Means, G. E., & Feeney, R. E. (1971) in *Chemical Modification of Proteins*, p 12, Holden-Day, San Francisco, CA.
Parkes, J. H., Liebman, P. H., & Pugh, E. N. (1979) *Invest. Ophthalmol. Visual Sci. 22*, ARVO Abstr.
Shinozawa, T., Sen, I., Wheeler, G., & Bitensky, M. (1979) *J. Supramol. Struct. 10*, 185–190.
Sternson, L. A., Stobaugh, J. F., & Repta, A. J. (1985) *Anal. Biochem. 144*, 233–246.
Stoeckenius, W., Lozier, R. H., & Bogomolni, R. A. (1979) *Biochim. Biophys. Acta 505*, 215–278.
Trepman, E., & Chen, R. F. (1980) *Arch. Biochem. Biophys. 204*, 524–532.
Wald, G. (1968) *Science (Washington, D.C.) 162*, 230–239.
Wong, C. G., & Rando, R. R. (1984) *Biochemistry 23*, 20–27.
Wong, W. S. D., Osuga, D. T., & Feeney, R. E. (1984) *Anal. Biochem. 139*, 58–67.

Mechanism of Inhibition of Protein Glycosylation by the Antiviral Sugar Analogue 2-Deoxy-2-fluoro-D-mannose: Inhibition of Synthesis of Man(GlcNAc)$_2$-PP-Dol by the Guanosine Diphosphate Ester†

William McDowell, Roelf Datema,* Pedro A. Romero, and Ralph T. Schwarz
Institut für Virologie, Justus Liebig-Universität, Giessen, D-6300 Giessen, FRG
Received June 11, 1985

ABSTRACT: 2-Deoxy-2-fluoro-D-mannose (2FMan), an antiviral mannose analogue, inhibited the dolichol cycle of protein glycosylation. To specifically inhibit oligosaccharide–lipid synthesis, and not (viral) protein synthesis in influenza virus infected cells, the addition of guanosine to the 2FMan-treated cells was required. Under these conditions an early step in the assembly of the oligosaccharide–lipid was inhibited, and as a consequence, the glycosylation of proteins was strongly inhibited. Low-molecular-weight, lipid-linked oligosaccharides accumulated in cells treated with 2FMan *plus* guanosine, although dolichol phosphate (Dol-P) and GDP-Man were still present in the treated cells, and membranes from these cells were not defective in assembly of lipid-linked oligosaccharides. Thus, the presence of a soluble inhibitor of oligosaccharide–lipid assembly in these cells was postulated, and GDP-2FMan and UDP-2FMan, two metabolites found in 2FMan-treated cells, were synthesized and used to study in cell-free systems the inhibition of oligosaccharide–lipid assembly. GDP-2FMan inhibited the synthesis of Man(GlcNAc)$_2$-PP-Dol from (GlcNAc)$_2$-PP-Dol and GDP-Man, and in addition, it caused a trapping of Dol-P as 2FMan-P-Dol, whereas UDP-2FMan only inhibited Glc-P-Dol synthesis. However, it is probable that neither trapping of Dol-P nor inhibition of Glc-P-Dol synthesis by UDP-2FMan contributed to inhibition of protein glycosylation in cells treated with 2FMan. Incorporation of 2FMan from GDP-2FMan or UDP-2FMan into dolichol diphosphate linked oligosaccharides and interference of GDP-2FMan with the latter steps of assembly of the dolichol diphosphate linked oligosaccharide could not be shown. It is concluded that 2FMan, via GDP-2FMan, inhibits protein glycosylation by blocking formation of Man(GlcNAc)$_2$-PP-Dol and, thus, further assembly of the oligosaccharide–lipid.

The mechanism of protein glycosylation and the biological roles of glycosylation of glycoproteins have received widespread attention (Hubbard & Ivatt, 1981; Kornfeld, 1982; Schwarz & Datema, 1982a). Thus, it has been firmly established that the biosynthesis of the asparagine-linked oligosaccharides of glycoproteins occurs in two discrete stages: (1) the assembly of the tetradecasaccharide Glc$_3$Man$_9$(GlcNAc)$_2$ on Dol-PP[1] (see Figure 1) and, following transfer to protein, (2) the processing of the protein-linked oligosaccharide to the complex-type and nonglucosylated, high-mannose-type oligosaccharides.

Several analogues of mannose and glucose interfere with glycosylation of proteins. They can be inhibitors of the assembly of the dolichol diphosphate linked oligosaccharide (2-deoxy-D-glucose, 2-deoxy-2-amino-D-glucose, 2-deoxy-2-fluoro-D-glucose, or 4-deoxy-4-fluoro-D-mannose; Schwarz & Datema, 1982a; Grier & Rasmussen, 1984), interfere with the processing of protein-linked oligosaccharides (bromoconduritol, *N*-methyl-1-deoxnojirimycin; Schwarz & Datema, 1982a; Elbein, 1984), or interfere with both stages (nojirimycin, 1-deoxynojirimycin; Datema et al., 1984).

The mannose analogue 2-deoxy-2-fluoro-D-mannose (2FMan) shows antiviral effects, probably because of inhibiting the glycosylation of viral glycoproteins (Schmidt et al., 1976). In chick embryo and yeast cells 2FMan is converted to both GDP-2FMan and UDP-2FMan (Schmidt et al., 1978). In

† This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 47, and a Heisenberg Stipendium to R.T.S.) and grants from the Stiftung Volkswagenwerk, the Fond der Chemie, and the Justus Liebig-Universität, Giessen. P.A.R. was supported by the Alexander von Humboldt-Stiftung and Zentrum für internationelle Migration und Entwicklung.
* Correspondence should be addressed to this author at the Department of Antiviral Chemotherapy, Research & Development Laboratories, Astra Läkemedel AB, S-151 85 Södertälje, Sweden.

[1] Abbreviations: Dol-P, dolichol phosphate; Dol-PP, dolichol diphosphate; EDTA, ethylenediaminetetraacetic acid; 2FMan, 2-deoxy-2-fluoro-D-mannose; Glc, glucose; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/85/0424-8145$01.50/0

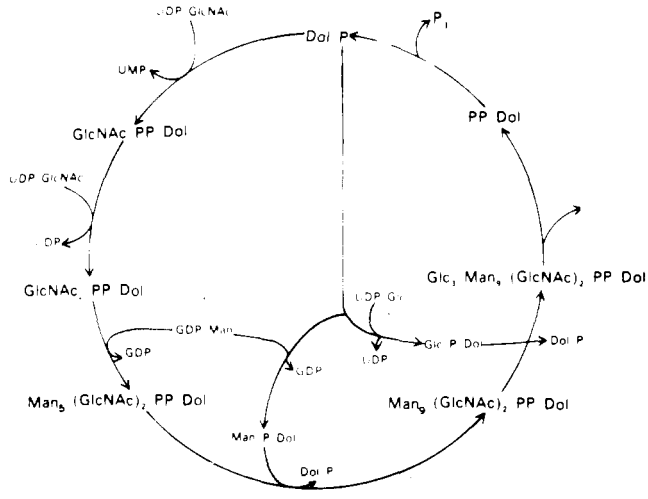

FIGURE 1: Pathway of the assembly of the lipid-linked oligosaccharide $Glc_3Man_9(GlcNAc)_2$-PP-Dol, the immediate precursor of Asn-linked oligosaccharides of glycoproteins. Based on Kornfeld (1982).

chick embryo cells 2FMan inhibits the assembly of the lipid-linked oligosaccharide (Datema & Schwarz, 1979) and is poorly, if at all, incorporated into glycoproteins (Schmidt et al., 1978). To help in designing glycosylation inhibitors not requiring prior metabolic conversion, we studied in some detail the mechanism of inhibition of oligosaccharide assembly by 2FMan. We show that the inhibitory effect can be attributed to an interference in the assembly of dolichol-linked oligosaccharides by a metabolite of 2FMan, GDP-2FMan.

