GLYCOSYLATION OF NASCENT POLYPEPTIDES IN *ASPERGILLUS NIGER*

A DISSERTATION

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We hereby recommend that the dissertation prepared under our supervision by Sheila Dawn Long entitled Glycosylation of Nascent Polypeptides in Aspergillus niger

be accepted as fulfilling this part of the requirements for the Degree of Doctor of Philosophy.

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CHAPTER I

INTRODUCTION

It has been reported that glycosylation is an essential step in the normal secretion of many proteins including hormones, enzymes, and immunoglobulins, and yet the mechanisms of carbohydrate attachment to proteins and the role this covalently bound unit plays is still a mystery. It has been determined through the use of inhibitors of glycosylation, that the attachment of carbohydrate to IgG1 in plasma cells is necessary for the transport of these protein molecules into rough and smooth membrane but is not necessary for final secretion from the cells[1]. On the other hand, a normally glycosylated k-type immunoglobulin light chain (k-46), secreted from a mouse myeloma tumor, treated with inhibitors of glycosylation, continues to be secreted in a non-glycosylated form[2]. In this case, glycosylation was not essential to the biosynthesis or secretion of k-46. Therefore, while the glycosylation of secretory proteins <u>in vivo</u> has been described for a number of systems with varying requirements for secretion, there is presently inadequate information concerning the initial steps in the transfer of the core oligosaccharide chain to the protein acceptor. Recent evidence suggests that the initial site of transfer occurs while the polypeptide is nascent[3,4]; however,

several investigators have shown that the carbohydrate can be transferred to the released protein[5,6]. The development of an efficient cell-free glycosylating system should help clarify this initial step by providing an adequate means for preparing non-glycosylated or partially glycosylated protein acceptors. In this way, the transfer of the core oligosaccharides can be manipulated *in vitro* and the nature of the transfer further elucidated. We have examined the *in vivo* and *in vitro* glycosylation of nascent polypeptides from *Aspergillus niger* (*A. niger*) in the presence of 2-deoxy-D-glucose (2DOG) and tunicamycin (TM), inhibitors of glycosylation.

It has been established that 2DOG will inhibit the glycosylation and often the secretion of several glycoproteins such as invertiase[7], acid phosphatase[8], alpha-glucosidase[8], and viral glycoprotein[9]. It appears that 2DOG specifically inhibits phosphoglucose isomerase[10] and phosphomannose isomerase[8], thereby reducing the availability of mannose to the core acceptors. In addition, 2DOG exerts an effect on multiple sites within the cell due to its inhibition of protein synthesis. On the other hand, TM is a glucosamine-containing antibiotic, which has been reported to inhibit the synthesis of glycoproteins and mannan peptides without decreasing protein synthesis[11,12]. Further work has demonstrated that the transfer of N-acetyl-glucosamine

from UDP-N-acetyl-glucosamine-1-P to dolichol phosphate can be inhibited by TM, but the transfer of GDP-mannose to dolichyl-phosphate is unaffected[13,14,15]. In this report we describe a procedure for the *in vitro* glycosylation of nascent polypeptides and demonstrate that 2DOG and TM inhibit the *in vivo* glycosylation of polypeptides in *A. niger* but have no effect on *in vitro* glycosylation.

CHAPTER II

EXPERIMENTAL PROCEDURE

Materials

GDP-(^{14}C)mannose (179 mCi/mmol), (^{14}C)mannose (59 mCi/mol) and (^{3}H)mannose (2.7 Ci/mmol) were obtained from Amersham Searle Corporation, Arlington Heights, Ill., (^{3}H)uridine (28 Ci/mmol) from Research Products International, and (^{3}H)leucine (60mCi/mmole) from ICN. Nonidet P-40 was purchased from Particle Data Laboratories, Elmhurst, Ill. Ethylenediaminetetraacetic acid, Ribonuclease A, Gum Guar, Cycloheximide, RNase-free sucrose, Diethylpyrocarbonate, and 2-deoxy-D-glucose were obtained from Sigma Chemical Co., St. Louis, Mo. Con A-Sepharose was from Pharmacia Fine Chemicals, Inc., Uppsala, Sweden and Bio-Gel P-10 was from Biorad Laboratories, Richmond, Calif. All other chemicals utilized were reagent grade from Baker Chemical Co., Phillipsburg, N.J. Reagents for the chick reticulocyte cell-free system, enzymatic fractions and amino acids were provided by Dr. John Lee, University of Texas at San Antonio Health Science Center. Tunicamycin was a gift of Professor G. Tamura, University of Tokyo, Tokyo, Japan.

Growth Conditions

A. niger was cultured as described previously by Rudick

and Elbein[16]. Twenty-four hr mycelial mats were harvested by filtration through cheesecloth, washed thoroughly with distilled water, frozen in liquid N_2, and ground within 10 sec for a duration of 5 min with a mortar and pestle.

Preparation of Polysomes

The ground mycelia were suspended in extraction buffer (0.01 M Tris-HCL pH 7.6, 0.3 M KCL, 0.005 M mercaptoethanol, 0.01 M spermidine, 0.014 M $MgCl_2$, 1% diethylpyrocarbonate, 1% NP-40) and centrifuged 20 min at 14,360 x g. The microsomal supernatent obtained from the 14,360 centrifugation was layered over a 12 ml 10/40% (w/v) linear sucrose gradient in extraction buffer lacking spermidine and NP-40 and centrifuged 2.5 hrs at 150,000 x g in a Beckman Model L5-65 ultracentrifuge with SW-27 rotor. The gradient tube was punctured from the bottom and absorbance of gradient fractions continually monitered at 259 nm in an Isco Model UA-5 Absorbance Monitor.

To obtain a polysomal pellet, the microsomal supernatent was layered over a 5 ml 40% sucrose cushion and centrifuged 1 hr at 177,700 x g in a Beckman 60 TI rotor.

Analytical Procedures

Radioactive samples were counted in a Beckman liquid scintillation system. Trichloracetic acid-precipitable (TCA) radioactivity was collected by filtration on 0.45 μm

Millipore filters, rinsed with three 50 ml volumes of 5% TCA, dried for 30 min at 160^{0}C and counted in 5 mls of a toulene-based scintillation fluor, or cell precipitates were dissolved in 0.3 mls of Beckman Bio-solv BBS-3 and counted in 5 mls of Bray's scintillation fluid. Protein concentration was determined by the method of Bradford[17].

