Control of N-Linked Oligosaccharide Synthesis: Cellular Levels of Dolichyl Phosphate Are Not the Only Regulatory Factor†

Y. T. Pan and Alan D. Elbein*
Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78284
Received March 26, 1990

ABSTRACT: When MDCK cells were incubated in the presence of the protein synthesis inhibitor puromycin or cycloheximide, there was a rapid and concentration-dependent inhibition in the incorporation of [2-^{3}H]mannose into lipid-linked oligosaccharide and into protein. However, mannose incorporation into dolichyl-P-mannose was not affected. Interestingly, these inhibitors did block [6-^{3}H]glucosamine incorporation into dolichyl-PP-GlcNAc as well as into lipid-linked oligosaccharides. Similar results were obtained when other cell lines were used and also when inhibitors of protein glycosylation such as β-hydroxynorvaline and β-fluoroasparagine were used. Cells incubated in puromycin did not show any changes in the levels of sugar nucleotides, GDP-mannose or UDP-GlcNAc, or in the in vitro activities of the glycosyltransferases that add mannose to the lipid-linked oligosaccharides. The inhibition of mannose incorporation into lipid-linked oligosaccharides could not be overcome by addition of dolichyl-P to the inhibited cells, even though the addition of dolichyl-P to control cells stimulated mannose incorporation into dolichyl-P-mannose, lipid-linked oligosaccharides, and protein from 3- to 5-fold. Thus, limitations in the levels of dolichyl-P do not appear to be a major factor in this inhibition. On the other hand, addition of the tripeptide acceptor *N*-acyl-Asn-Try-Thr did overcome the puromycin inhibition to some extent, suggesting that accumulation of some intermediate such as lipid-linked oligosaccharides might be involved in the inhibition.

The biosynthesis of N-linked oligosaccharides involves an initial series of reactions whereby the sugars GlcNAc, mannose, and glucose are sequentially transferred, either from their nucleoside diphosphate sugar derivatives or from the dolichyl-linked sugar derivatives, to dolichyl-P to form the lipid-linked oligosaccharide $Glc_3Man_9(GlcNAc)_2$-PP-dolichol (Elbein, 1979; Struck & Lennarz, 1979; Hubbard & Ivatt, 1981; Kornfeld & Kornfeld, 1985). Although many of the reactions in this lipid-linked saccharide pathway have been demonstrated, relatively little is known about the properties of the enzymes involved, and there is also a lack of information on how this pathway is regulated in the cell. For example, how does the cell modify the rate of lipid-linked oligosaccharide synthesis in response to demands for increases or decreases in the rate of glycoprotein synthesis?

Previously, we (Schmitt and Elbein, 1979) and others (Spiro et al., 1976; Hubbard & Robbins, 1980; White & Speake, 1980; Grant & Lennarz, 1983) demonstrated in cell culture systems that when protein synthesis was blocked with cycloheximide or puromycin or when RNA synthesis was inhibited with actinomycin (Hubbard & Robbins, 1980), the incorporation of mannose into lipid-linked oligosaccharides was also inhibited. However, mannose incorporation into dolichyl-P-mannose was not inhibited under these circumstances. While one explanation for these results could be that the levels of dolichyl-P are limiting and that no further lipid-linked oligosaccharide can be formed since all of the available dolichyl-P is tied up in the form of lipid-linked saccharides, several other experiments indicate that this explanation is not sufficient to explain the results. First of all, the formation of dolichyl-P-mannose does not stop under inhibitory conditions, indicating that there is dolichyl-P available as a sugar acceptor (Schmitt & Elbein, 1979). Thus, unless the formation of dolichyl-P-mannose utilized different pools of dolichyl-P or is in different compartments than is the production of lipid-linked oligosaccharides, there should be sufficient amounts of dolichyl-P available. Second, the addition of exogenous dolichyl-P to LM cells inhibited with cycloheximide could not overcome the inhibition of mannose incorporation into lipid-linked oligosaccharides, even though the addition of dolichyl-P to uninhibited cells greatly stimulated the incorporation of mannose into lipid-linked oligosaccharides (Grant & Lennarz, 1983). Such experiments suggest another level of control of the pathway of N-linked oligosaccharide assembly, perhaps by a feedback inhibition mechanism.

In the experiments described here, we have expanded our earlier studies to show that inhibitors of protein synthesis, or amino acid analogues that prevent glycosylation, inhibit mannose incorporation into lipid-linked oligosaccharides, without affecting mannose incorporation into dolichyl-P-mannose. Similar results were observed with a number of different cell lines, including Madin-Darby canine kidney cells, HT-29 intestinal carcinoma cells, and Chinese Hamster ovary cells, as well as with two CHO cell mutants that are altered in the biosynthesis of lipid-linked oligosaccharides. On the other hand, the inhibitors of protein synthesis did inhibit the incorporation of GlcN into dolichyl-PP-GlcNAc. Although the addition of exogenous dolichyl-P to these cells could not overcome the inhibition, the addition of a tripeptide that has been shown to act as an acceptor of oligosaccharide from lipid-linked oligosaccharide (Wieland et al., 1987) did show some reversal of the inhibition. Since levels of various key enzymes or intermediates did not appear to be altered in puromycin-treated cells, it seems likely that the formation of $Glc_3Man_9(GlcNAc)_2$-PP-dolichol is subject to feedback control, perhaps at the level of GlcNAc-1-P transferase.

Experimental Procedures

Materials. Dulbecco's modified medium, F-12 nutrient mixture, and fetal calf serum were from Gibco Labs. MEM α-medium was from Hazelton Co., and FTA hemagglutination

† This research was supported by a grant from the Robert A. Welch Foundation and by Grant HL-17783 from the National Heart and Lung Institute.

0006-2960/90/0429-8077$02.50/0

buffer was from BBL Microbiology Systems. [2-^{3}H]Mannose (10 Ci/mmol) and [6-^{3}H]glucosamine (25 Ci/mmol) were purchased from American Radiolabeled Co. or from ICN. UDP-[^{3}H]GlcNAc (283 mCi/mmol), GDP-[^{14}C]mannose (269 mCi/mmol), and [4,5-^{3}H]leucine (50 Ci/mmol) were obtained from New England Nuclear Co. Bio-Gel P-4 (200–400 mesh) was from Bio-Rad, endoglucosaminidase H (Endo H) from Miles Labs, and Pronase from Calbiochem. Cycloheximide, puromycin, and β-hydroxynorvaline were obtained from Sigma Chemical Co., and β-fluoroasparagine (Rathod et al., 1986) was generously supplied by Drs. P. Rathod and R. Abeles, Brandeis University. Dolichyl-P was kindly supplied by Dr. Roy Keenan of this department, who isolated it from pig liver. The tripeptide *N*-acyl-Asn-Tyr-Thr-NH_2, which was used as an acceptor (Wieland et al., 1987), was graciously donated by Drs. Wieland and J. Rothman, Stanford University.