Materials and Methods

Sugars, Oligosaccharides, Sugar Nucleotides, and Lipid-Linked Sugars. [2-^{3}H]Mannose (2–5 Ci/mmol), GDP-[U-^{14}C]mannose and UDP-[U-^{14}C]glucose (each at 300 Ci/mol), and UDP-[6-^{3}H]glucose (3.1 Ci/mmol) were from Amersham. New England Nuclear supplied GDP-[1-^{3}H]mannose (12.6 Ci/mmol), UDP-*N*-acetyl[U-^{14}C]glucosamine (230 Ci/mol), and [^{3}H]Glc-P-Dol (6.5 Ci/mmol). Tunicamycin and 2-deoxy-2-fluoro-D-mannose (2FMan) were from Calbiochem., San Diego (CA). 2-Deoxy-2-fluoro-D-[U-^{14}C]mannose (343 Ci/mol) was a custom synthesis by New England Nuclear. [^{14}C]Man-P-Dol, ([^{14}C]GlcNAc)$_2$-PP-Dol, Glc_3([^{3}H]-Man)$_9$(GlcNAc)$_2$-PP-Dol, [^{3}H]Man(GlcNAc)$_2$-PP-Dol, ([^{3}H]Man)$_2$(GlcNAc)$_2$-PP-Dol, ([^{3}H]Man)$_3$(GlcNAc)$_2$-PP-Dol, and ([^{3}H]Glc)$_3$Man$_9$(GlcNAc)$_2$-PP-Dol were prepared as described (Datema et al., 1980; Schwarz & Datema, 1982b). The radiolabeled oligosaccharides Glc_xMan_y-$(GlcNAc)_2$ were prepared from the above mentioned lipid-linked oligosaccharides (Schwarz & Datema, 1982b). Unlabeled sugar nucleotides were obtained from Boehring-Mannheim.

Other Materials. The ^{14}C-labeled amino acids (U-^{14}C-labeled protein hydrolysate) were from Amersham. Endoglucosaminidase H (Endo H; from *Streptomyces griseus*) was from Miles; α-mannosidase (type III, from jack beans), dolichol phosphate (Type III), 2,3-dimercaptopropanol, guanosine 5′-monophosphomorpholidate and uridine 5′-monophosphomorpholidate (as their dicyclohexylcarboxamide salts) were brought from Sigma. [G-^{3}H]Adenosine was from New England Nuclear. Thin-layer plates of silica G-60, or cellulose, were from Merck, Darmstadt, and DEAE-cellulose (DE-52) was from Whatman; Bio-Gel P-4 (−400 mesh) was from Bio-Rad. Boehringer Mannheim supplied AMP, guanosine, and uridine. All other chemical reagents were of the highest purity available.

Synthesis of Nucleoside Diphosphate Esters of 2-Deoxy-2-fluoro Sugars. The 2-deoxy-2-fluoromannose 1-phosphates were prepared by a microscale modification of the procedure of MacDonald (1961) as follows.

(i) The 2-deoxy-2-fluoro sugar (10 mg or 100 μCi) was dried over P_2O_5 and dissolved in dry, redistilled pyridine (4 mL). Freshly distilled acetic anhydride (refluxed over dry potassium acetate before distillation) was added (1.5 mL), the temperature was kept at 10 °C, and the mixture was stirred for 1 h. The reaction mixture, left overnight at room temperature, was then poured over a mixture of 20 g of crushed ice (prepared from distilled water) and water (20 mL) and stirred for 1 h. This material was extracted with chloroform (3 × 10 mL), and the chloroform layer was extracted with 7% HCl (3 × 20 mL), with 0.9% NaCl (2 × 20 mL), and finally with a saturated $NaHCO_3$ solution (10 mL). The organic layer was dried overnight (Na_2SO_4) and filtered, and the residue was washed with chloroform. After evaporation of chloroform, the resulting syrup was kept over P_2O_5.

The peracetates of 2FMan ran as one discrete spot on silica gel thin-layer plates (solvent: 1-butanol/acetic acid/10% ammonia/water, 35/15/7.5/22.5 by volume). The NMR analysis showed the presence of an anomeric mixture of the α- and β-anomers (yield, 80–90%).

(ii) The peracetylated sugars (8 mg; 80 μCi) were dissolved in 1 mL of tetrahydrofuran (dried and freshly distilled) and added to crystalline phosphoric acid dissolved in 1 mL of tetrahydrofuran (the crystalline phosphoric acid had been dried overnight over P_2O_5). The solvent was distilled off in vacuo. This syrup was kept at 8 × 10^{-3} torr for 45 min and then heated at 80 °C for 30 min. To the reaction mixture 1 N LiOH (10 mL) was then added with stirring, and after 1 h at room temperature and 2 h at 4 °C, the mixture was centrifuged. The supernatant and the wash of the residue were treated with Dowex 50W×2 (H^+, form) until the pH dropped to 9–10. The solution was then filtered, neutralized with acetic acid, concentrated, and applied to a column (1 × 20 cm) of Dowex 1×8 (Et_3N–H_2CO_3 form). The column was eluted with a 400-mL linear gradient of Et_3N–H_2CO_3 from 0.05 to 0.3 M. Fractions (5 mL) were collected and assayed for radioactivity or phosphorus (the nonlabeled material). Fractions corresponding to the elution position of sugar phosphates were pooled and freeze-dried after dilution with water (36% yield). NMR analysis revealed that predominantly (>90%) the α-anomer was formed.

The synthesis of the nucleotide esters was by reaction of 2FMan 1-phosphate with the guanosine or uridine monophosphomorpholidates as described by Elbein (1966). The sugar nucleotides were isolated after paper chromatography (Whatman No. 3) using solvent 4 (see below) and desalted by paper chromatography (25–50% yield). The products were characterized by chromatography on columns of Dowex 1×8 (1 × 20 cm) in the Et_3N–H_2CO_3 form, using a linear gradient of Et_3N–H_2CO_3, and by enzymatic analysis using phosphodiesterase and phosphatase (Schmidt et al., 1978).

Tissue Culture, Virus Infection, and Labeling Procedures. The infection of monolayers of secondary chick embryo fibroblasts with fowl plague virus (an influenza A virus) was as described (Datema & Schwarz, 1981). The cells were maintained in Earle's medium containing 10 mM sodium pyruvate. Labeling of infected or uninfected cells with [2-^{3}H]Man or ^{14}C-labeled amino acids was as described (Datema & Schwarz, 1981). The dolichol diphosphate linked oligosaccharides were obtained after extraction with $CHCl_3$/MeOH (2/1 by volume), water, and $CHCl_3$/MeOH/H_2O (10/10/3

by volume) as described (Schwarz & Datema, 1982b). The lipid-free residue contains the viral glycoproteins (Schwarz & Datema, 1982b).