Preparation of Smooth Membranes

Smooth membranes from A. niger were obtained by disrupting 48 hr mycelia for 5 min at 4^{0}C in a VirTis homogenizer, suspended in 0.1 M Tris-HCl pH 7.6, 0.3 M KCl, and 0.014 M $MgCl_2$, and centrifuged at 390 x g for 10 min and the resulting supernatent centrifuged at 14,360 x g for 20 min. The supernatent was layered over a 10/30% (w/v) discontinuous sucrose gradient and centrifuged 3 hrs at 150,000 x g in a Beckman SW-27 rotor. The membrane collected at the interface was immediately frozen in liquid N_2 and stored at -70^{0}C.

In Vitro Incorporation of (^{14}C)Mannose into Polysomes

In all experiments, polysomes that had been labelled with (^{3}H)leucine for 1 min in vivo, were pelleted as described above and incubated 1 hr at 30^{0}C in a glycosylation system (250 µl) containing at final concentrations, smooth membranes from A. niger (200 µg of protein), 1.25 µCi GDP(^{14}C)mannose, 10mM $MnCl_2$, and 0.05 M Tris-HCl pH 7.0. At the end of the incubation period, NP-40 and PPi were added at a final

concentration of 1% and 1mM respectively to stop the reaction. The membrane was then removed by centrifugation at 14,360 x g for 20 min and the ribosomes were concentrated by centrifugation through 40% sucrose and nascent chains released by addition of EDTA and pancreatic ribonuclease A at a final concentration of 10 mM and 10 µg/ml respectively for 1 hr at 37°C. The ribosome pellet was discarded after centrifugation at 177,700 x g for 1 hr, and the supernatent (3 ml volume) containing released nascent chains was desalted on Bio-gel P-2 (column ht 13 cm x 0.9 cm) and lyopholized. The resulting material was resuspended in 2 mls of H_2O and further purified on Bio-gel P-10 (column ht 57 cm x 1.5 cm) in 0.1 M acetic acid and the (^3H)leucine and (^{14}C)mannose labelled fractions were pooled, lyophilized, resuspended in 0.5 mls H_2O and passed over Con A-Sepharose (column ht 13 cm x 0.9 cm) in 0.1 M sodium acetate, pH 6.0. The bound radioactivity was released from the column with 100 mM alpha-D-methylglucopyranoside.

Immunoprecipitation of Released Nascent Polypeptides

Antibodies against alpha-glucosidase were prepared as described previously [18]. (^3H)leucine labelled polysomes were allowed to glycosylate in vitro as described in the preceeding section and the nascent polypeptides were isolated. The lyophilized protein was resuspended in 130 µl

of phosphate buffered saline with 120 µl anti-alpha-glucosidase and 120 µg carrier alpha-glucosidase for 1 hr at 37°C and the incubation continued overnight at 4°C. The precipitate was washed 3 times with phosphate buffered saline, dissolved in 0.3 mls of Beckman Biosolv BBS-3, and counted in 5 mls of a toulene based scintillation fluid. Background was adjusted by a concurrent control precipitation utilizing ovalbumin.

CHAPTER III

RESULTS

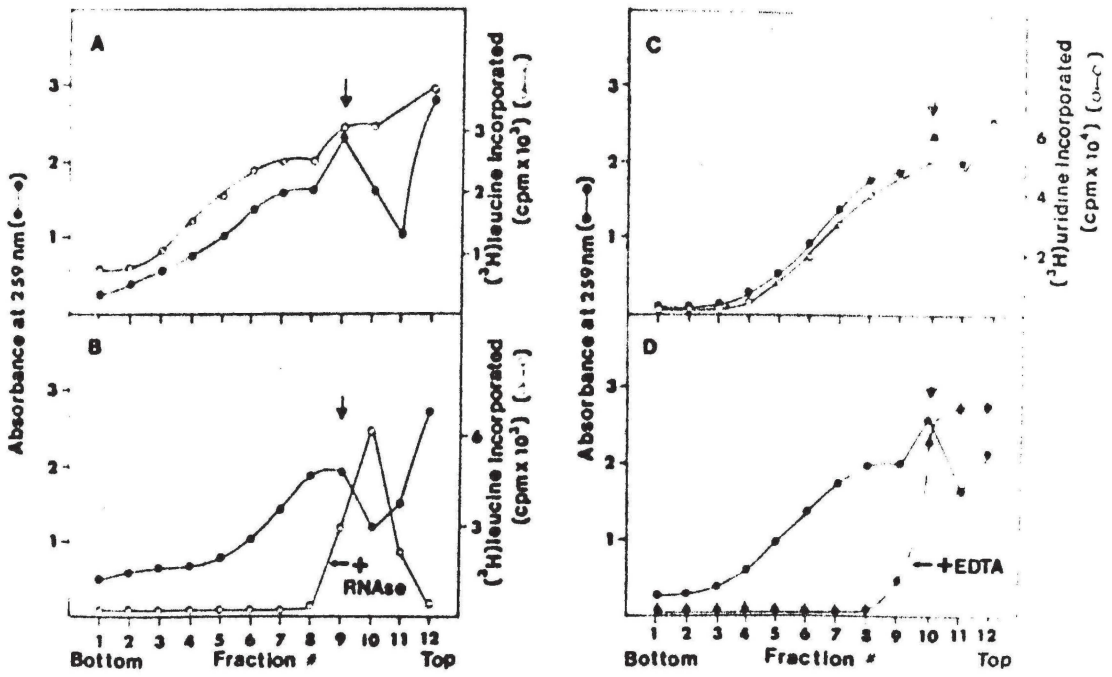
Polysome Characterization

Polysomes were isolated as described in Experimental Procedures and centrifuged on 10/40% sucrose gradients as shown in Fig. 1. To verify that the UV-absorbing material present in the gradient was polysomal, the following tests were performed:

1. RNAse treatment- As indicated in Fig. 1B, 5 mg/ml of pancreatic RNAse A was added to the extraction buffer during polysome isolation and the polysomes analyzed on sucrose gradients. In the control profile, the trichloroacetic (TCA) precipitable (^{3}H)leucine activity is seen in polysomal and monosomal areas, however, in the presence of RNAse, the (^{3}H)-leucine activity shifted exclusively to the monosome area, indicating that the messenger strands of the polysomes had been hydrolyzed.

2. EDTA treatment- 0.02 M EDTA was added to the extraction buffer and the polysomes analyzed on sucrose gradients (Fig. 1D). The control absorption profile displays distinct polysomal and monosomal areas. In the presence of EDTA, all of the absorbing material remained in the top of the gradient suggesting that the chelation of Mg by EDTA effected a

Fig. 1. Polysome analysis in 10/40% sucrose gradients. The arrow indicates the monosome peak and the area to the left includes the polysomes. (^{3}H)leucine (0.025 uCi/ml) was added to 24 hr mycelia 1 min prior to cycloheximide treatment (A). 5 mg/ml of pancreatic RNAse A was added to the extraction buffer during polysome isolation and allowed to incubate 40 min at 4^{o} C before analyzing on sucrose gradients (B). (^{3}H)uridine (5 x 10^{-4} mCi/ml) was added to 24 hr mycelia for 30 min prior to polysome isolation and subsequent gradient analysis (C). 0.02 M EDTA was added to the extraction buffer containing polysomes for 10 min prior to polysome analysis on sucrose gradients (D). Each type of experiment was repeated 3 times.

