan was readily formed *in vitro* when labelled GDP-4dMan was incubated with the microsomal membrane preparation. Likewise, when GDP-4dMan alone was incubated with membranes saturated with Dol-*PP*-$(GlcNAc)_2$, 4dMan was incorporated into the first five mannose positions of the lipid-linked oligosaccharide (presumably those derived directly from GDP-Man). Dol-*P*-4dMan is unlikely to be a donor of 4dMan residues because the formation of lipid-linked oligosaccharides larger than the heptasaccharide was not observed (Fig. 4*d*). In agreement with the observation that Dol-*PP*-$(GlcNAc)_2Man_9$ is formed in 4dMan-treated cells, large lipid-linked oligosaccharides were formed *in vitro* in the presence of GDP-Man and GDP-4dMan together (Fig. 4*c*). It might be expected that *in vivo* the formation of Dol-*P*-4dMan could lead to a trapping of

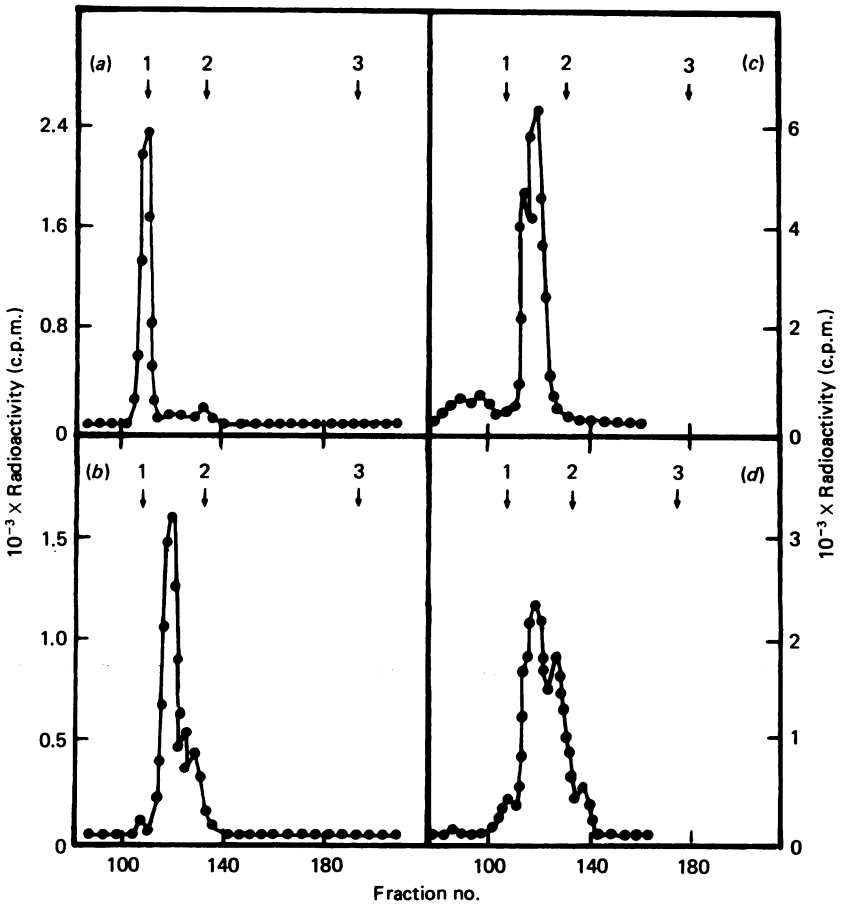

Fig. 5. Bio-Gel P4 chromatography of oligosaccharides derived from the CMW extracts and Pronase-digested glycoproteins of BHK cells labelled with [2-^{3}H]mannose in the absence and in the presence of 4dMan

BHK 21 cells untreated (*a* and *c*) or treated (*b* and *d*) with 10 mM-4dMan for 240 min were labelled with [2-^{3}H]mannose (700 μCi/plate) for 30 min. The cells were extracted with CM and CMW. The lipid-linked oligosaccharides present in the CMW extract were released by mild acid hydrolysis and then analysed by gel filtration on a column (1 cm × 105 cm) of Bio-Gel P4. (*a*) CMW extract from untreated cells. (*b*) CMW extract from treated cells. The lipid-free residue from the above cells was digested with Pronase, desalted by passage through Bio-Gel P2 and then treated with endo-*N*-acetylglucosaminidase H. The released high-mannose, oligosaccharides and complex glycopeptides were then analysed on a column of Bio-Gel P4. (*c*) Untreated cells. (*d*) 4dMan-treated cells. The arrows 1, 2 and 3 indicate the elution positions of $Glc_3Man_9(GlcNAc)_2$, $Man_5(GlcNAc)_2$ and [^{14}C]mannose respectively. The column void volume was at fraction 76.