Preparation of Microsomal Membrane Fractions. A crude microsomal preparation was isolated from chick embryo cells by the procedure of Krag & Robbins (1977) as in Schwarz & Datema (1982b). This preparation, containing 15–20 mg of protein/mL, was either used directly or treated, to complete partially assembled lipid-linked oligosaccharides, by incubation at 37 °C with 1% by volume each of 0.2 mM GDP-Man and 0.2 mM UDP-Glc, for 10 min. Further incubation with 1% by volume of 2 mM GDP and 2 mM UDP for 10 min was then used to deplete the membranes of unused Man-P-Dol and Glc-P-Dol. The membranes were then diluted with 50 volumes of ice-cold 20 mM Tris·HCl buffer (pH 7.5) containing 150 mM NaCl and sedimented by centrifugation at 100000*g* (60 min), at 4 °C, and the pellet was suspended in 20 mM Tris·HCl, 150 mM NaCl, 0.4 mM $MgCl_2$, and 0.4 mM $MnCl_2$ (pH 7.5) as above. This microsome fraction will be referred to as "treated microsomes".

Membranes enriched in $(GlcNAc)_2$-PP-Dol were prepared by incubating the original microsome preparation with 22 μM UDP-GlcNAc (Datema & Schwarz, 1978). Membranes enriched in $Man_9(GlcNAc)_2$-PP-Dol were prepared by incubating the original microsome preparation with 22 μM UDP-GlcNAc for 20 min, followed by 14 μM GDP-Man for 10 min (Datema et al., 1981). After dilution with 10 volumes of ice-cold 20 mM Tris·HCl, pH 7.5, containing 0.1 M mannono-1,5-lactone (to inhibit α-mannosidase activity), the microsomes were pelleted by centrifugation at 100000*g* for 60 min at 4 °C. The pellet was then suspended in 20 mM Tris·HCl, 150 mM NaCl, 0.4 mM $MgCl_2$, and 0.4 mM $MnCl_2$ (pH 7.5) at the same protein concentration as above. A similar procedure was used to obtain membranes enriched in Man_x-$(GlcNAc)_2$-PP-Dol (x = 5, 6; Datema et al., 1980). However, EDTA (10 mM) was present during the incubation with GDP-Man, which was extended to 60 min. EDTA inhibits Man-P-Dol synthesis, preventing elongation to Man_9-$(GlcNAc)_2$-PP-Dol (Chambers et al., 1977).

Synthesis of Lipid-Linked Oligosaccharides in Cell-Free Systems. Cell-free synthesis of lipid-linked oligosaccharides was performed as described by Schwarz & Datema (1982b), but with the addition of ATP (0.14 mM) and 2,3-dimercaptopropanol (5 mM) as by Faltynek et al. (1981) to prevent degradation of fluoro sugar nucleotides (Datema & Schwarz, 1984).

Separation Methods. Thin-layer plates of silica G-60, or cellulose, and paper chromatograms (Whatman 3MM) were developed by using either of the following solvent systems (all solvent ratios are by volume): (1) chloroform/methanol/ammonia/water (65/35/4/4); (2) chloroform/methanol/water (60/39/4); (3) ethyl acetate/pyridine/acetic acid/water (5/5/1/3); (4) 1 M ammonium acetate, pH 7.5/ethanol (2/5). Radioactive material was located with a Berthold LB 2821 automatic scanner. Columns (1 × 150 cm) of Bio-Gel P-4 (−400 mesh) were used for the separation of oligosaccharides derived from lipid-linked oligosaccharides, released by mild acid hydrolysis (Datema & Schwarz, 1981), and subsequently treated with Endo H (Datema & Schwarz, 1981). The columns were calibrated with lipid-derived oligosaccharides obtained from fluoroglucose-treated cells (Datema et al., 1980).

Dolichol monophosphate-linked sugars were isolated by ion-exchange chromatography on columns (1 × 2 cm) of DEAE-cellulose (acetate form) equilibrated with 99% methanol. Samples were applied in 1 mL of butanol, and dolichol monophosphate derivatives were eluted with 0.2 M ammonia acetate in 99% methanol (Dankert et al., 1966). Dolichol diphosphate linked saccharides were purified on columns (1 × 2 cm) of DEAE-cellulose (acetate form) equilibrated with chloroform/methanol/water (10/10/3). Samples were applied in 3 mL of the same solvent and the dolichol diphosphate linked sugars eluted with 0.12 M ammonium acetate in the same solvent.

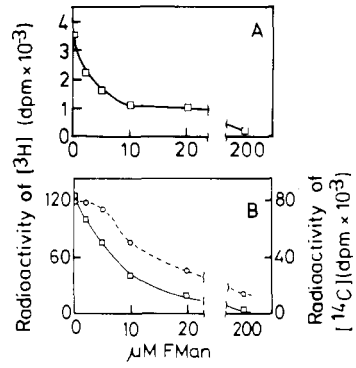

FIGURE 2: Effects of 2FMan treatment on the incorporation of (A) [^{3}H]mannose into lipid-linked oligosaccharides and (B) [^{3}H]mannose and ^{14}C-labeled amino acids into viral glycoproteins. Fowl plague virus infected cells were treated immediately after infection for 4 h with the indicated concentrations of 2FMan and then labeled for 2 h with 10 μCi of [2-^{3}H]mannose and 0.5 μCi of a ^{14}C-labeled amino acid mix without removing the fluoro sugar. The cells were harvested in $CHCl_3$/MeOH (2/1) and extracted. Symbols: □, [^{3}H]mannose; ○, ^{14}C-labeled amino acids.

Other Procedures. The pool sizes of UDP-GlcNAc and GDP-Man were determined by enzymatic procedures (Datema et al., 1980) after purification of the sugar nucleotides by paper chromatography, as by Schmidt et al. (1978). The energy charge was determined by labeling cells with [^{14}C]adenosine as by Datema & Schwarz (1981). The mild acid hydrolysis and treatments with Endo H and α-mannosidase have also been described (Schwarz & Datema, 1982b).

RESULTS

2FMan Inhibition of Protein Glycosylation. Cells infected with the influenza A virus fowl plague virus were treated from 0–6 h postinfection with different concentrations of 2FMan and labeled with [2-^{3}H]Man and ^{14}C-labeled amino acids between 4 and 6 h postinfection. The radioactivity incorporated into dolichol diphosphate linked oligosaccharides and into glycoproteins was determined. As shown in Figure 2, 10–20 μM 2FMan causes a strong inhibition of both lipid-linked oligosaccharide synthesis and protein glycosylation. However, in addition, labeling of proteins was also inhibited. At high concentrations (200 μM) protein glycosylation and lipid glycosylation were almost completely abolished. This block was achieved 30 min after the addition of the drug to the infected cells (not shown).

Previous studies have shown that 2FMan is metabolized (Schmidt et al., 1978), and as a consequence the energy status of the cell may be altered. Energy depletion, in turn, leads to inhibition of Man-P-Dol formation (Datema & Schwarz, 1981), resulting in an inhibition of protein glycosylation. However, the energy charge of 2FMan-treated cells did not differ from that of untreated cells (Table I).

The formation of the 2FMan metabolites UDP-2FMan and GDP-2FMan (Schmidt et al., 1978) might result in depletion of UTP or GTP pools, or both, thus inhibiting protein synthesis. Attempts to reverse the inhibition of protein synthesis with exogenous uridine were unsuccessful. However, addition of guanosine to the culture medium reversed the inhibition of

Table I: Parameters of Nucleotide Metabolism in 2FMan-Treated Cells[a]

additions	energy charge	pool sizes (nmol/mg of protein) of UDP-GlcNAc	GDP-Man
none	0.85	1.00	0.28
200 μM 2FMan	0.85		
500 μM 2FMan		3.01	0.40

[a] Chick embryo cells were incubated with the indicated concentrations of 2FMan. The energy charge was determined after labeling cells with [^{3}H]adenosine; the pool sizes of UDP-GlcNAc and GDP-Man were determined by enzymatic procedures after isolation of the sugar nucleotides.