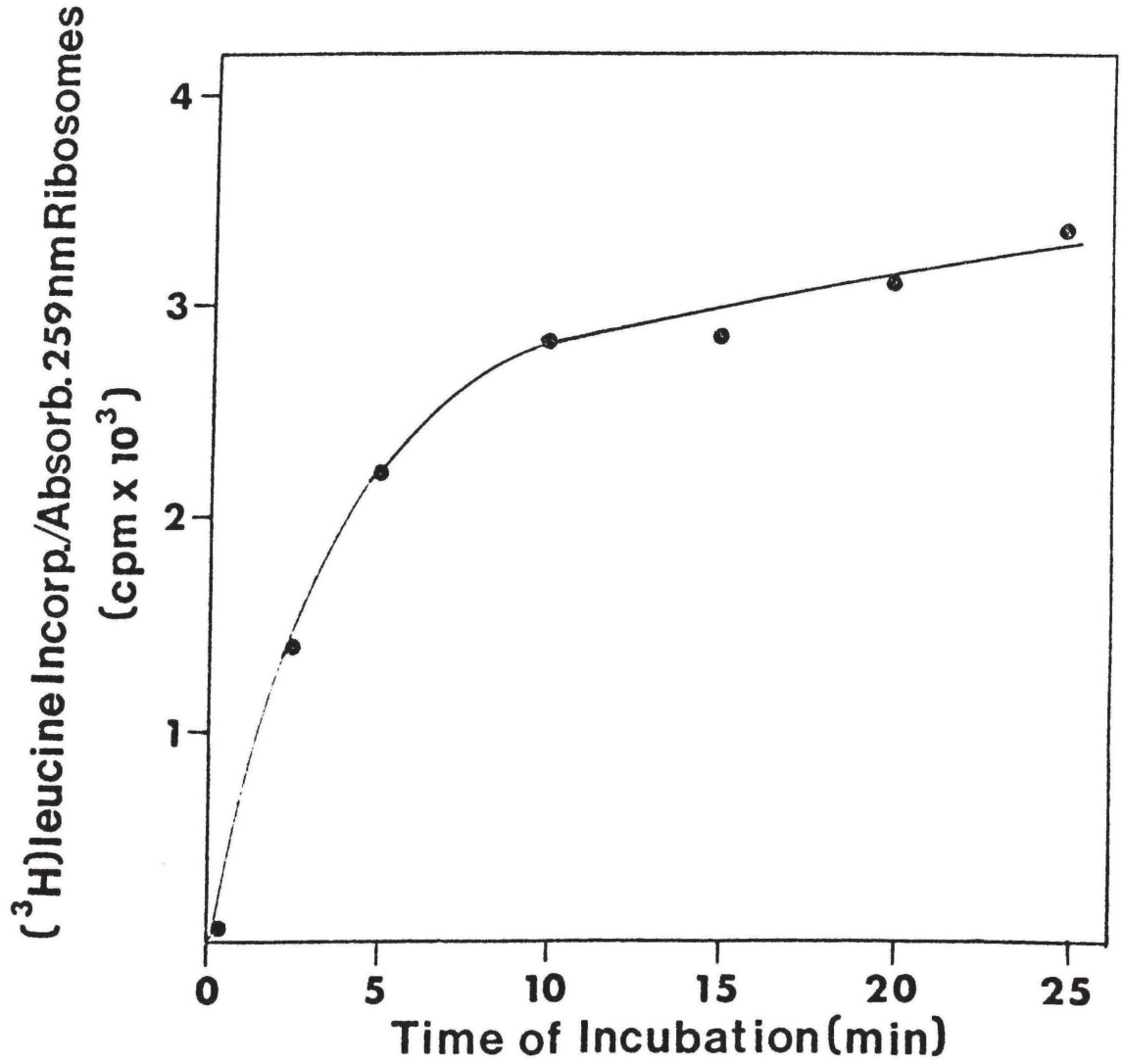

A
B
C
D
Absorbance at 259 nm (●—●)
(³H)leucine incorporated (cpm x 10³) (○---○)
(³H)uridine incorporated (cpm x 10⁴) (○—○)
+
RNAse
+EDTA
1 2 3 4 5 6 7 8 9 10 11 12
Bottom
Fraction #
Top

complete dissociation of the polysome fraction.

3. Cell-free protein synthesis- A modification of the chick reticulocyte system of Lee[19] was utilized to measure the ability of the polysome fraction to incorporate amino acids in a cell-free system without the addition of polysomes from the reticulocyte system. Polysomal pellets were gently homogenized and incubated with the cell-free constituents for 0 to 25 min in a 50 µl total volume and the reaction stopped by the addition of 0.15 mls of 1 M KOH. The TCA precipitable material was counted as indicated in Fig. 2 and the resulting curve indicates a linear incorporation of (^{3}H)leucine into polysomes for 5 min followed by a saturation of incorporation. This demonstrates the ability of the ribosome fraction to support protein synthesis, which is further evidence that a proportion of the ribosomes are polysomal.

In order to demonstrate specific labelling in the polysomal fraction, the incorporation of (^{3}H)leucine and (^{3}H)uridine was observed. (^{3}H)leucine was added at a final concentration of 0.025 µCi/ml to 24 hr mycelia 1 min prior to polysome preparation and analyzed on 10/40% sucrose gradients. The radioactive pattern obtained showed leucine incorporation in the polysomal area as well as the monosome peak, indicating minor breakage of the polysome chains during centrifugation (Fig. 1a). A 30 min pulse of (^{3}H)uridine at a final concentration of 5×10^{-4} mCi/ml was added to 24 hr cultures. The uridine-

Fig. 2. Stimulation of (^{3}H)leucine uptake in a cell-free chick reticulocyte protein synthesizing system. Ribosomes were added to the cell-free system and allowed to undergo protein synthesis for up to 25 min at 5 min intervals. Each sample was then TCA precipitated, washed, and counted as described in the Results. This experiment was repeated 3 times with the same results.