Growth and Labeling of Cells. Madin-Darby canine kidney (MDCK) cells were grown to confluency in six-well Corning plates as previously described (Pan & Elbein, 1982). Cultures were preincubated with various amounts of inhibitors for the times indicated in the figures, and then [2-^{3}H]mannose, [6-^{3}H]glucosamine, or [4,5-^{3}H]leucine was added. After a second incubation, the cell monolayers were washed well with phosphate-buffered saline (PBS), scraped from the plates, and extracted with organic solvents to isolate lipid-linked monosaccharides and lipid-linked oligosaccharides.

Addition of Dolichyl-P to Cell Cultures. In some experiments, dolichyl-P was added to control and inhibited cell cultures to determine if it could overcome the effect(s) of the various inhibitors on the synthesis of lipid-linked saccharides. Confluent monolayers of MDCK cells were placed in 2% MEM, and various amounts of the appropriate inhibitor were added. After an incubation of 1 h, dolichyl-P, at several different concentrations, was added to the cells. The dolichyl-P was suspended either in methanol or in dimethyl sulfoxide and was added directly to the medium while the plates were being agitated. Control plates were also done in which the same amount of solvent, but without dolichyl-P, was added to the medium. The amount of solvent added was kept under 5% of the total volume of the medium. No effect of solvent alone was observed with regard to mannose or glucosamine incorporation into lipid-linked saccharides or glycoproteins. The cells were usually incubated in the presence of inhibitor and dolichyl-P for 1 h before the addition of the [2-^{3}H]mannose, although in some experiments [3H]mannose and dolichyl-P were added simultaneously.

Isolation of Lipid-Linked Saccharides and Glycoproteins. The cell suspensions, harvested after various times of incubation with or without inhibitor, were extracted with different mixtures of $CHCl_3$–CH_3OH–H_2O to isolate the lipid-linked monosaccharides, lipid-linked oligosaccharides, and glycoproteins (Chambers & Elbein, 1975). Briefly, the lipid-linked monosaccharides were first extracted from cell pellets with $CHCl_3$–CH_3OH–H_2O (1:1:1), and then lipid-linked oligosaccharides were extracted from the cell pellet with $CHCl_3$–CH_3OH–H_2O (10:10:3). The residue remaining after these extractions was digested with Pronase to obtain the glycopeptides.

Characterization of the Oligosaccharides. The oligosaccharides were released from the lipid-linked oligosaccharides by mild acid hydrolysis. Oligosaccharides and glycopeptides obtained were identified by chromatography on a 1.5 × 100 cm column of Bio-Gel P-4 (200–400 mesh), equilibrated and run in 0.35% acetic acid. Oligosaccharide or glycopeptide peaks emerging from the column were pooled, concentrated to dryness, taken up in 50 mM citrate buffer, pH 6.0, and digested with 10 milliunits of endoglucosaminidase H. Products of this digestion were identified on the Bio-Gel P-4 columns. For additional characterization, oligosaccharides were treated with jack bean α-mannosidase as previously described (Pan & Elbein, 1982). Products were again identified by gel filtration on columns of Bio-Gel P-4.

Determination of Levels of Sugar Nucleotides. To determine whether any of the various treatments altered the levels of sugar nucleotides, confluent MDCK cells were incubated for several hours in the presence of various amounts of puromycin, and then either [2-^{3}H]mannose or [6-^{3}H]glucosamine was added to label the sugar nucleotides. After an incubation of 1 h, cells were harvested and extracted with cold 5% trichloroacetic acid. Trichloroacetic acid was removed by extraction with ethyl ether, and sugar nucleotides were identified by paper chromatography on Whatman 3MM paper in ethanol–1 M ammonium acetate, pH 7.5 (7:3), and in isobutyric acid–NH_4OH–H_2O (57:4:39). The amount of radioactivity in GDP-mannose or UDP-GlcNAc was compared in control cells and in cells incubated with various amounts of puromycin.

Assay of Glycosyltransferases. In order to determine whether the inhibitors of protein synthesis would also affect the activities of the various glycosyltransferases involved in the biosynthesis of lipid-linked saccharides, MDCK cells were incubated for various times in the presence of the inhibitors, then cells were harvested and gently broken by homogenization or sonication, and membrane preparations were tested for various glycosyltransferases. GlcNAc-1-P transferase (i.e., transfer of GlcNAc-1-P from UDP-GlcNAc to dolichyl-P) was assayed by measuring the transfer of [^{3}H]GlcNAc from UDP-[^{3}H]GlcNAc into $CHCl_3$–CH_3OH–H_2O (1:1:1) in the presence of exogenous dolichyl-P (Kaushal & Elbein, 1985). Dolichyl-P-mannose synthase was measured by determining the incorporation of [^{14}C]mannose from GDP-[^{14}C]mannose into dolichyl-P, in the presence and absence of added dolichyl-P (Pan et al., 1983). Other mannosyl transferases involved in the synthesis of lipid-linked oligosaccharides were also assayed by following the transfer of mannose from GDP-mannose into lipid-linked oligosaccharides that are soluble in $CHCl_3$–CH_3OH–H_2O (10:10:3).

Results

Inhibitors of Protein Synthesis Also Inhibit Mannose Incorporation into Lipid-Linked Oligosaccharides. The effect of cycloheximide, or puromycin, on the incorporation of [2-^{3}H]mannose into dolichyl-P-mannose, lipid-linked oligosaccharides, and glycoprotein was examined as demonstrated in Figures 1 and 2. Cells were plated in six-well plates and grown until they had just reached confluency. The media was removed, and 2 mL of 2% MEM was added to each well along with the appropriate amount of one of the inhibitors. Control cells did not receive inhibitor, but in the case of the cycloheximide experiments, they received the same volume of 50% methanol. After an incubation of 1 h, 20 μCi of [2-^{3}H]-mannose was added to each well. At the times shown in Figure 1, the media was aspirated from the appropriate wells, and the monolayers were washed three times with PBS. The cells were then removed from the plates by scraping with a plastic paddle and immediately placed in $CHCl_3$–CH_3OH–H_2O (1:1:1). The other extractions for lipid-linked oligosaccharides were done as described under Experimental Procedures. Since there was a possibility that changes in some of the intermediates could occur during the time that it took to scrape the cells and get them into the extraction medium, in several