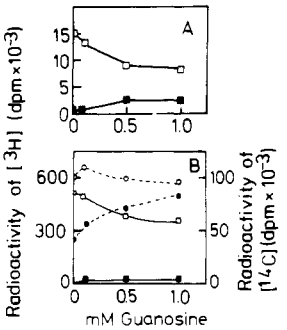

FIGURE 3: Effect of guanosine on incorporation of (A) [^{3}H]mannose into lipid-linked oligosaccharides and (B) [^{3}H]mannose and ^{14}C-labeled amino acids into viral glycoproteins, in the absence or presence of 2FMan. Fowl plague virus infected cells were treated immediately after infection for 4 h with 0.2 mM 2FMan (filled symbols) or no fluoro sugar (open symbols) and the indicated concentrations of guanosine. The cells were then labeled with 50 μCi of [2-^{3}H]mannose and 0.5 μCi of ^{14}C-labeled amino acids for 2 h without removing the drugs. The cells were harvested in $CHCl_3$/MeOH (2/1) and extracted. Symbols: □ and ■, [^{3}H]mannose; ○ and ●, ^{14}C-labeled amino acids.

incorporation of amino acids into protein, without inhibiting protein glycosylation (Figure 3). Thus, in subsequent experiments 1 mM guanosine was routinely included in the culture medium when studies were done on the effects of 2FMan on protein glycosylation.

2FMan inhibits dolichol-linked oligosaccharide synthesis before it inhibits protein glycosylation (Datema & Schwarz, 1979). This is also true for cells treated with 2FMan in the presence of guanosine (results not shown).

2FMan Inhibition of Lipid-Linked Oligosaccharide Synthesis. Infected cells treated with 200 μM 2FMan *plus* 1 mM guanosine from 0–5 h postinfection were labeled with [2-^{3}H]Man from 3–5 h postinfection. The dolichol diphosphate linked oligosaccharides present in the $CHCl_3$/MeOH (2/1) and $CHCl_3$/MeOH/H_2O (10/10/3) extracts were then isolated and mild acid hydrolyzed, and the resulting oligosaccharides were analyzed, after treatment with Endo H, on columns of Bio-Gel P-4. Figure 4 shows that in the presence of 2FMan only small lipid-linked oligosaccharides were made. The amount of [^{3}H]Man incorporated into oligosaccharides was 28% of the amount incorporated into oligosaccharides from nontreated cells.

To assess whether lipid-linked oligosaccharides smaller than those shown in Figure 4 accumulate in 2FMan-treated cells, microsomes from 2FMan-treated and nontreated cells were assayed from their capacities to synthesize lipid-linked oligosaccharides in the presence of tunicamycin and GDP-[^{14}C]Man. The results in Table II show that microsomes from 2FMan-treated cells have an increased capacity to synthesize lipid-linked oligosaccharides per milligram of protein. When UDP-GlcNac is added to the microsomes instead of tunicamycin, the microsomal preparations have similar synthetic capacities (Table II). The oligosaccharides from the incubation with UDP-GlcNAc and GDP-[^{14}C]Man were released from the lipid, treated with Endo H, and analyzed by chromatography on columns of Bio-Gel P-4. This result showed that the products from either type of microsome were $Man_9GlcNAc$ and $Glc_xMan_9GlcNAc$ (x = 1, 2, 3), with 89% of the radioactivity in $Man_9GlcNAc$. Hence, membranes from 2FMan-treated cells do not contain an inhibitor of oligosaccharide–lipid assembly but accumulate small lipid-linked oligosaccharides, which can serve as precursors to $Man_9GlcNAc_2$-PP-Dol when incubated with GDP-[^{14}C]Man.

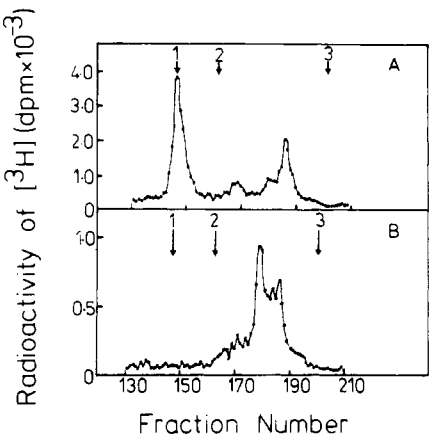

FIGURE 4: Bio-Gel P-4 chromatography of oligosaccharides formed in cells in the absence (A) and presence (B) of 2FMan. Fowl plague virus infected cells either untreated (A) or treated (B) with 0.2 mM 2FMan *plus* 1 mM guanosine, added 1 h postinfection, were labeled with 100 μCi of [2-^{3}H]Man (in 2.5 mL of medium) from 4 to 6 h postinfection. The $CHCl_3$/MeOH (2/1) and $CHCl_3$/MeOH/H_2O (10/10/3) extracts were prepared and combined, and the dolichol diphosphate linked oligosaccharides were purified by passage through a column of DEAE-cellulose. The oligosaccharides were released by mild acid hydrolysis, treated with Endo H, and chromatography over a calibrated column of Bio-Gel P-4. Arrows indicated the following: 1, $Glc_3Man_9GlcNAc$; 2, $Man_5(GlcNAc)_2$; 3, $(GlcNAc)_2$.

Table II: Capacity of Membranes To Synthesize Lipid-Linked Saccharides[a]

membrane source	additions	radioactivity (cpm) incorporated into $CHCl_3$/MeOH extract	$CHCl_3$/MeOH/H_2O extract	residue
nontreated cells	tunicamycin	25 400	1 280	1240
treated cells	tunicamycin	25 400	8 760	5000
nontreated cells	UDP-GlcNAc	18 700	31 800	8400
treated cells	UDP-GlcNAc	24 100	27 900	7900

[a] Virus-infected cells were treated or not treated with 200 μM 2FMan *plus* 1 mM guanosine. After 4 h, the crude membrane fraction was prepared, and membranes (1.6 mg of protein per tube) were incubated with 1.2 μM GDP-[^{14}C]Man and then extracted. Tunicamycin was present at 1 μg/mL and UDP-GlcNAc at 20 μM.

The radioactive material extracted with $CHCl_3$/MeOH (2/1) in these experiments was identified as [^{14}C]Man-P-Dol (not shown). The membranes from either source have identical capacities to synthesize [^{14}C]Man-P-Dol from exogenous GDP-[^{14}C]Man (Table II). This suggests that Dol-P was not limiting in the assay. Indeed, when the rate of formation of [^{14}C]Man-P-Dol was studied with membranes with 2FMan-treated or nontreated cells, no differences were found (not shown). Furthermore, treatment of cells with 0.5 mM 2FMan does not deplete these cells of UDP-GlcNAc and GDP-Man (Table I), which, together with Dol-P, are needed for the

Table III: Inhibition of Lipid-Linked Mono- and Disaccharide Synthesis by Nucleotide Esters of 2FMan[a]

product synthesized	IC_{50} (μM)[b]	
	UDP-2FMan	GDP-2FMan
Man-P-Dol	>150 (5%)[c]	32
Glc-P-Dol	3	24
$(GlcNAc)_2$-PP-Dol	>150 (20%)	35

[a] Microsomes were incubated with UDP-[^{14}C]Glc, GDP-[^{14}C]Man, or UDP-[^{14}C]GlcNAc, and the lipid-linked sugars were extracted with $CHCl_3$/MeOH (2/1). The products formed were identified by thin-layer chromatography. [b] The concentration giving 50% inhibition of the incorporation of the radiolabeled sugar into the $CHCl_3$/MeOH (2/1) soluble product. [c] Values in parentheses give the percent inhibition at 150 μM inhibitor.

assembly of the lipid-linked oligosaccharide to the heptasaccharide–lipid stage, $Man_5(GlcNAc)_2$-PP-Dol. This, in turn, suggests that in cells treated with 2FMan an inhibitor is present that prevents the assembly of the lipid-linked oligosaccharides. In cells 2FMan is metabolized to UDP-2FMan and GDP-2FMan. These sugar nucleotides were therefore synthesized and tested in cell-free systems as inhibitors of lipid-linked oligosaccharide synthesis.