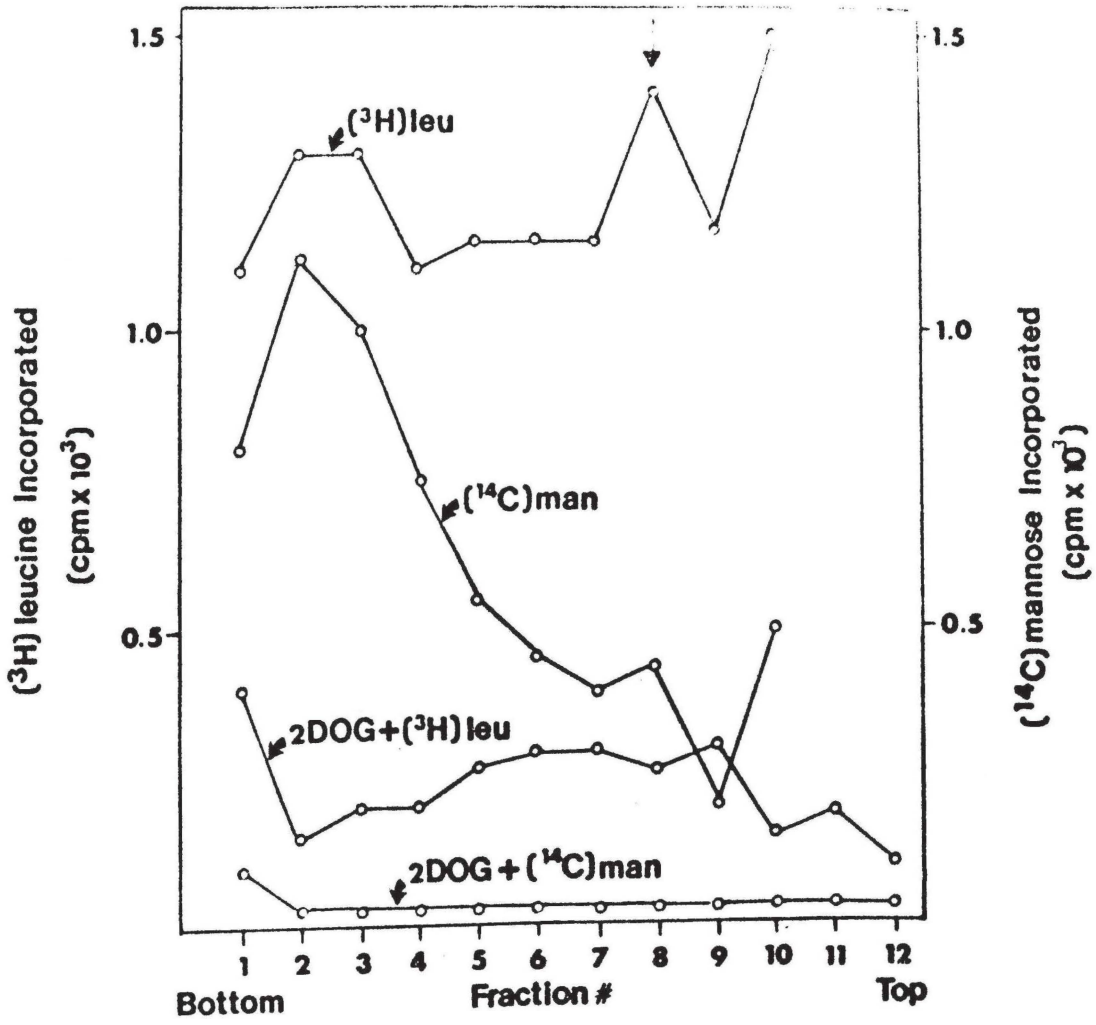

(³H)leucine Incorp./Absorb. 259nm Ribosomes
(cpm x 10³)
4
3
2
1
0
5
10
15
20
25
Time of Incubation (min)

labelling pattern (Fig. 1c) closely follows the same pattern in the absorption curve indicating the absorbing material contained RNA.

In Vivo Glycosylation and the Effects of TM and 2DOG

To determine the initial site of glycosylation in vivo, 24 hr mycelia were labelled 1 min with (^{3}H)leucine and (^{14}C)-mannose at a final concentration of 0.025 μCi/ml and 0.01 μCi/ml respectively. Cycloheximide (100 μg/ml) was added and polysomes immediately prepared. The mannose labelling pattern of the control in Fig. 3 demonstrates a definitive association of mannose with the polysome region. If the cells were treated with cycloheximide (100 μg/ml) initially, and then labelled with (^{3}H)leucine and (^{14}C)mannose for 1 min as just described, no significant amount of label appeared associated with the polysomes. When the cells were preincubated with 0.05% 2DOG for 10 min prior to a 1 min pulse of (^{3}H)leucine and (^{14}C)mannose, and polysomes prepared, the (^{3}H)leucine and (^{14}C)mannose incorporation fell. This indicated an almost complete cessation of protein synthesis, and therefore, cessation of glycosylation (Fig. 3). When the cells were preincubated with 2.5 μg/ml of TM for 10 min, and then given a 1 min pulse of (^{3}H)leucine and (^{14}C)mannose, the mannose incorporation in the treated polysomes fell sharply while the leucine incorporation in the polysome

Fig. 3. *In vivo* polysome gradient labelling patterns. One culture was pre-incubated for 10 min with 2DOG (0.05%). Then the 2DOG-treated culture and a control culture without 2DOG preincubation were labelled with (^{14}C)-mannose (0.01 µCi/ml) and (^{3}H)leucine (0.025 µCi/ml) for 1 min. Polysomes were isolated and centrifuged on 10/40% sucrose gradients. Fractions were collected, TCA precipitated, washed, and counted. This experiment was repeated 3 times with the same results. Equal weights of treated and non-treated mycelia were utilized for all comparison experiments.


1.5
1.0
0.5
(³H)leu
(¹⁴C)man
2DOG+(³H)leu
2DOG+(¹⁴C)man
(³H) leucine Incorporated (cpm x 10³)
(¹⁴C) mannose Incorporated (cpm x 10³)
1 2 3 4 5 6 7 8 9 10 11 12
Bottom
Fraction #
Top

region remained constant (Fig. 4). This indicated that TM caused little or no inhibition of protein synthesis, but greatly reduced the amount of glycosylation occurring.