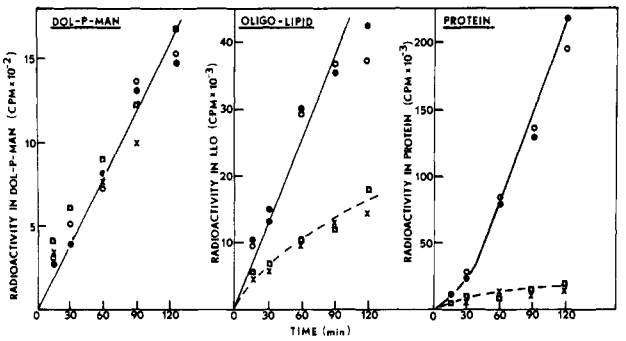

FIGURE 1: Effect of puromycin on the incorporation of [2-^{3}H]mannose into dolichyl-P-mannose, lipid-linked oligosaccharides, and protein in MDCK cells. Cells were grown to near confluency in six-well Limbro dishes. At the start of the experiment, the media were removed by aspiration, and the monolayers were washed several times with PBS. One milliliter of MEM containing 2% fetal calf serum was added to each well, and then various amounts of puromycin were added (0.02–2 mM) as indicated. Three wells from each plate were used for each experimental point. After an incubation of 1 h with inhibitor, [^{3}H]mannose was added to each well, and the incubations were continued for the times shown. At each time, the media were removed by aspiration, and the monolayers were washed three or four times with PBS. The monolayers were then scraped from the plates with a plastic paddle, and the three similar wells were pooled in one centrifuge tube. The cells were isolated by centrifugation and sequentially extracted as described to obtain the various lipid and protein fractions. Curves are identified as follows: (●) control cells; (○) cells in 0.02 mM puromycin; (□) cells in 0.2 mM puromycin; (×) cells in 2 mM puromycin.

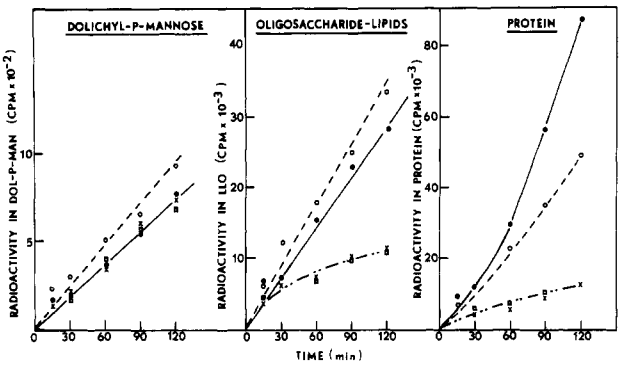

FIGURE 2: Effect of cycloheximide on the incorporation of mannose into dolichyl-P-mannose, lipid-linked oligosaccharides, and protein in MDCK cells. The protocol for this experiment was the same as for that shown in Figure 1 except that cycloheximide was used instead of puromycin. Symbols are as follows: (●) control cells; (○) cells in 10^{-7} M cycloheximide; (×) cells in 10^{-5} M cycloheximide; (□) cells in 10^{-4} M cycloheximide.

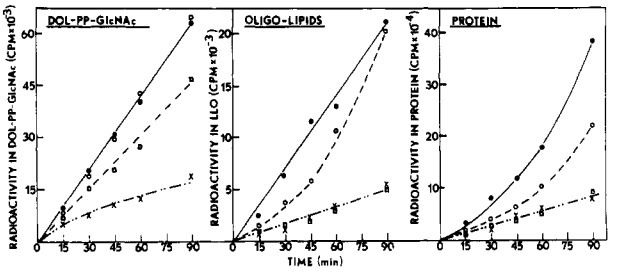

FIGURE 3: Effect of puromycin on the incorporation of [6-^{3}H]-glucosamine into dolichyl-PP-GlcNAc, lipid-linked oligosaccharides, and protein in MDCK cells. The experimental protocol was similar to that in Figures 1 and 2 except that [^{3}H]glucosamine was used as the label instead of mannose. Symbols are as follows: (●) control cells; (○) cells in 0.02 mM puromycin; (□) cells in 0.2 mM puromycin; (×) cells in 2 mM puromycin.

experiments we added 50% CH_3OH to the washed monolayers to rapidly stop metabolism and then scraped the cells from the plates. The results were essentially the same as when cells were scraped in PBS.

It can be seen from the results in Figure 1 that puromycin, at levels of up to 2 mM, had no effect on the synthesis of dolichyl-P-mannose over a 2-h incubation (left panel). However, there was a considerable inhibition of mannose incorporation into lipid-linked oligosaccharides (middle panel) and into glycoprotein (right panel), at both 0.2 and 2 mM concentrations of inhibitor. This inhibition was seen at the earliest (30 min) time point and continued throughout the course of the experiment. It appeared from these time course studies that mannose incorporation into glycoprotein was inhibited first and to the greatest extent, while incorporation into lipid-linked oligosaccharides continued at a very low rate for the 2-h experiment.

A similar experiment using cycloheximide rather than puromycin is shown in Figure 2. In this experiment also, there was no inhibition of mannose incorporation into dolichyl-P-mannose even at cycloheximide concentrations that almost completely inhibited protein synthesis. However, the incorporation of mannose into lipid-linked oligosaccharides and into protein were almost completely inhibited at cycloheximide concentrations of 10^{-5} M, or above. It should be noted that in these cells the amount of radioactivity in dolichyl-P-mannose, at any time, was less than 10% of that found in lipid-linked oligosaccharides or in glycoprotein, probably because the dolichyl-P-mannose is rapidly turning over. Therefore, the sampling of dolichyl-P-mannose is somewhat less reliable. Nevertheless, in repeated experiments (at least four with each inhibitor), the results were similar to those seen in Figures 1 and 2. In addition, similar results were obtained with CHO cells or with HT-29 carcinoma cells when these cells were treated in the same way as the MDCK cells.