Inhibition of Lipid-Linked Mono- and Disaccharides. UDP-2FMan in concentrations up to 150 μM did not inhibit the synthesis of [^{14}C]Man-P-Dol or ([^{14}C]GlcNAc)$_2$-PP-Dol from GPD-[^{14}C]Man and UDP-[^{14}C]GlcNAc, respectively. Synthesis of [^{14}C]Glc-P-Dol from UDP-[^{14}C]Glc was inhibited 50% by 3 μM UDP-2FMan (Table III). This inhibition could not be reversed by exogenous Dol-P (300 μg/mL).

GDP-2FMan, on the other hand, inhibited the synthesis of [^{14}C]Glc-P-Dol, [^{14}C]Man-P-Dol, and ([^{14}C]GlcNAc)$_2$-PP-Dol from the respective sugar nucleotides (Table III). These inhibitions were reversed by adding Dol–P in the assay (300 μg/mL). This suggests that GDP-2FMan can trap Dol-P to form 2FMan-P-Dol. To identify such a product, microsomes were incubated with 0.05 μCi (1.2 μM) of GDP-[^{14}C]2FMan, and the radioactivity extracted with $CHCl_3$/MeOH (2/1) was determined. Incorporation was linear for 15 min, and 2.3 pmol of [^{14}C]2FMan/mg of protein was transferred in 15 min from GDP-[^{14}C]2FMan. Incubations with UDP-[^{14}C]2FMan did not give rise to radioactive material extractable with $CHCl_3$/MeOH (2/1).

The evidence that the $CHCl_3$/MeOH extract from incubations with GDP-[^{14}C]2FMan contains [^{14}C]2FMan-P-Dol is as follows: (1) on thin-layer chromatography (silica gel G-60, solvent 1) one peak migrating slightly ahead of Man-P-Dol is seen (Figure 5); (2) the material binds to DEAE-cellulose (acetate form) in 99% MeOH and is quantitatively eluted with 0.2 M ammonium acetate, characteristic of dolichol monophosphate linked sugars; (3) 2FMan is released after hydrolysis in 1 M HCl in 50% 1-propanol for 30 min at 100 °C (inset in Figure 5); (4) the formation of the lipid-linked sugar is stimulated 3-fold by addition to the microsomes of 0.17 mg/mL Dol-P; (5) the synthesis of the lipid-linked sugar is inhibited by 10 mM EDTA, known to also inhibit the synthesis of Man-P-Dol (Chambers et al., 1977). Thus, in cell-free systems GDP-2FMan can trap Dol-P, inhibiting the de novo synthesis of lipid-linked sugars.

Inhibition of the Early Stage of Lipid-Linked Oligosaccharide Assembly. For these investigations treated microsomes (see Materials and Methods) were first enriched in $(GlcNAc)_2$-PP-Dol and then incubated with GDP-[^{3}H]Man (0.46 mM). GDP-2FMan inhibited the formation of [^{3}H]-Man-labeled material extractable with $CHCl_3$/MeOH (2/1) maximally 69% (not shown). The lipid-linked oligosaccharides

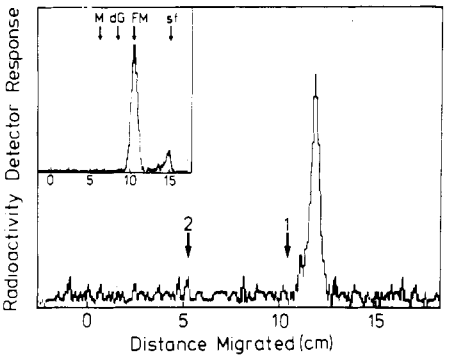

FIGURE 5: Thin-layer chromatography of the $CHCl_3$/MeOH (2/1 v/v) extract derived from incubating GDP-[^{14}C]2FMan with chick embryo cell membranes. GDP-[^{14}C]2FMan was incubated with chick embryo cell membranes for 20 min at 37 °C and a $CHCl_3$/MeOH (2/1) extract prepared. This was subjected to thin-layer chromatography on silica G-60 using solvent 1. The arrows indicate the positions of Man-P-Dol (1) and $(GlcNAc)_2$-PP-Dol (2). Inset: Mild acid hydrolysate of the product chromatographed on a cellulose thin-layer plate with solvent 3. M, dG, and FM indicate the positions of mannose, 2-deoxyglucose, and 2FMan reference compounds, respectively, and sf indicates the position of the solvent front.

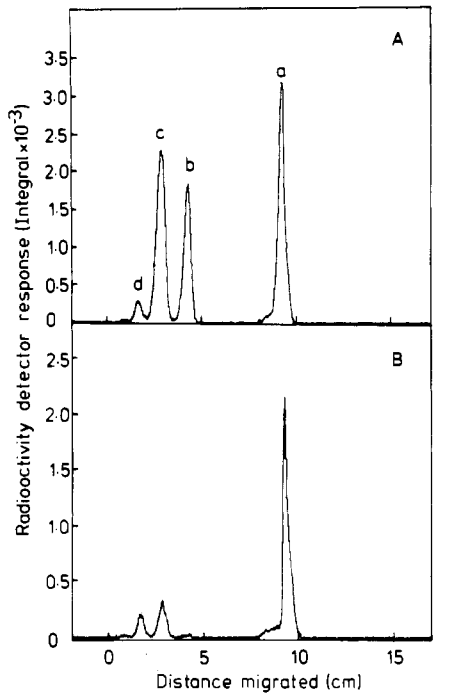

FIGURE 6: Thin-layer chromatography of dolichol-linked saccharides extracted with $CHCl_3$/MeOH (2/1 v/v) synthesized in microsomes in the absence (A) and presence (B) of GDP-2FMan. Microsomes enriched in $(GlcNAc)_2$-PP-Dol were incubated with GDP-[^{3}H]Man (0.7 μCi; 60 pmol) for 10 min in absence (A) or presence (B) of 150 μM GDP-2FMan. The $CHCl_3$/MeOH (2/1 v/v) extracts were prepared and subjected to silica gel G-60 thin-layer chromatography using solvent 1. The peaks marked a–d show the same mobility as reference compounds Man-P-Dol, $Man(GlcNAc)_2$-PP-Dol, Man_2-$(GlcNAc)_2$-PP-Dol, and $Man_3(GlcNAc)_2$-PP-Dol, respectively. The solvent front is at 16 cm.

formed in the absence of GDP-2FMan, and present in the $CHCl_3$/MeOH (2/1) extract, were analyzed by thin-layer chromatography (silica gel G-60, solvent 1) and, after mild acid hydrolysis, by paper chromatography (Whatman 3MM, solvent 2). The thin-layer chromatography showed four peaks of radioactivity (Figure 6). From the mobility of reference compounds, it is deduced that peak a contains Man-P-Dol; peak b, $Man(GlcNAc)_2$-PP-Dol; peak c $Man_2(GlcNAc)_2$-PP-Dol; peak d, $Man_3(GlcNAc)_2$-PP-Dol. Paper chroma-