Characterization of In Vitro Glycosylation

To demonstrate that the in vitro glycosylation detected was not merely a result of membrane glycosylation and release, the ribosome concentration was progressively increased while the membrane concentration remained constant. As can be seen in Fig. 5, there is a linear relationship between the amount of ribosomal material present and the incorporation of (^{14}C)-mannose. This indicates that the polysomes were the component chiefly responsible for mannose incorporation, and not the membranes. In addition, the reconcentrated ribosomal material could be layered on a 10/40% linear sucrose gradient after performing in vitro glycosylation and centrifuged 2.5 hr at 150,000 x g. In the ribosomal profile obtained (Fig. 6), polysomes were still present and labelled with (^{14}C)mannose. Puromycin (100 µg/ml) had no effect on in vivo glycosylation, but at the same concentration, in vitro would release 88% of the (^{14}C)mannose and (^{3}H)leucine label associated with the polysomes (Table 1). RNAse (10 µg/ml) and EDTA (10 mM) would release 72% of the (^{14}C)mannose and (^{3}H)leucine activity (Table 1). It was specifically shown that the ^{14}C-radioactivity was still in mannose. The mannose-labelled polysomal

Fig. 4. In vivo polysome gradient labelling patterns. One culture was preincubated for 10 min with TM (2.5 µg/ml) and then a control and the TM-treated cultures were labelled with (^{14}C)mannose (0.01 µCi/ml) and (^{3}H)leucine (0.025 µCi/ml) for 1 min prior to polysome isolation. The polysomes were centrifuged on 10/40% sucrose gradients, fractions collected, precipitated with TCA, washed, and counted. The experiment was repeated 3 times.

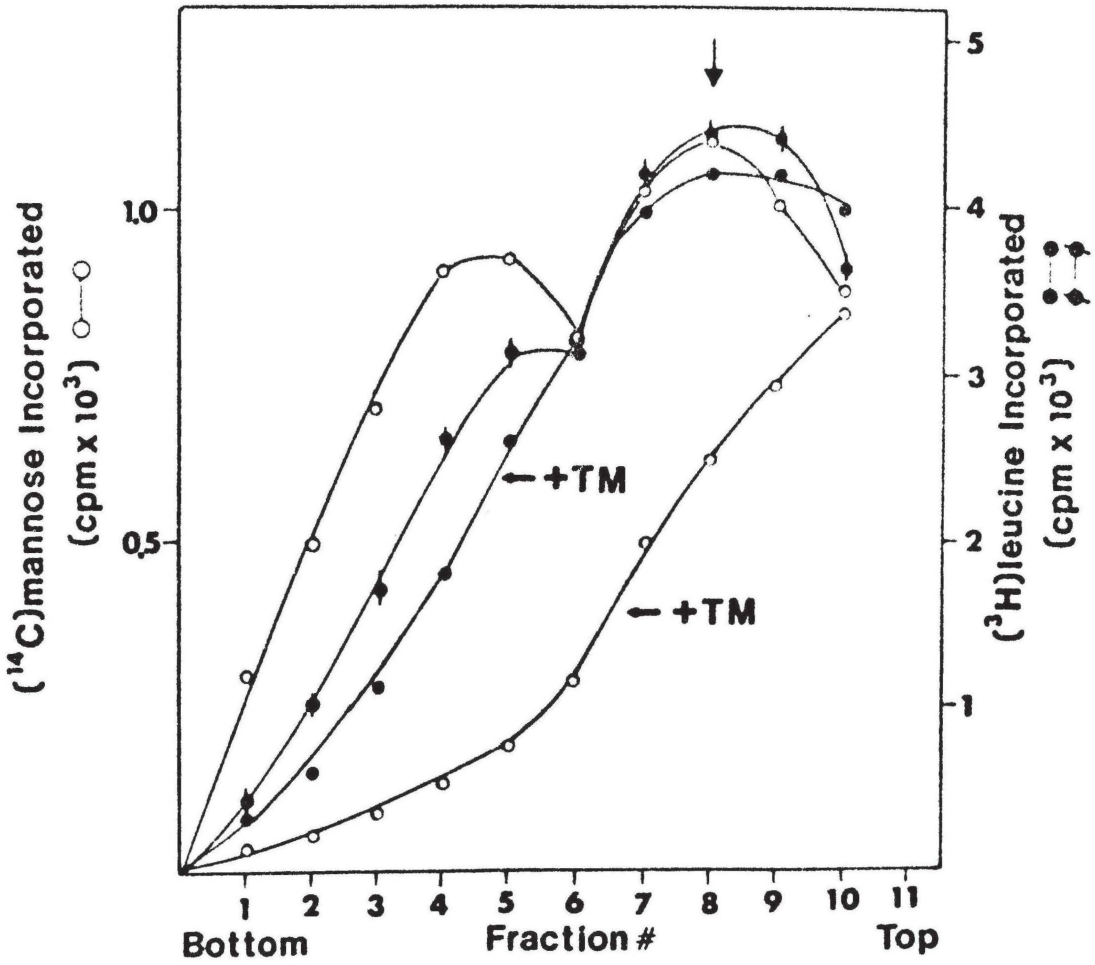

(14C)mannose Incorporated
(cpm x 10³)
1.0
0.5
(3H)leucine Incorporated
(cpm x 10³)
5
4
3
2
1
+TM
+TM
1 2 3 4 5 6 7 8 9 10 11
Bottom
Fraction #
Top

Fig. 5. (^{14}C)mannose incorporation into an *in vitro* glycosylation system as a function of ribosomal concentration. The concentration of ribosomes allowed to glycosylate *in vitro* was gradually increased from 0-24 absorbance units while all other constituents including membrane concentration remained the same. This experiment was repeated twice.