Effect of Inhibitors of Protein Synthesis on GlcN Incorporation into Dolichyl-PP-GlcNAc, Lipid-Linked Oligosaccharides, and Glycoproteins. Since cycloheximide and puromycin showed such a pronounced effect on mannose incorporation into lipid-linked oligosaccharides, it was of interest to examine their effects on the incorporation of [^{3}H]glucosamine into the various lipid intermediates and into protein. Figure 3 shows the results of one experiment where puromycin was used as the inhibitor with MDCK cells. It can be seen that puromycin, at 0.2 mM concentrations or higher, strongly inhibited the incorporation of glucosamine into both lipid-linked oligosaccharides (middle panel) and glycoproteins (right panel). However, in contrast to results seen in Figures 1 and 2 with mannose incorporation, in these experiments puromycin also inhibited the incorporation of glucosamine into GlcNAc-PP-dolichol (left panel). At 0.2 mM puromycin, the inhibition was about 30–40%, whereas at 2 mM concentration it was greater than 80%. Similar results were observed when cycloheximide was used in place of puromycin (data not shown). The fact that the formation of dolichyl-PP-GlcNAc was inhibited (Figure 3), whereas no inhibition of dolichyl-P-mannose was observed (Figures 1 and 2), suggested a control point in the biosynthetic pathway somewhere along the pathway of GlcNAc incorporation into lipid-linked saccharides. Although glucosamine is also incorporated into proteoglycan by MDCK cells, most of the radioactivity found in the residue in these experiments (Figure 3, right panel) appeared to be in glycoproteins since it was converted to water-soluble materials by treatment with Pronase, and the glycopeptides were similar in size to those labeled with mannose. Furthermore, addition of various amounts of cetylpyridinium chloride to the Pronase digests to precipitate the proteoglycans followed by the addition of carrier chondroitin sulfate to ensure precipitation only removed about 20% of the radioactivity in the

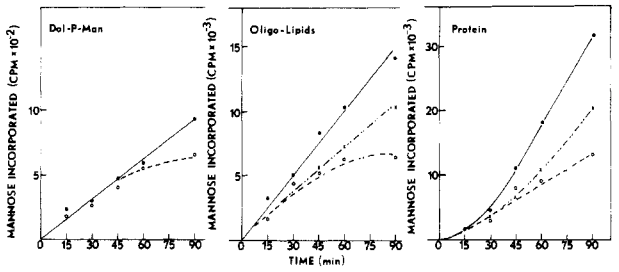

FIGURE 4: Effect of fluoroasparagine on the incorporation of mannose into dolichyl-P-mannose, lipid-linked oligosaccharides, and protein in MDCK cells. The protocol for this experiment followed the same procedure as described in other figures except that fluoroasparagine was used instead of puromycin or cycloheximide. Symbols are as follows: (●) control cells; (×) cells in 1 mM fluoroasparagine; (○) cells in 5 mM fluoroasparagine.

sample, and this loss of radioactivity was the same in control cell extracts and in those cells treated with either puromycin or cycloheximide.

Effect of Other Inhibitors on Mannose Incorporation into Lipid-Linked Saccharides and Glycoproteins. Since the above inhibitors all block protein synthesis, it was of interest to test some inhibitors that might instead affect the glycosylation of the protein, to see whether their effects on the synthesis of lipid-linked oligosaccharides would be similar. β-Hydroxynorvaline is an amino acid analogue that substitutes for the threonine in the Asn-X-Ser (Thr) tripeptide that is required for glycosylation to occur. This substitution prevents glycosylation of those sites having threonine rather than serine in the consensus sequence (Docherty & Aronson, 1985). Fluoroasparagine is another amino acid analogue that inhibits glycosylation by substituting for asparagine residues in the protein (Rathod et al., 1986). MDCK cells were incubated for several hours with various amounts of the above inhibitors, and then, [2-^{3}H]mannose was added to label the lipid-linked saccharides and the glycoprotein. Figure 4 shows the results of an experiment using fluoroasparagine as the inhibitor, at 1 or 5 mM concentrations. At 1 mM there was about a 25% inhibition of mannose incorporation into lipid-linked oligosaccharides (middle panel) and into glycoproteins (right panel), especially after 45 min of incubation or longer. Increasing the concentration of inhibitor to 5 mM caused a greater degree of inhibition, amounting to about 50%. On the other hand, as shown by the panels on the left of Figure 4, there was essentially no inhibition of mannose incorporation into dolichyl-P-mannose, except perhaps at the longest time point. Similar results were observed when hydroxynorvaline was used in place of fluoroasparagine. Although neither of these inhibitors gives complete inhibition of protein glycosylation, probably due to the presence of endogenous protein that is available for glycosylation, the results nevertheless complement those shown above and indicate that levels of dolichyl-P are not the only factor that controls the rate of glycoprotein biosynthesis.

Comparison of Lipid-Linked Saccharides in Control and Inhibited Cells. The lipid-linked monosaccharides formed in the presence and absence of cycloheximide and labeled with either [^{3}H]mannose or [^{3}H]glucosamine were isolated by solvent extraction and identified by thin-layer chromatography on silica gel plates in either $CHCl_3$–CH_3OH–H_2O (65:25:4) or $CHCl_3$–CH_3OH–acetic acid–H_2O (25:15:4:2). In the presence or absence of inhibitors, the major (>90%) mannose-labeled lipid corresponded to dolichyl-P-mannose, while the major glucosamine-containing lipid corresponded to dolichyl-PP-GlcNAc (data not shown). The oligosaccharide portion of the lipid-linked oligosaccharides was also compared in control and inhibited cells. In both cases, on Bio-Gel P-4 columns, the major oligosaccharide released from the lipid by mild acid hydrolysis eluted earlier than the $Glc_3Man_9GlcNAc_1$ standard. However, after treatment of these oligosaccharides with Endo H, both samples migrated with the $Glc_3Man_9GlcNAc_1$ standard (data not shown). These data indicate that the major oligosaccharide associated with the dolichyl-PP in control or inhibited cells is the $Glc_3Man_9(GlcNAc)_2$ structure.

Effect of Inhibitors of Protein Synthesis on CHO Cell Mutants. We examined the effects of puromycin and cycloheximide on mannose incorporation into dolichyl-P-mannose, lipid-linked-oligosaccharide, and glycoprotein in several mutant cell lines of CHO cells. One of these cell lines is called CHO-B421 and is missing the dolichyl-P-mannose synthase (Stoll et al., 1982). This mutant makes a truncated lipid-linked oligosaccharide of the $Man_5(GlcNAc)_2$ structure. The second mutant (SRW) was isolated as a castanospermine-resistant type, and while the genotype is not known, it also makes a truncated lipid that has a $Man_5(GlcNAc)_2$ oligosaccharide (Lehrman & Zeng, 1989). In both of these mutants, puromycin and cycloheximide caused an inhibition of mannose incorporation into lipid-linked oligosaccharide and glycoprotein at essentially the same concentrations and in the same manner as described above with MDCK cells or wild-type CHO cells.

Effect of Exogenous Dolichyl-P on Inhibition of Mannose Incorporation. Since one possible explanation for the results described above could be that dolichyl-P levels are limiting due to the accumulation of lipid-linked oligosaccharides, we examined the effects of adding exogenous dolichyl-P to the inhibited cell cultures to determine whether this could overcome the inhibition. Previous studies by Grant and Lennarz (1983) showed that the addition of dolichyl-P to mouse LM cells resulted in a 300% stimulation in mannose incorporation into lipid-linked saccharides. However, dolichyl-P was not able to overcome the cycloheximide inhibition. Since those studies were done in LM cells in the absence of glucose and without preincubation, we repeated these experiments in MDCK cells under a variety of conditions.