Table IV: Inhibition of Lipid-Linked Oligosaccharide Assembly by GDP-2FMan, Early Stage

product	radioactivity (cpm) incorporated into lipid-linked sugars[a]	
	no GDP-2FMan	150 μM GDP-2FMan
Man-P-Dol	27965	16716
$Man(GlcNAc)_2$-PP-Dol	16492 (0%)[b]	446 (0%)[b]
$Man_2(GlcNAc)_2$-PP-Dol	24380 (41%)	3344 (72%)
$Man_3(GlcNAc)_2$-PP-Dol	2868 (61%)	2007 (82%)

[a] Treated microsomes (see Materials and Methods) were saturated with $(GlcNAc)_2$-PP-Dol and incubated with 0.46 mM GDP-[^{3}H]Man, 150 μM GDP-2FMan, or no GDP-2FMan. [b] Values in parentheses are the percentage of the radioactivity in the oligosaccharide released after α-mannosidase treatment as [^{3}H]Man. To this end, oligosaccharides were isolated by paper chromatography after mild acid hydrolysis of the $CHCl_3$/MeOH (2/1) extract (see text). Note: The amounts of radioactivity incorporated into protein-linked oligosaccharides were 2315 (no GDP-2FMan) and 360 cpm (150 μM GDP-2FMan).

tography of the mild acid hydrolysates showed [^{3}H]Man and, deduced from mobility of reference compounds, radiolabeled $(GlcNAc)_2Man$, $(GlcNAc)_2Man_2$, and $(GlcNAc)_2Man_3$. Treatment of the isolated oligosaccharides with α-mannosidase released [^{3}H]Man, as shown in Table IV, in agreement with the general structure of the oligosaccharides b, c, and d as $(GlcNAc)_2\beta Man(\alpha Man)_n$ with n = 0, 1, and 2, respectively.

From incubations in presence of maximally inhibitory concentrations of GDP-2FMan (150 μM), the same series of compounds is obtained (Figure 6), albeit in smaller amounts (Table IV) and in different proportions (Figure 6). Thus in the presence of GDP-2FMan the synthesis of dolichol diphosphate linked oligosaccharides is inhibited 88% and the synthesis of Man-P-Dol only 45%. This suggests that, under these conditions, GDP-2FMan preferentially inhibits the incorporation of [^{3}H]Man into lipid-linked oligosaccharides. Specifically, incorporation of [^{3}H]Man into Man-$(GlcNAc)_2$-PP-Dol is inhibited most strongly, namely, 98% (Table IV). In the presence of GDP-2FMan no increased incorporation of [^{3}H]Man into the protein-linked oligosaccharides was found (see footnote *b* in Table IV).

To find out whether inhibition of trisaccharide–lipid synthesis is caused by formation of $2FMan(GlcNAc)_2$-PP-Dol, GDP-[^{3}H]Man was omitted from the assay, and GDP-[^{14}C]2FMan (0.05 μCi; 42 μM) was used instead. Under these conditions no radioactivity was incorporated into Dol-PP-linked oligosaccharides (not shown). When the membranes were saturated with $([^{14}C]GlcNAc)_2$-PP-Dol and then incubated with GDP-2FMan (17 μM), $([^{14}C]GlcNAc)_2$-PP-Dol was not elongated to a lipid-linked trisaccharide, as shown by analysis of the saccharides released by mild acid hydrolysis (Figure 7A,C). It should be noted that under these conditions 2-deoxyglucose (dGlc) from GDP-dGlc is incorporated into the trisaccharide–lipid $dGlc(GlcNAc)_2$-PP-Dol (Datema & Schwarz, 1978), and Man from GDP-Man is incorporated into $Man_9(GlcNAc)_2$-PP-Dol (Figure 7B) and protein-linked oligosaccharides. Inclusion in the assay of a small amount of GDP-Man (0.8 or 1.7 μM) in addition to GDP-2FMan (17 μM) does not cause elongation of preformed ($[^{14}C]$-$GlcNAc)_2$-PP-Dol either (not shown). Thus GDP-2FMan is not a donor of 2FMan residues in the early steps of lipid-linked oligosaccharide synthesis.

Inhibition of the Late Steps in Lipid-Linked Oligosaccharide Assembly. For these investigations membranes were enriched in either $Man_{5,6}(GlcNAc)_2$-PP-Dol, or Man_9-$(GlcNAc)_2$-PP-Dol and then incubated with GDP-[^{3}H]Man or UDP-[^{3}H]Glc, respectively, with or without GDP-2FMan

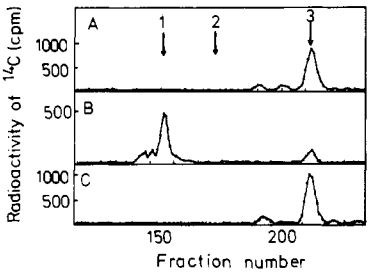

FIGURE 7: Bio-Gel P-4 chromatography of oligosaccharides labeled with UDP-[^{14}C]GlcNAc in a cell-free system, in the absence or presence of GDP-Man or GDP-2FMan. Chick embryo membranes were incubated with UDP-[^{14}C]GlcNAc for 20 min and then further incubated 20 min with (A) buffer, (B) GDP-Man (17 μM), or (C) GDP-2FMan (17 μM). The reaction was stopped by adding $CHCl_3$/MeOH (2/1 by volume), and the lipid-linked oligosaccharides present in the $CHCl_3$/MeOH (2/1) and $CHCl_3$/MeOH/H_2O (10/10/3) extracts were combined. the oligosaccharides were released by mild acid hydrolysis. Arrow 1 points to the elution position of $Man_9(GlcNAc)_2$, arrow 2 to $Man_5(GlcNAc)_2$, and arrow 3 to $(GlcNAc)_2$. The void volume was 97 fractions in (A), 94 fractions in (B), and 93 fractions in (C).

Table V: Inhibition of Lipid-Linked Oligosaccharide Assembly by GDP-2FMan, Late Stage

expt	product	radioactivity (dpm) incorporated into lipid-linked sugars[a]	
		no GDP-2FMan	150 μM GDP-2FMan
1	$Man_{7,8,9}(GlcNAc)_2$-PP-Dol	36784	9380
1	$Man_9(GlcNAc)_2$-PP-Dol	23910	5347
1	Man-P-Dol	59867	16890
2	$Glc_{1,2,3}Man_9(GlcNAc)_2$-PP-Dol	7503	5873
2	$Glc_3Man_9(GlcNAc)_2$-PP-Dol	4352	2995
2	Glc-P-Dol	42840	15080

[a] Microsomes were saturated with $Man_{5,6}(GlcNAc)_2$-PP-Dol and incubated with GDP-[^{3}H]Man (experiment 1) or saturated with Man_9-$(GlcNAc)_2$-PP-Dol and incubated with UDP-[^{3}H]Glc (experiment 2). The lipid-linked oligosaccharides were present in the $CHCl_3$/MeOH/H_2O (10/10/3) extract and the dolichol phosphate linked sugars in the $CHCl_3$/MeOH (2/1) extract. Note: A part of the radioactivity in these experiments was associated with proteins. In experiment 1 the protein-bound radioactivity was 23678 (without GDP-2FMan) or 11681 dpm (with GDP-2FMan); in experiment 2 the numbers were 8186 and 6800 dpm, respectively. This material was not characterized.