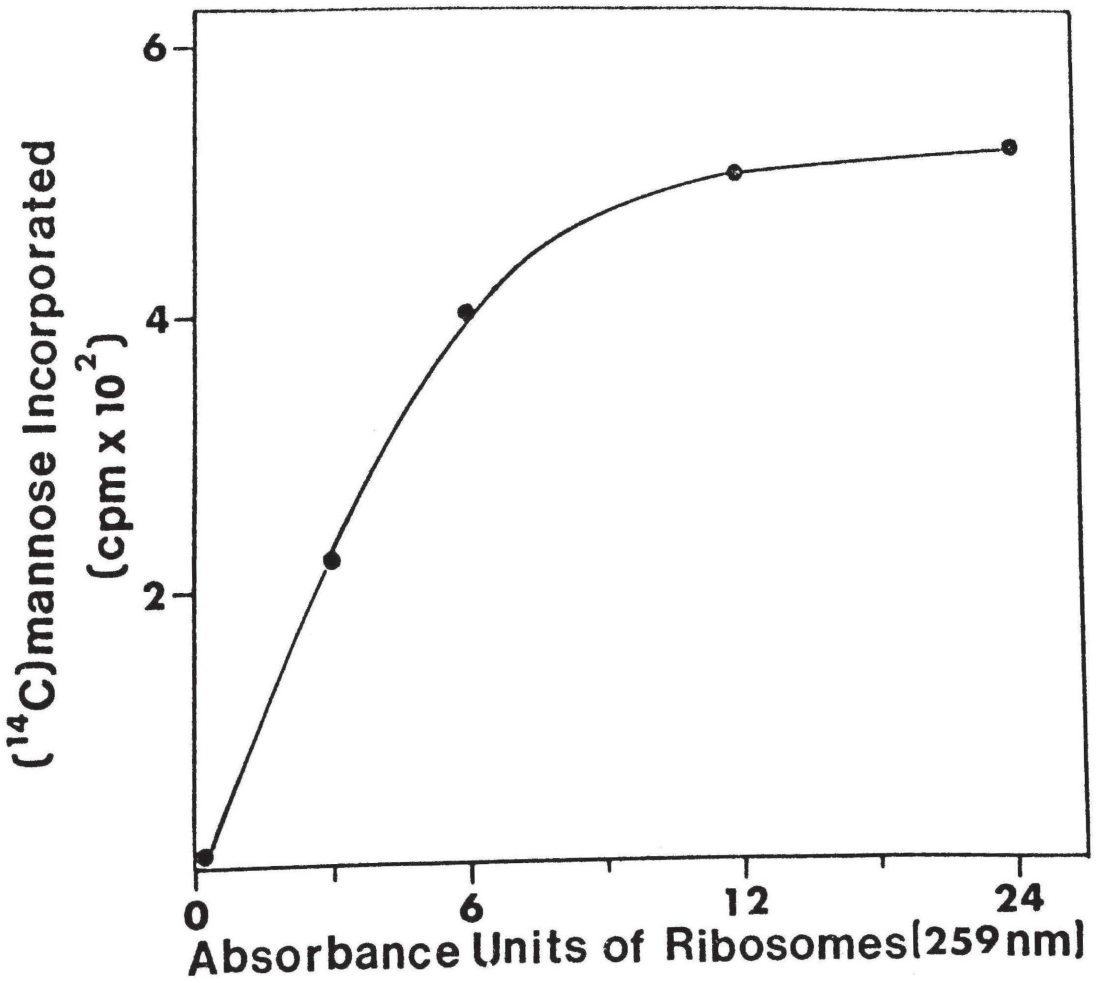

6
4
2
0
6
12
24
(14C)mannose Incorporated
(cpm x 10^2)
Absorbance Units of Ribosomes (259 nm)

Fig. 6. A ribosomal profile of polysomes allowed to glycosylate *in vitro* in the presence of GDP-(^{14}C)mannose for 1 hr at 37° C, reconcentrated by centrifugation, and then centrifuged on a 10/40% sucrose gradient. Fractions were collected, TCA-precipitated, washed, and counted.

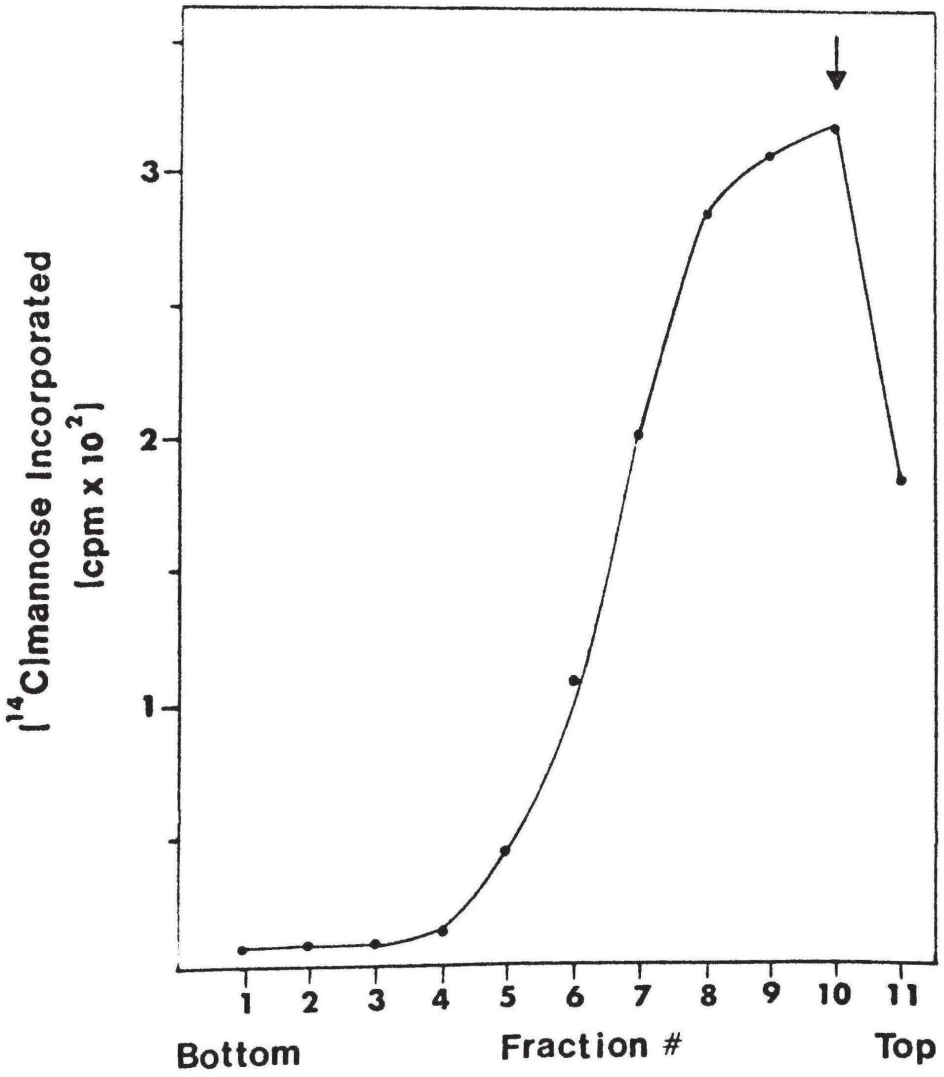

[^{14}C]mannose Incorporated
[cpm x 10^2]
3
2
1
1
2
3
4
5
6
7
8
9
10
11
Bottom
Fraction #
Top

Table 1. (^{3}H) leucine activity in polysomes. (^{3}H) leucine containing polysomes were incubated in the presence of puromycin (100 µg/ml) for 30 min at 30° C or 10 µg/ml RNAse and 10 mM EDTA for 15 min at 37° C. After TCA precipitation overnight, the samples were washed and counted. The experiment was repeated 3 times and the values vary by no more than 10%.

(^{3}H) LEUCINE ACTIVITY IN POLYSOMES (CPM)

	RELEASED BY:	
INTACT (%)	PUROMYCIN (%)	EDTA-RNASE (%)
787 (100%)	687 (88%)	566 (72%)

material was subjected to hydrolysis and subsequent paper chromatography according to the technique of Spiro and Spiro[20].