When dolichyl-P, suspended either in methanol or in DMSO, was added to uninhibited MDCK cells incubated in 2% MEM, it caused a significant stimulation in the incorporation of [2-^{3}H]mannose into dolichyl-P-mannose, into lipid-linked oligosaccharides, and into glycoprotein. One such experiment is shown in Figure 5. It can be seen that the addition of 5 μg of dolichyl-P to each well almost doubled the incorporation of mannose into all three components, whereas 20 μg of dolichyl-P gave a 3-fold or more stimulation in the incorporation. On the other hand, when dolichyl-P was replaced with a different type of phospholipid, such as phosphatidylcholine, there was no stimulation in mannose incorporation into any of these compounds. These studies indicate that dolichyl-P is able to enter the cells and to act as an acceptor of mannose.

In other experiments not shown, the incubations with dolichyl-P were extended to 2 h. Although the formation of dolichyl-P-mannose had almost leveled off in control cells and in cells supplemented with dolichyl-P by this time, the incorporation of mannose into lipid-linked oligosaccharides and into glycoproteins was still stimulated by dolichyl-P and was considerably enhanced over that of control cells.

Since the addition of dolichyl-P did cause a significant stimulation in glycoprotein synthesis, we examined the effect of adding dolichyl-P to cells inhibited with puromycin or cycloheximide. That is, if inhibition of lipid-linked oligo-

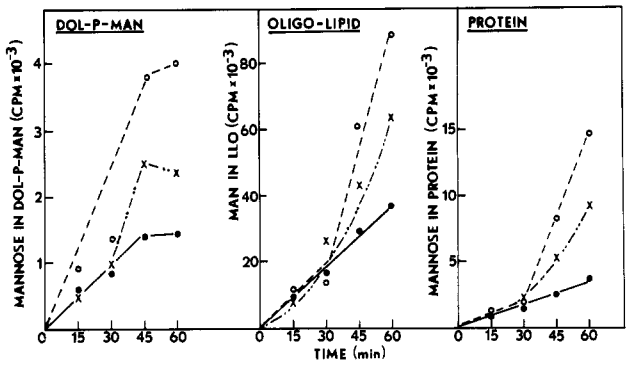

FIGURE 5: Effect of adding dolichyl-P to MDCK cell monolayers on the incorporation of [^{3}H]mannose into dolichyl-P-mannose, lipid-linked oligosaccharides, and protein. In this experiment, dolichyl-P was suspended in methanol and added to the media with vigorous swirling. The volume of methanol added was always less than $^1/_{20}$ of that of the medium. After an incubation of 30 min, during which time the plates were occasionally swirled, [^{3}H]mannose was added, and the incubations were continued for the times shown. Cells were removed and extracted as described. Symbols are as follows: (●) control cells; (×) cells incubated with 5 μg of dolichyl-P; (○) cells incubated with 20 μg of dolichyl-P.

Table I: Effect of Addition of Dolichyl-P on Puromycin Inhibition

			radioactivity incorporated (cpm) into		
				lipid-linked	
sample	inhibitor	dolichyl-P (μmol)	Dol-P-Man	oligo	protein
1		0	1268	4314	16090
2		1	1993	7176	22949
3		2	3404	12888	36979
4		4	4877	21559	50934
5	puromycin	0	1476	1423	947
6	puromycin	1	4624	1689	953
7	puromycin	2	5396	1858	927
8	puromycin	4	10378	2834	866

saccharide synthesis were due to limiting levels of dolichyl-P, exogenous dolichyl-P should overcome the inhibition. Cells were preincubated for 1 or 2 h in the presence of different amounts of puromycin or cycloheximide, and then different amounts of dolichyl-P were added. After an incubation of 1 h, [2-^{3}H]mannose was added, and the incubations were continued for various times. Table I shows the results obtained in one experiment where cells were harvested 1 h after the addition of labeled mannose. The upper four incubations (i.e., samples 1–4) are controls and show that, in the absence of inhibitor, increasing amounts of dolichyl-P cause increased incorporation of mannose into dolichyl-P-mannose, lipid-linked oligosaccharide, and glycoprotein. Maximum stimulation in this experiment was 3–4-fold at about 4 μmol of dolichyl-P/well. On the other hand, as shown by the lower four incubations (samples 5–9), addition of dolichyl-P to cells inhibited by puromycin did not stimulate, or only slightly stimulated, mannose incorporation into lipid-linked oligosaccharide and into glycoprotein. Interestingly enough, even under these conditions of inhibition, mannose incorporation into dolichyl-P-mannose was still stimulated by addition of dolichyl-P, and in fact, the stimulation was almost twice that of control cells. Thus, it seems likely that dolichyl-P is able to enter the cells and act as a mannose acceptor. The fact that it is not able to overcome the puromycin inhibition strongly suggests that inhibition of lipid-linked oligosaccharide synthesis is not due to limitations in the amount of available dolichyl-P. Results similar to those described above were obtained when radioactive mannose and dolichyl-P were added at the same time, or when dolichyl-P was added in DMSO rather than in methanol (data not shown). In addition, the results were similar when cycloheximide was used in place of puromycin.

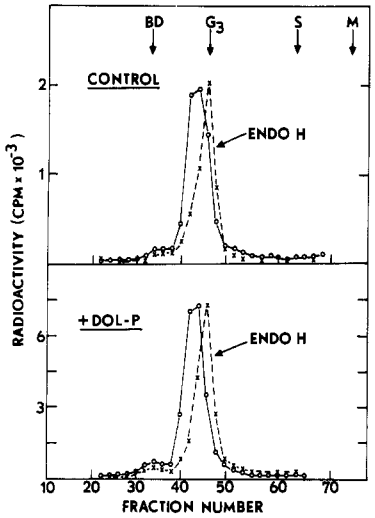

FIGURE 6: Identification of the oligosaccharide portion of the lipid-linked oligosaccharide produced in the presence of dolichyl-P. MDCK cells were grown in a 150-cm flask to near confluency, and dolichyl-P was added to one flask. After 15 min, [^{3}H]mannose was added to label the lipid-linked saccharides. After an incubation of 30 min, cells were harvested, and the lipid-linked oligosaccharides were isolated. The oligosaccharide portion was released by mild acid hydrolysis and separated on a column of Bio-Gel P-4 (○—○). The radioactive peak was pooled, treated with endoglucosaminidase H, and rechromatographed on the Bio-Gel P-4 column (×--×). The upper curve is that of control cells while the lower profiles are from cells treated with dolichyl-P. Standards shown by the arrows are BD = blue dextran, G_3 = $Glc_3Man_9GlcNAc_1$, S = stachyose, and M = mannose.