(150 μM). The lipid-linked oligosaccharides were isolated, subjected to mild acid hydrolysis, and treated with Endo H and the oligosaccharides analyzed on calibrated columns of Bio-Gel P-4. From incubations of $Man_{5,6}(GlcNAc)_2$-PP-Dol with GDP-[^{3}H]Man radioactivity was found in $Man_7GlcNAc$, $Man_8GlcNAc$, and $Man_9GlcNAc$ in the proportion 1:4.6:7.4. This proportion was unchanged when the incubation was performed in the prsence of GDP-2FMan, but the incorporation of [^{3}H]Man into the oligosaccharides was inhibited 75%. In the same experiment the formation of [^{3}H]Man-P-Dol was inhibited 72% by GDP-2FMan (Table V). This indicates that GDP-2FMan does not block the assembly of the lipid-linked oligosaccharide from $Man_5(GlcNAc)_2$-PP-Dol to Man_9-$(GlcNAc)_2$-PP-Dol. The decreased labeling of Man_9-$(GlcNAc)_2$-PP-Dol (Table V) is probably caused by lack of Man-P-Dol. Addition of GDP-2FMan did not "chase" the radioactivity from lipid-linked into protein-linked oligosaccharide (see footnote *a* to Table V).

From the incubations of $Man_9(GlcNAc)_2$-PP-Dol with UDP-[^{3}H]Glc, radioactivity was, after analysis as above, found in $Glc_1Man_9GlcNAc$, $Glc_2Man_9GlcNac$, and $Glc_3Man_9GlcNAc$ in the proportion 1:1.6:3.5. This proportion

was not significantly changed when GDP-2FMan was added to the reaction mixture, and the incorporation of [^{3}H]Glc into the oligosaccharides was inhibited only slightly (22%). In the same experiment formation of [^{3}H]Glc-P-Dol was inhibited 65%. Thus, GDP-2FMan, did not block the assembly from $Man_9(GlcNAc)_2$-PP-Dol to $Glc_3Man_9(GlcNAc)_2$-PP-Dol, but inhibition of formation of Glc-P-Dol only partially affected formation of $Glc_3Man_9(GlcNAc)_2$-PP-Dol. The decrease in radioactivity in the lipid-linked oligosaccharide fraction after addition of GDP-2FMan is not due to increased incorporation into the protein fraction (Table V).

Incorporation of radioactivity from GDP-[^{14}C]2FMan into material with the properties of dolichol diphosphate linked oligosaccharides, under the conditions used in these experiments, could not be shown (results not presented).

Discussion

Earlier work (Schmidt et al., 1976) has shown that 2FMan is an antiviral agent. This mannose analogue could also cause lysis of yeast cells (Biely et al., 1973). Here we have shown that 2FMan inhibits an early step in the assembly of the dolichol diphosphate linked tetradecasaccharide, precursor of the Asn-linked oligosaccharides of glycoproteins, and that GDP-2FMan may be exterting this inhibition.

To circumvent the inhibition by 2FMan of protein synthesis, or at least the incorporation of amino acids into viral proteins, guanosine has to be added to the culture medium (Figure 3), in which case only protein glycosylation, but not protein synthesis, was inhibited. Guanosine itself decreased the incorporation of [2-^{3}H]Man into lipid-linked oligosaccharides, but with no effect on the formation of $Glc_3Man_9(GlcNAc)_2$-PP-Dol (our unpublished results). The inhibition of protein glycosylation by 2FMan (Datema & Schwarz, 1979) or by 2FMan plus guanosine was a consequence of inhibition of dolichol diphosphate glycosylation. Hence, the inhibition of oligosaccharide–lipid assembly by 2FMan was analyzed in more detail.

For the analysis two complementary approaches were used, namely, (1) an analysis of inhibition of lipid-linked oligosaccharide synthesis in cells treated with 2FMan *plus* guanosine and (2) an analysis of inhibition of synthesis of lipid-linked oligosaccharides in cell-free systems by GDP-2FMan and UDP-2FMan. The analysis in intact cells showed that assembly of lipid-linked oligosaccharides was inhibited (Figure 4) and that cells treated with 2FMan *plus* guanosine accumulated small lipid-linked oligosaccharides (Table II). Furthermore, despite the possibility of GDP-2FMan to trap Dol-P as 2FMan-P-Dol, membranes from treated cells could still synthesize de novo Man-P-Dol, Glc-P-Dol, and $(GlcNAc)_2$-PP-Dol from endogenous Dol-P and exogenous sugar nucleotides. Also, the formation of GDP-2FMan did not decrease the pool of GDP-Man, a phenomenon that was also observed in 2FGlc-treated cells, where GDP-2FGlc is formed (Datema et al., 1980). Therefore, our results imply that despite the presence of Dol-P, GDP-Man, and $(GlcNAc)_2$-PP-Dol, the assembly of the lipid-linked oligosaccharides was blocked in 2FMan-treated cells. Membranes from 2FMan-treated cells were, however, fully capable of synthesizing lipid-linked oligosaccharides from exogenous GDP-Man and endogenous $(GlcNAc)_2$-PP-Dol and Glc-P-Dol. As the energy charge (primarily affecting Man-P-Dol synthesis; Datema & Schwarz, 1981) of 2FMan-treated cells was not different from untreated cells, we reasoned that soluble metabolites of 2FMan might inhibit oligosaccharide–lipid assembly. Such metabolites are GDP-2FMan and UDP-2FMan, and as mannose more effectively reversed the antiviral effects of 2Fman than did glucose (Schmidt et al., 1976), it is likely that GDP-2FMan, and not UDP-2FMan, was responsible for inhibition of viral protein glycosylation. Studies with cell-free systems confirmed this.

Using these nucleoside diphosphate esters of 2FMan, we found that the following steps in oligosaccharide–lipid synthesis could be inhibited: (1) Glc-P-Dol formation from UDP-Glc and Dol-P; (2) Man-P-Dol formation from GDP-Man and Dol-P; (3) $(GlcNAc)_2$-PP-Dol formation from UDP-GlcNAc and Dol-P; (4) $Man(GlcNAc)_2$-PP-Dol formation from $(GlcNAc)_2$-PP-Dol and GDP-Man. Reaction 1 was inhibited by both UDP-2FMan and GDP-2FMan, whereas only GDP-2FMan inhibited reactions 2, 3, and 4. Other steps in the assembly of the dolichol diphosphate linked oligosaccharide were not directly inhibited by GDP-2FMan. Inhibition of reactions 1, 2, and 3 by GDP-2FMan was caused by formation of 2FMan-P-Dol from GDP-2FMan and Dol-P. In this respect GDP-2FMan resembles GDP-dGlc (Schwarz & Datema, 1982a). The inhibition of reaction 4 was not due to incorporation of 2FMan in place of Man into the trisaccharide–lipid. In this respect GDP-2FMan differs from GDP-dGlc (Schwarz & Datem, 1982a).

The inhibition of reactions 1, 2, and 3 by GDP-2FMan in cell-free systems is probably not the prime target of the inhibition of oligosaccharide–lipid assembly by 2FMan in vivo, since (a) the inhibition of assembly (Figure 4) occurs before the involvement of Glc-P-Dol and Man-P-Dol in the pathway (see Figure 1), (b) $(GlcNAc)_2$-PP-Dol formation does not seem to be inhibited (see above), and (c) membranes from 2FMan-treated cells are not depleted in Dol-P (Table II), and if 2FMan-P-Dol were present in these membranes, it does not inhibit oligosaccharide–lipid assembly (Table II). Nevertheless, the inhibition of Man-P-Dol formation may certainly contribute to inhibition, as shown in Table V for the cell-free assembly. We, therefore, conclude that inhibition of reaction 4, the formation of $Man(GlcNAc)_2$-PP-Dol, by GDP-2FMan contributes most strongly to the inhibition of oligosaccharide–lipid synthesis in 2FMan-treated cells.