In Vitro Glycosylation in the Presence of 2DOG or TM

The incorporation of (^{14}C)mannose from GDP(^{14}C)mannose into nascent polypeptides was analyzed in vitro in the presence and absence of 2DOG or TM. When 0.05% or 0.025% 2DOG was added to the glycosylation mixture containing nascent chains labelled with (^{3}H)leucine and allowed to proceed as described earlier, there was no significant difference in the ratio of (^{3}H)leucine to (^{14}C)mannose incorporated as compared to that of control values. The background was computed in all cases by concurrently incubating glycosylation mixtures with membranes in the absence of ribosomes. When 2.5 ug/ml of TM was added to glycosylation mixtures the same results were obtained; i.e. there was no significant difference in the ratio of (^{3}H)leucine/(^{14}C)mannose incorporated into the nascent polypeptides.

Characterization of Nascent Polypeptide Chains

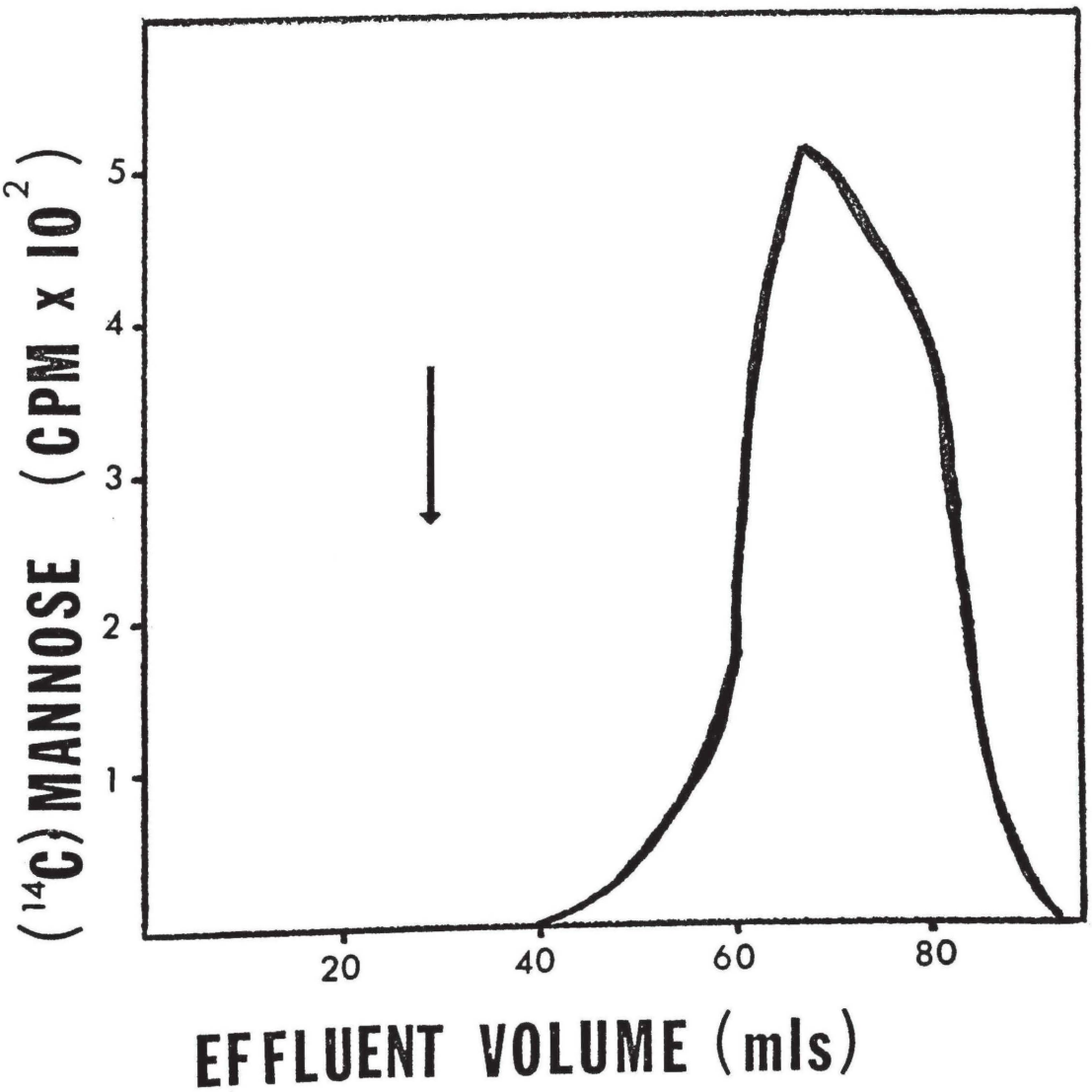
To verify that the polypeptide chains isolated were nascent and glycosylated in vitro, the released chains were first desalted on Biogel P-2 and lyophilized. This material was then subjected to analyses. First, the nascent chains

were further separated on Biogel P-10 and the fractions containing (^{3}H)leucine and (^{14}C)mannose label were pooled. After lyophilization, these fractions were passed through Con A-Sepharose in 0.1 M acetic acid. One-sixth of the label remained attached to the column and was eluted with 100 mM D-methylglucopyranoside. The eluted fraction contained both ^{3}H and ^{14}C.

To approximate the size of the nascent chains, the desalted P-2 lyophilized material from nascent chains glycosylated *in vivo* and from nascent chains glycosylated *in vitro* were separated on Biogel P-30 in 0.1 M acetic acid. The ^{3}H- and ^{14}C-labelled fractions were eluted after the void volume indicating that the chains have a molecular weight of less than 30,000 (Fig. 7). This is consistent with the observation that the chains are included in the void volume of a P-10 column. There seemed to be little distinction between the size of the chains from *in vitro* or *in vivo* experiments.

To demonstrate that a specific protein could be glycosylated by the *in vitro* process, the nascent chains were allowed to react with antibody to alpha-glucosidase, a major extracellular glycoprotein, as described earlier. Four-tenths percent of both the ^{3}H and ^{14}C label was precipitated over that of a control ovalbumin anti-ovalbumin precipitation indicating that a small portion of nascent chains present were

Fig. 7. Elution pattern of released nascent polypeptide chains on a Biogel P-30 column. Released nascent polypeptides that had been glycosylated *in vitro* were concentrated on Biogel P-2, lyophilized, and separated on Biogel P-30 in 0.1 M acetic acid. The arrow indicates the void volume.


(14C) MANNOSE (CPM x IO2)
5
4
3
2
1
20
40
60
80
EFFLUENT VOLUME (mls)

composed of alpha-glucosidase, and that alpha-glucosidase had been glycosylated *in vitro* (Table 2).