Effect of Exogenous Dolichyl-P on Glucosamine Incorporation. While dolichyl-P had a great stimulatory effect on mannose incorporation into dolichyl-P-mannose, lipid-linked oligosaccharides, and glycoproteins when added to MDCK cell cultures, it failed to stimulate glucosamine incorporation into dolichyl-PP-GlcNAc or into lipid-linked oligosaccharides (data not shown). The reason for this lack of stimulation is not known, but it may be related to the orientation or "accessibility" of the GlcNAc-1-P transferase to added dolichyl-P, as compared to that of dolichyl-P-mannose synthase. That is, there is some evidence to indicate that some of these endoplasmic reticulum glycosyltransferases have a cytoplasmic orientation whereas others face the lumen of the ER (Hirschberg & Snider, 1987).

Structure of the Oligosaccharide Moiety Produced in the Presence of Dolichyl-P. Since dolichyl-P stimulated mannose incorporation into lipid-linked oligosaccharides 3–4-fold, it was of interest to determine whether the oligosaccharide portion of the lipid-linked oligosaccharides was the same in the presence or absence of added dolichyl-P. A large-scale preparation was done with confluent MDCK cells grown in a 150-cm flask. Dolichyl-P (100 μg) was added to one flask and solvent alone to the control flask. After a preincubation of 1 h in the presence of dolichyl-P, [^{3}H]mannose was added to each flask, and the cells were incubated for another hour. The lipid-linked oligosaccharides were isolated from control cells and from cells incubated with exogenous dolichyl-P, and the oligosaccharides were released from the lipid by mild acid hydrolysis. The oligosaccharides were then chromatographed on a column of Bio-Gel P-4 as shown in Figure 6. The solid lines show that in both cases the oligosaccharides emerged from the column prior to the $Glc_3Man_9GlcNAc$ standard, suggesting that they contained two GlcNAc residues [i.e., $Glc_3Man_9(GlcNAc)_2$]. Additional proof of this structure was shown by the fact that when either oligosaccharide was treated with endoglucosaminidase H, there was a shift in their migration to the same position as the standard, $Glc_3Man_9GlcNAc$. It

Table II: Effects of Puromycin on Glycosyltransferase Activity

	radioactivity incorporated (cpm) from			
	GDP-mannose		UDP-GlcNAc	
control enzyme	−Dol-P	+Dol-P	−Dol-P	+Dol-P
control enzyme				
50 μL	1079	42058	561	919
100 μL	2069	52239	663	813
puromycin enzyme				
50 μL	1032	39647	659	652
100 μL	1851	42410	572	728

should be noted that the amount of radioactivity in the lipid-linked oligosaccharides formed in the presence of dolichyl-P was 3–4 times that of control cells in keeping with the results presented in Table I. Nevertheless, the product produced in the cells stimulated by dolichyl-P was the same as that found in control cells. As indicated above, we also compared the structures of the oligosaccharides produced in the presence of cycloheximide or puromycin to those of control cells. In these cases also, the major oligosaccharide was $Glc_3Man_9(GlcNAc)_2$.

Effect of Inhibitors on Formation of GDP-mannose and UDP-GlcNAc. To determine whether the inhibitors of protein synthesis affected the levels of key sugar nucleotides, MDCK cells were grown in 150-cm flasks to near confluency, and then puromycin, at 0.1 or 1 mM concentration, or cycloheximide, at 10^{-5} or 10^{-3} M concentration, was added to the flasks. After an incubation of several hours to allow the inhibitor to take effect, [^{3}H]mannose or [^{3}H]glucosamine was added to label the cells. Cells were harvested by scraping after various periods of time in the isotopes, and the cell pellet was extracted with ice-cold TCA to extract the acid-soluble nucleotides. After removal of the TCA with ether, the aqueous phase was subjected to chromatography to separate GDP-mannose and UDP-GlcNAc. After a second chromatography, the radioactivity in the sugar nucleotides from control cells or cells grown in the various inhibitors was determined. Essentially no difference was observed in the amount of radioactivity found in GDP-mannose or UDP-GlcNAc in inhibited or control cells, indicating that these inhibitors of protein synthesis do not affect the levels of these sugar nucleotides (data not shown).

Effect of Puromycin on Glycosyltransferase Activities. To determine whether the inhibitors of protein synthesis affected the levels of some of the enzymes in the lipid-linked saccharide pathway, MDCK cells were grown in 150-cm flasks to near confluency and then incubated for several hours in the presence of 2 mM puromycin. The cells were then removed from the plates by scraping, washed well with buffer, and homogenized to prepare a microsomal enzyme fraction. This microsomal fraction was then compared to that from control cells in terms of its ability to transfer mannose from GDP-mannose and to transfer GlcNAc from UDP-GlcNAc into lipid-linked monosaccharides. The results from this experiment are presented in Table II. These assays were done with and without added dolichyl-P to be certain that any effects were not due to limitations in dolichyl-P levels. It can be seen from the data in the table that the puromycin extract was slightly less effective in terms of its transfer of mannose and GlcNAc, especially in the presence of exogenous dolichyl-P. This is probably due to some turnover of these glycosyltransferases during the 2-h incubation and the inability of the cells to synthesize new proteins. However, the results indicate that the effect of puromycin on the formation of the lipid-linked saccharides is not due to changes in the amount of glycosyltransferases, although it could still be due to a feedback type of inhibition which is relieved when cells are disrupted. In addition, we examined the incorporation of mannose from GDP-mannose (and dolichyl-P-mannose) into lipid-linked oligosaccharides in control and puromycin-treated cells (data not shown). Again, no differences were observed in these cell-free extracts, indicating that the mannosyltransferases that add additional mannose residues to GlcNAc-GlcNAc-PP-dolichol were not inhibited. Finally, we added various amounts of puromycin to control cell extracts to see whether it would affect any of these reactions. No decrease in mannose or GlcNAc incorporation was observed.