The studies of the mode of action of the antiviral sugar analogues 2-deoxyglucose (Datema & Schwarz, 1978), 2-deoxy-2-fluoroglucose (Datema et al., 1980), and 2-deoxy-2-fluoromannose (this study) have shown that the guanosine diphosphate esters of the sugar analogues inhibit lipid-linked oligosaccharide synthesis, which leads to inhibition of viral protein glycosylation. However, the precise mode of action of each ester differs.

Acknowledgments

We acknowledge the technical assistance of Kirstin Schallner and Ulrike Klein. We thank Drs. R. Rott and C. Scholtissek for encouragement and Drs. Bleihauer and Schwarzmann (Institut für Organische Chemie und Biochemie, Universität Bonn) for kindly recording the NMR spectra. We also thank Merit Kropp for secretarial assistance.

References

Biely, P., Kovaritz, I., & Bauer, S. (1973) *J. Bacteriol. 115*, 1108–1120.

Chamber, J., Forsee, W. T., & Elbein, A. D. (1977) *J. Biol. Chem. 252*, 2498–2506.

Dankert, M., Wright, A., Kelley, W. S., & Robbins, P. W. (1966) *Arch. Biochem. Biophys. 166*, 425–435.

Datema, R., & Schwarz, R. T. (1978) *Eur. J. Biochem. 90*, 505–516.

Datema, R., & Schwarz, R. T. (1979) *Biochem. J. 184*, 113–123.

Datema, R., & Schwarz, R. T. (1981) *J. Biol. Chem. 256*, 11191–11198.
Datema, R., & Schwarz, R. T. (1984) *Biosci. Rep. 4*, 213–221.
Datema, R., Schwarz, R. T., & Jankowski, A. W. (1980) *Eur. J. Biochem. 109*, 331–341.
Datema, R., Pont-Lezica, R., Robbins, P. W., & Schwarz, R. T. (1981) *Arch. Biochem. Biophys. 206*, 65–71.
Datema, R., Romero, P. A., Rott, R., & Schwarz, R. T. (1984) *Arch. Virol. 81*, 25–39.
Elbein, A. D. (1966) *Methods Enzymol. 8*, 142–145.
Elbein, A. D. (1984) *CRC Crit. Rev. Biochem. 16*, 21–49.
Faltynek, C. P., Silbert, J. E., & Hof, L. J. (1981) *J. Biol. Chem. 256*, 7139–7141.
Grier, T. J., & Rasmussen, J. R. (1984) *J. Biol. Chem. 259*, 1027–1030.
Hubbard, S. C., & Ivatt, R. J. (1981) *Annu. Rev. Biochem. 50*, 555–583.
Kornfeld, S. (1982) in *Glycoconjugates* (Horowitz, M. I., Ed.) Vol. III, pp 3–23, Academic Press, New York.
Krug, S. S., & Robbins, P. W. (1977) *J. Biol. Chem. 252*, 2621–2629.
MacDonald, D. L. (1961) *J. Org. Chem. 26*, 908–911.
Schmidt, M. F. G., Schwarz, R. T., & Ludwig, H. (1976) *J. Virol. 18*, 819–823.
Schmidt, M. F. G., Biely, P., Kratky, Z., & Schwarz, R. T. (1978) *Eur. J. Biochem. 87*, 55–68.
Schwarz, R. T., & Datema, R. (1982a) *Adv. Carbohydr. Chem. Biochem. 40*, 287–379.
Schwarz, R. T., & Datema, R. (1982b) *Methods Enzymol. 83*, 432–443.

Interaction of Calmodulin and a Calmodulin-Binding Peptide from Myosin Light Chain Kinase: Major Spectral Changes in Both Occur as the Result of Complex Formation†

Rachel E. Klevit,*,‡ Donald K. Blumenthal,§ David E. Wemmer,‡ and Edwin G. Krebs§
Department of Chemistry and Howard Hughes Medical Institute, University of Washington, Seattle, Washington 98195
Received May 28, 1985

ABSTRACT: Many different enzymes are activated by direct interaction with calmodulin; this interaction is thought to occur through a distinct calmodulin-binding domain in each of these enzymes. We have recently reported the sequence of a 27-residue peptide (denoted M13), derived from skeletal muscle myosin light chain kinase (MLCK), that exhibits the properties expected of a calmodulin-binding domain [Blumenthal, D. K., Takio, K., Edelman, A. M., Charbonneau, H., Titani, K., Walsh, K. A., & Krebs, E. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3187–3191]. The interaction between chemically synthesized M13 peptide and calmodulin has been studied by circular dichroism (CD) and proton nuclear magnetic resonance (NMR) spectroscopy. In the presence of Ca^{2+}, the observed ellipticity of an equimolar mixture of M13 and calmodulin is much greater than the sum of the ellipticities of the two isolated proteins. In the absence of Ca^{2+}, the measured ellipticity of the mixture is approximately the sum of the two components. Addition of the peptide to calmodulin causes dramatic changes in the proton NMR spectrum; at a 1:1 molar ratio, no evidence of either free peptide or free calmodulin is observed. Moreover, these data demonstrate that a unique species of the M13–calmodulin complex is formed, indicating that the peptide binds to calmodulin in only one way. The many resonances affected by M13 binding include residues in both halves of the calmodulin molecule. The observed CD and NMR effects suggest that secondary and tertiary conformational changes occur both in M13 and in calmodulin upon complex formation. Thus, changes in calmodulin tertiary structure following protein binding may represent an additional step in the presently accepted mechanism for calmodulin-dependent activation of MLCK and other target proteins.

The Ca^{2+}-binding protein calmodulin regulates a wide variety of enzymes and processes [for reviews, see Manalan & Klee (1984) and Klee & Vanaman (1982)]. Although the general scheme for the mechanism of regulation of many enzymes by calmodulin (eq 1) has been accepted for some time, little is known at the structural level about the interactions that occur between calmodulin and its various target proteins.

$$CaM + 4Ca^{2+} \rightleftarrows Ca^{2+}_4\cdot CaM + enzyme_{inactive} \rightleftarrows Ca^{2+}_4\cdot CaM\cdot enzyme_{active} \quad (1)$$

Recently we reported the sequence of a 27-residue peptide derived from skeletal muscle myosin light chain kinase (MLCK)[1] that shows the properties expected of a calmodulin-binding domain (Blumenthal et al., 1985). The studies of Edelman et al. (1985) involving limited proteolysis of the intact enzyme also point to this region in MLCK as being involved in calmodulin binding. The peptide, called M13, is isolated

† This research was supported by National Institutes of Health Grant AM35187-01 (R.E.K.) and a Muscular Dystrophy Association postdoctoral fellowship (D.K.B.). The 500-MHz spectrometer is supported by instrumentation grants from the Murdock Foundation, the National Science Foundation (PCM80-18053), and the National Institutes of Health (GM-28764-0151).
‡ Department of Chemistry.
§ Howard Hughes Medical Institute.

[1] Abbreviations: Aoc, *tert*-amyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; CaM, calmodulin; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HPLC, high-performance liquid chromatography; MLCK, myosin light chain kinase; MOPS, 4-morpholinepropanesulfonic acid; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

0006-2960/85/0424-8152$01.50/0