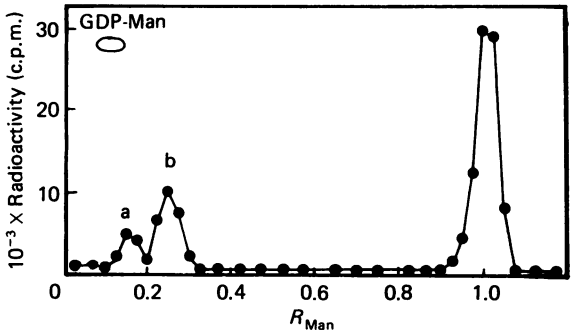

Fig. 6. Paper-chromatographic analysis of the aqueous extract after labelling of BHK cells with [1-^{14}C]4FMan

BHK cells were labelled with [1-^{14}C]4FMan (43 μCi) for 240 min and then extracted to give aqueous, CM and CMW fractions (Grier & Rasmussen, 1984). The aqueous (4 mM-$MgCl_2$) fraction was subjected to paper chromatography with solvent B. The radioactivity in 1 cm strips was measured by liquid-scintillation counting.

Table 3. Characterization of [1-^{14}C]4FMan metabolites

Peaks a and b (Fig. 6) were eluted with water from the chromatogram of [1-^{14}C]4FMan metabolites and treated with acid, alkaline phosphatase (AP) or phosphodiesterase and alkaline phosphatase together (AP+PDE) (Grier & Rasmussen, 1983). 4FMan was identified as the released product in each case after paper-chromatographic analysis with solvent B. + signifies that the sample was susceptible to the treatment used, and − signifies that the sample was resistant to the treatment used. Abbreviation: N.D., not determined.

Peak	Susceptibility to treatment			Designation
	Acid	AP	AP+PDE	
a	+	−	+	GDP-4FMan
b	−	+	N.D.	4FMan-6-*P*

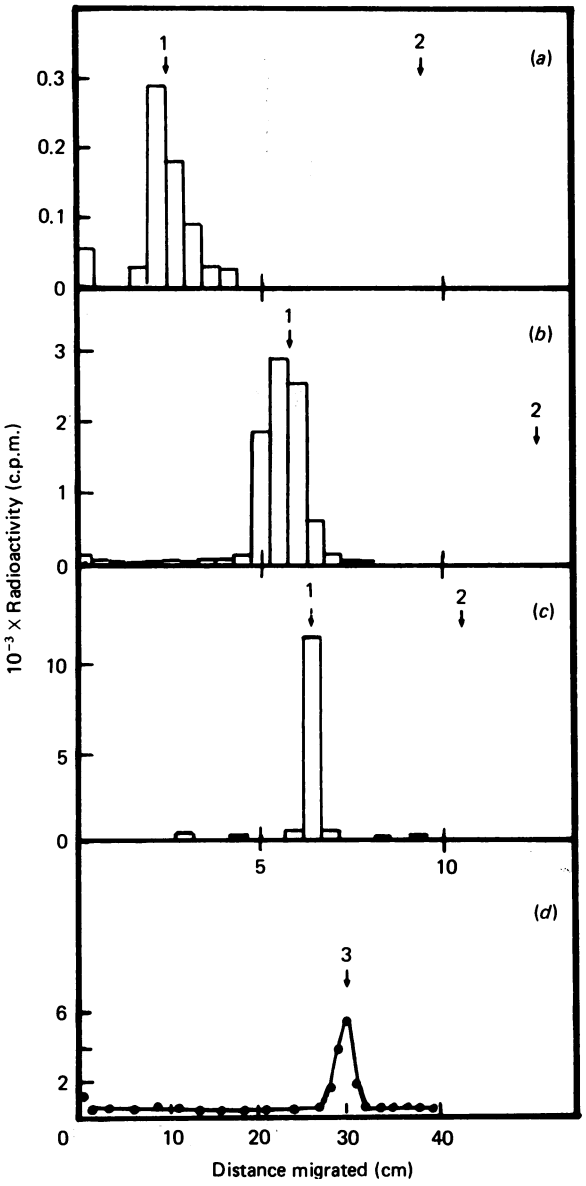

Fig. 7. Analysis of the CM fraction after labelling BHK cells with [1-^{14}C]4FMan