Addition of Tripeptide Acceptor Partially Overcomes Cycloheximide Inhibition of Lipid-Linked Oligosaccharide Synthesis. Since the inhibition of protein synthesis by puromycin or cycloheximide removes the final oligosaccharide acceptor, it was of interest to determine what would happen to lipid-linked oligosaccharide synthesis in the presence of an artificial acceptor. Wieland et al. (1987) have shown that the tripeptide *N*-acyl-Asn-Tyr-Thr-NH_2, when added to cells, is glycosylated in the ER and then secreted into the medium. In the experimental results shown in Table III, we incubated MDCK cells in the presence of three different concentrations of cycloheximide (10^{-6}, 10^{-4}, 10^{-3}) for 1 h and then added about 2 μg of tripeptide to each well of cells. After another incubation of 1 h, [2-^{3}H]mannose was added, and the cells were incubated for a final hour. The cells were then scraped from the plates and extracted as described above to obtain the various glycolipid and glycoprotein fractions. The results shown in the table indicate that the addition of the tripeptide to the cells does relieve the inhibition of lipid-linked oligosaccharides to a considerable extent, although the incorporation of mannose into 10:10:3 soluble material does not return to control levels. On the other hand, as expected the addition of tripeptide does not affect mannose incorporation into the pellet (i.e., glycoprotein), and this incorporation is inhibited in either the presence or absence of tripeptide. Likewise, the incorporation of mannose into dolichyl-P-mannose (i.e., 1:1:1), while quite low, is not altered either by the addition of cycloheximide or by the addition of tripeptide. In one other experiment, cells were incubated with cycloheximide at 1 mM concentration, and then three different concentrations of tripeptide were added. In the control incubation (cells inhibited with cycloheximide but without tripeptide), 1508 cpm of mannose was found in the lipid-linked oligosaccharides. However with 200 ng of tripeptide, the radioactivity in the lipid-linked oligosaccharides was 1690 cpm, and this increased to 2533 at 500 ng of tripeptide and to 3211 at 800 ng. These studies suggest that the accumulation of lipid-linked oligosaccharides, presumably the $Glc_3Man_9(GlcNAc)_2$-PP-dolichol, somehow signals the cells to stop the synthesis of additional lipid-linked saccharides. Thus, when an artificial glycosylation acceptor is added, the levels of lipid-linked oligosaccharides

Table III: Effect of Tripeptide Acceptor on Inhibition of Lipid-Linked Oligosaccharides

	radioactivity in 1:1:1 (cpm)		radioactivity in 10:10:3 (cpm)		radioactivity in pellet	
treatment of cells	−tripeptide	+tripeptide	−tripeptide	+tripeptide	−tripeptide	+tripeptide
control	236	244	8789	11567	9523	10357
10^{-6} M cycloheximide	214	223	6987	9355	5264	5492
10^{-4} M cycloheximide	218	241	4210	4075	1879	1727
10^{-3} M cycloheximide	223	344	3309	6008	1128	2177

fall, and therefore, inhibition is lifted.

Discussion

Although there is a fair amount of information available about the reactions involved in the biosynthesis of N-linked oligosaccharides, relatively little is known about how this pathway is controlled in the cell. Earlier studies have suggested that alterations in the levels of dolichyl-P regulate the rate of N-linked oligosaccharide biosynthesis in vivo (Lucas & Levin, 1977; Harford & Waechter, 1977, 1980; Lucas & Nevar, 1978). Thus, in a variety of in vitro studies, the addition of dolichyl-P substantially stimulated the glycosylation activity, suggesting that the level of dolichyl-P was limiting. Similar results were obtained with a highly organized multienzyme system from thyroid rough endoplasmic reticulum that is capable of performing N-linked glycosylation of newly synthesized proteins. In isolated ER vesicles, glycosylation reactions were found to be controlled to a large extent by the availability of a key substrate, dolichyl-P, as well as by the amount of endogenous polypeptide acceptor present (Spiro & Spiro, 1986). Furthermore, dolichyl-P levels do fluctuate at various times during the cell cycle or during development, indicating that this may be an important factor in N-linked glycosylation. For example, in the case of the developing sea urchin embryo, it has been shown that there is a 4–5-fold increase in the amount of total dolichols during the compactin-sensitive period prior to gastrulation (Carson & Lennarz, 1981). Most of this increase was in dolichyl-P. The level of dolichyl-P dehydrodolichyl-diphosphate synthase increases in parallel between day 7 and day 23 of spermatogenesis in semineferous tubules of immature rats (Chen et al., 1989). Thus clearly, the biosynthesis and turnover of dolichyl-P in the cell and the level of this sugar acceptor are important factors in the rate and extent of N-linked glycosylation. However, it seems likely that there are other control points in the pathways of N-linked glycosylation that are perhaps exerted at the level of specific enzymes in the pathway.

In the present study, we have examined the factors that regulate the glycosylation of proteins in various cultured cells. Since previous studies had demonstrated that the inhibition of protein synthesis (or RNA synthesis) also resulted in an inhibition of mannose incorporation into lipid-linked oligosaccharides, we used puromycin, cycloheximide, and several glycosylation inhibitors to try and determine whether this inhibition of lipid-linked oligosaccharides was due to a lack of sufficient amounts of dolichyl-P to act as a sugar acceptor. With fluoroasparagine and β-hydroxynorvaline, the glycosylation site is altered, and therefore, there is diminished glycosylation, but the synthesis of the proteins is apparently normal. However, in the presence of these inhibitors, as well as with puromycin and cycloheximide, mannose incorporation into lipid-linked oligosaccharide, but not into dolichyl-P-mannose, was inhibited.

Of course, these results could be explained if the amount of dolichyl-P were limiting as a result of it being tied up in the form of lipid-linked oligosaccharides. However, this explanation is apparently not tenable since the addition of exogenous dolichyl-P to the cell cultures did not overcome the inhibition of mannose incorporation into lipid-linked oligosaccharides, even though the addition of dolichyl-P to control cells did stimulate mannose incorporation into dolichyl-P-mannose, lipid-linked oligosaccharides, and protein by 3–5-fold. Thus, the dolichyl-P does appear to be taken up by these MDCK cells and to be able to reach the site of N-linked oligosaccharide synthesis. Similar results were reported previously by Grant and Lennarz (1983), who also added dolichyl-P to cells to overcome the cycloheximide inhibition. Interestingly enough, we could overcome the puromycin-induced inhibition of mannose incorporation into lipid-linked oligosaccharides to some extent by adding a tripeptide to the inhibited cells. This tripeptide has been shown to act as an acceptor of oligosaccharide in other systems (Wieland et al., 1987). Although we did not have sufficient amounts of material to show that tripeptide did act as an acceptor, its addition to inhibited cells reversed the inhibition by as much as 50%. These experiments might suggest that the accumulation of lipid-linked oligosaccharides, as a result of the inhibition of protein synthesis or protein glycosylation, leads to the formation of some intermediate (perhaps lipid-linked oligosaccharide itself or some other compound) that inhibits further biosynthesis of lipid-linked oligosaccharides. Grant and Lennarz suggested that GTP might be the cause of this inhibition, and in fact, they showed that GTP does inhibit mannose transfer, from GDP-[^{14}C]mannose into lipid-linked oligosaccharides. Interestingly enough, Spiro and Spiro using intact and disrupted thyroid ER vesicles to study dolichyl-P effects on glycosylation found that GTP was a good inhibitor of mannosyltransferase activity that synthesizes dolichyl-P-mannose (Spiro & Spiro, 1986).