Table 2. Immunoprecipitation of *in vitro* glycosylated nascent polypeptide chains. Nascent polypeptide chains glycosylated *in vitro* were immunoprecipitated by the addition of anti-alpha-glucosidase. Background was adjusted by concurrent ovalbumin/anti-ovalbumin precipitation. The experiment was repeated twice.

IMMUNOPRECIPITATION OF IN VITRO GLYCOSYLATED NASCENT POLYPEPTIDE CHAINS WITH ANTI-α-GLUCOSIDASE SERUM

	INITIAL CPM	$(^3H)/(^{14}C)$ RATIO	ANTI-α-GLUCOSIDASE PRECIPITABLE CPM (%)	$(^3H)/(^{14}C)$ RATIO
(^3H)LEUCINE	161,550		684 (0.4)	
		> 1.8		> 1.8
(^{14}C)MANNOSE	89,450		358 (0.4)	

CHAPTER IV

DISCUSSION

Under normal conditions A. niger secretes a variety of extracellular enzymes including alpha-glucosidase, beta-glucosidase, and beta-galactosidase. The carbohydrate composition and oligosaccharide linkage of these glycoproteins have been previously characterized and each possesses a single oligosaccharide chain consisting of mannose and glucosamine[18]. Electron microscopy evidence has suggested that glycosylation of these enzymes, in particular alpha-glucosidase, occurs at the plasma membrane[21]. Beyond this structural and pictorial characterization little information has been obtained. The primary objective of this study was to examine the location and nature of glycosylation in A. niger by comparing a series of in vivo and in vitro experiments. Evidence is presented which suggests that carbohydrate units in our system are initially attached to nascent polypeptide chains and this process occurs through use of a lipid-intermediate.

To our knowledge polysomes had not been previously isolated from A. niger. It was, therefore, necessary to develop an isolation procedure and to verify the polysomal nature of the isolated fraction. RNAse treatment, EDTA

treatment, stimulation of a cell-free protein synthesizing system, and specific labelling with (^{3}H)leucine and (^{3}H)uridine confirmed the polysomal nature of the material. Next, the presence of nascent polypeptide chains in association with the polysomes was verified. Ribosomes isolated from mycelia which had been labelled 1 min with (^{3}H)leucine lost their label after exposure to RNAse or EDTA indicating that a 1 min pulse did not label ribosomal proteins, suggesting that the released label was in nascent polypeptide chains. Furthermore, when (^{3}H)leucine-labelled polysomes were treated with puromycin, which is specific for release of nascent polypeptide chains, 80% of the leucine label was released.

It was now possible to perform <u>in vivo</u> experiments examining glycosylation specifically of nascent chains. After pulsing mycelia for 1 min with (^{3}H)leucine and (^{14}C)mannose it was found that the polysomes were double-labelled. This suggests that the initial steps of glycosylation occur while the polypeptide is still associated with the ribosome. In fact, the mannose label was usually associated with the longer polysomes. This suggests that the longer chains are better acceptors of carbohydrate units. These results are consistent with current reports on the initial location of glycosylation. For example, Kiely et. al.[3] has demonstrated that nascent ovalbumin chains incorporate mannose and glucosamine, but the majority of the carbohydrate is attached as the peptide is nearing release from the polysome complex.

The *in vivo* labelling experiments were modified by preincubating the mycelia with 2DOG or TM prior to labelling with (^{3}H)leucine or (^{14}C)mannose. In the *in vivo* 2DOG experiments, protein synthesis was inhibited in the mycelia to such an extent that it was not possible to determine what effect, if any, 2DOG had on the glycosylation process. But when the mycelia were preincubated 15 min with TM, there was a definite inhibition of (^{14}C)mannose incorporation into polysomes, while the amount of (^{3}H)leucine incorporated closely paralleled the control. These results indicate that the oligosaccharide chain in this system is probably transferred via a lipid-intermediate as has been reported for a variety of systems[11,12,13]. Further experiments were performed to determine if mycelia exposed to TM for longer periods of time would cease secretion of glycoproteins. It was found that when cells were incubated with TM and assayed at hourly intervals for up to 8 hr for the secretion of active enzyme, alpha-glucosidase was secreted in normal quantities and was normally glycosylated. This suggests that the cells are only temporarily sensitive to TM and can overcome or bypass its effects.

An *in vitro* glycosylating system was established in order to more precisely control and manipulate the conditions for glycosylation. Through this system (^{14}C)mannose from GDP(^{14}C)-mannose was incorporated into nascent polypeptides as verified by stimulation of mannose incorporation in the cell free system

when the polysome quantity was increased. In fact, ribosomes, reconcentrated after being allowed to glycosylate *in vitro* could be centrifuged on 10/40% gradients and the resulting polysomal profile showed a definative polysomal region that was (^{14}C)-labelled.

It was of interest to show that specific extracellular enzymes were included in the nascent chain population. Therefore, immunoprecipitation with anti-alpha-glucosidase was tried. The results demonstrated that at least 0.4% of the released nascent chains were alpha-glucosidase polypeptides.

It was difficult to precisely size the released nascent chains and it soon became apparent that there was a broad peak of size ranges from less than 10,000 molecular weight units to 30,000 molecular weight units. Also noteworthy is the fact that chains released from polysomes glycosylated *in vivo* or *in vitro* were of approximately similar lengths.

Next, an attempt was made to determine whether glycosylation could proceed in the presence of 2DOG or TM. 2DOG had no effect on the kinetics of our cell-free system which is not surprising since 2DOG has never been implicated in exerting its effects at the level of carbohydrate transfer to the nascent chain, but rather by decreasing the availability of mannose to the transglycosidases[8]. On the other hand, TM does directly inhibit carbohydrate transfer to the nascent polypeptide *in vivo*, yet TM had no effect on *in vitro*

glycosylation. This occurs because the membrane present in the reaction mixture probably contains oligosaccharide-lipid which can be transferred in the presence of TM. We are presently interested in examining stimulation of the in vitro system with UDP-N-acetyl-glucosamine and dolichol phosphate and in determining whether purified oligosaccharide chains can be transferred in the cell-free system.

Unlike the many membrane glycosylation systems established, this study deals exclusively with the glycosylation of nascent chains. One other in vitro glycosylation system has been developed which reports specific glycosylation of post-translational proteins[3]. This procedure needed extremely high concentrations of carbohydrate donor, was cumbersome, and resulted in low yields. From our data it appears that glycosylation occurs more readily in nascent proteins, indicating that this is the normal in vitro acceptor. In addition, this system has potential for use use in further analysis of carbohydrate transfer and as a system for screening potential inhibitors of glycosylation.

CHAPTER V

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CHAPTER VI

VITA

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