BHK 21 cells were labelled and extracted with CM as described in the legend to Fig. 6. The CM extract was analysed by t.l.c. on silica gel G-60 with (*a*) solvent C, (*b*) solvent D, or (*c*) solvent E, and the radioactivity in 1 cm portions of each plate was measured by liquid-scintillation counting. The labelled material was eluted from the silica gel and adsorbed on DEAE-cellulose (acetate form) equilibrated with CMW. The label eluted with 2 mM-ammonium acetate in CMW was hydrolysed with acid under mild conditions and then subjected to paper chromatography with solvent B (*d*). The arrows indicate the positions of 1, Dol-*P*-Man, 2, solvent front, and 3, 4FMan.

Dol-*P* and a subsequent inhibition of lipid-linked oligosaccharide biosynthesis, as has been proposed for other sugar analogues (Schwarz & Datema, 1982*a*; McDowell *et al.*, 1985, 1986). The observations that *in vivo* most glycoproteins synthesized in the presence of 4dMan are glycosylated and that a fully mannosylated dolichol intermediate is synthesized suggest that the trapping, if it is occurring, is only partial. The greatly diminished amounts of the fully glucosylated lipid-linked oligosaccharide indicate that Dol-*P*-Glc synthesis may be selectively impaired by 4dMan. *In vitro* it was found that GDP-4dMan more effectively inhibited Dol-*P*-Glc synthesis than it did that of Dol-*P*-Man (Table 2). Taken together, the results obtained *in vitro* suggest that Dol-*P*-Glc synthesis is inhibited in 4dMan-treated cells, perhaps through a decrease in Dol-*P* availability because of Dol-*P*-4dMan formation. As a result, the non-glucosylated intermediate Dol-*PP*-$(GlcNAc)_2Man_9$ is the predominant lipid-linked oligosaccharide synthesized *in vivo*.

It is not clear whether, in spite of the low steady-state amount of Dol-*PP*-$(GlcNAc)_2Man_9Glc_3$, a sufficient amount of this intermediate is synthesized to glycosylate newly synthesized proteins. If so, then the inhibition of normal processing is most probably a consequence of incorporation of 4dMan residues into the oligosaccharides. An alternative explanation is that $Man_9(GlcNAc)_2$ is transferred directly to the protein (Romero & Herscovics, 1986) and processed abnormally, either because of interference from incorporated 4dMan residues or because of impaired transport from the endoplasmic reticulum to the Golgi apparatus. It is also possible that 4dMan itself may be an inhibitor of the processing mannosidases. However, this remains to be tested.

It is interesting that two mannose analogues containing modifications at position C-4 can have such differing effects. Presumably the higher electronegativity of the fluorine atom in GDP-4FMan is responsible for its inhibitory effects, since GDP-4dMan is an alternative substrate. In a study of the glycosyltransferases involved in the biosynthesis of *Salmonella* O-specific polysaccharides (Shibaev, 1978) it was found for GDP-Man that the 2-, 3- and 6-hydroxy groups of the D-mannosyl group do not participate in the interaction with the *Salmonella* mannosyltransferase. The effects of modification of the C-4 hydroxy group were not investigated with the *Salmonella* mannosyltransferase, but GDP-Man analogues containing 3-deoxy or 6-deoxy substituents are good substrates of the GDP-Man:Dol-*P* mannosyltransferase in chick-embryo cells (W. McDowell & R. T. Schwarz, unpublished work), indicating similarities between bacterial and avian mannosyltransferases. Thus it is possible that the C-4 position of mannose is an important determinant for enzyme–substrate recognition in mannosyltransferases.

The technical assistance of Kirstin Schallner and Ulrike Klein is gratefully acknowledged. Dr. R. Rott and Dr. C. Scholtissek are thanked for their encouragement. This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 47), Fonds der Chemischen Industrie and the National Science Research Foundation (Grant PCM 8208288). During part of this work R. T. S. was a recipient of a Heisenberg Fellowship of the Deutsche Forschungsgemeinschaft. R. T. S. also thanks Dr. J. Montreuil for his encouragement of this co-operation.

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Received 30 March 1987/12 June 1987; accepted 10 August 1987