In our experiments, we have not been able to pinpoint the exact site of inhibition in the lipid-linked saccharide pathway nor the intermediate involved in the inhibition. When cell-free extracts were prepared from MDCK cells that had been incubated for 2–3 h in puromycin, these extracts incorporated mannose, from GDP-mannose, and GlcNAc, from UDP-GlcNAc, into lipid-linked monosaccharides and lipid-linked oligosaccharides to the same extent as control cells. Thus, in disrupted cells, at least, the various glycosyltransferases are apparently normal. Furthermore, the addition of puromycin or cycloheximide to control cell extracts did not affect the activity of any of these glycosyltransferases. We also examined the levels of the sugar nucleotides, GDP-mannose and UDP-GlcNAc, in cells inhibited with puromycin or cycloheximide and could find no differences from those of control cells. We have not yet looked at the levels of other nucleotides such as GTP, GDP, ATP, etc. but will do so in the near future.

Perhaps the major clue to the site of inhibition is the observation that glucosamine incorporation into GlcNAc-PP-dolichol is inhibited by puromycin or cycloheximide. This is in contrast to mannose incorporation into dolichyl-P-mannose, which, white quite low in these cells, is consistently not affected by these inhibitors. Since the formation of GlcNAc-PP-dolichol is the first step in the lipid-linked oligosaccharide pathway, this enzyme would seem to be a likely point for control. In fact, studies by Kean (1982) with a microsomal enzyme preparation have shown that addition of dolichyl-P-mannose inhibits the incorporation of GlcNAc, from UDP-[^{3}H]GlcNAc into GlcNAc-PP-dolichol. We have observed similar results with a partially purified GlcNAc-1-P transferase from pig aorta (Kaushal & Elbein, 1985). It is interesting to note that studies by Chapman and Calhoun on the effects of glucose starvation of CHO cells on the synthesis of lipid-linked oligosaccharides and N-linked glycoproteins found that cycloheximide and puromycin did not inhibit the formation of lipid-linked oligosaccharides in glucose-starved cells. In fact, glucose-starved cells incubated in puromycin produced the usual $Glc_3Man_9(GlcNAc)_2$-PP-dolichol, whereas glucose-starved cells in the absence of puromycin produced mostly the $Man_5(GlcNAc)_2$-PP-dolichol (Chapman & Calhoun, 1988). The authors suggest that starved cells may lack a cytoplasmic factor (such as GTP) that is responsible for shutting off the

lipid-linked oligosaccharide synthesis in glucose-fed cells.

ACKNOWLEDGMENTS

We thank Drs. Rathod and Abeles for their generous sample of β-fluoroasparagine and Drs. Wieland and Rothman for the tripeptide. We also thank Dr. Sharon Krag for the CHO-B421 culture and Dr. Mark Lehrman for the CHO-SRW cell line.

Registry No. Dolichyl-PP-GlcNAc, 56938-89-7; dolichyl-P, 12698-55-4; dolichyl-P-mannose, 55598-56-6; GlcNAc-1-P transferase, 70431-08-2; dolichyl-P-mannose synthase, 62213-44-9; mannosyl transferase, 9055-06-5.

REFERENCES

Carson, D. D., & Lennarz, W. J. (1981) *J. Biol. Chem. 256*, 4679–4684.

Chambers, J. P., & Elbein, A. D. (1975) *J. Biol. Chem. 250*, 6504–6513.

Chapman, A. E., & Calhoun, J. C., IV (1988) *Arch. Biochem. Biophys. 260*, 320–333.

Chen, Z., Romrell, L. J., & Allen, C. M. (1989) *J. Biol. Chem. 264*, 3849–3853.

Docherty, P. A., & Aronson, N. N., Jr. (1985) *J. Biol. Chem. 260*, 10847–10855.

Elbein, A. D. (1979) *Annu. Rev. Plant Physiol. 30*, 239–272.

Grant, S. R., & Lennarz, W. J. (1983) *Eur. J. Biochem. 134*, 575–583.

Harford, J. B., & Waechter, C. W. (1980) *Biochem. J. 188*, 481–490.

Harford, J. B., Waechter, C. W., & Earl, F. L. (1977) *Biochem. Biophys. Res. Commun. 76*, 1036–1043.

Hirschberg, C., & Snider, M. D. (1987) *Annu. Rev. Biochem. 56*, 63–88.

Hubbard, S. C., & Robbins, P. W. (1980) *J. Biol. Chem. 255*, 11782–11793.

Hubbard, S. C., & Ivatt, R. J. (1981) *Annu. Rev. Biochem. 50*, 555–583.

Kaushal, G. P., & Elbein, A. D. (1985) *J. Biol. Chem. 260*, 16303–16309.

Kean, E. L. (1982) *J. Biol. Chem. 257*, 7952–7954.

Kornfeld, R., & Kornfeld, S. (1985) *Annu. Rev. Biochem. 54*, 631–664.

Lehrman, M. A., & Zeng, Y. (1989) *J. Biol. Chem. 264*, 1584–1593.

Lucas, J. J., & Levin, E. (1977) *J. Biol. Chem. 252*, 4330–4336.

Lucas, J. J., & Nevar, C. (1978) *Biochim. Biophys. Acta 528*, 475–482.

Pan, Y. T., & Elbein, A. D. (1982) *J. Biol. Chem. 257*, 2795–2801.

Pan, Y. T., Hori, H., Saul, R., Sanford, B. A., Molyneux, R. J., & Elbein, A. D. (1983) *Biochemistry 22*, 3975–3984.

Rathod, P. K., Tasjian, A. H., Jr., & Abeles, R. H. (1986) *J. Biol. Chem. 261*, 6461–6469.

Schmitt, J. W., & Elbein, A. D. (1979) *J. Biol. Chem. 254*, 12291–12294.

Spiro, M. J., & Spiro, R. G. (1986) *J. Biol. Chem. 261*, 14725–14732.

Spiro, M. J., Spiro, R. G., & Bhoyroo, V. D. (1976) *J. Biol. Chem. 251*, 6400–6408.

Stoll, J., Robbins, A., & Krag, S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2296–2300.

Struck, D. J., & Lennarz, W. J. (1979) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W. J., Ed.) pp 35–73, Plenum Press, New York.

White, D. A., & Speake, B. K. (1980) *Biochem. J. 192*, 297–301.

Wieland, F. T., Gleason, M. L., Serafini, T. A., & Rothman, J. E. (1987) *Cell 50*, 289–300.