INTRACELLULAR LOCATION OF PROTEIN GLYCOSYLATION IN *ASPERGILLUS NIGER*

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN MOLECULAR BIOLOGY IN THE GRADUATE SCHOOL OF THE TEXAS WOMAN'S UNIVERSITY

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We hereby recommend that the Dissertation prepared under our supervision by Smita Vaidya

entitled Intracellular Location of Protein Glycosylation in Aspergillus niger.

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

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ABSTRACT

Protein glycosylation was measured as a function of time in various subcellular organelles of Aspergillus niger. Initiation of glycosylation appeared to take place in the ribosomes, and its completion may take place in the smooth endoplasmic reticulum (SER) in which the highest mannosyl transferase activity was found. Solubilization of the SER fraction yielded two glycoproteins after SDS polyacrylamide gel electrophoresis suggesting that once the glycosylation of secretory proteins is completed, all the glycoproteins except those bound to the membrane of SER are immediately released from it. An inhibitor of cytoplasmic protein synthesis prevented glycosylation in all but the mitochondrial fraction, indicating a one to one relationship between protein synthesis and glycosylation. Since small amounts of mannosyl transferase were detected in the mitochondria, they may glycosylate some of their own proteins as well as receive glycoproteins from the SER. A considerable amount of glycoprotein was detected in the high speed supernatant (HSS) of the cell, in which a possible precursor of a secretory glycoprotein, α-glucosidase, was detected by immune precipitation.

The HSS may contain several precursors of secretory glycoproteins and the plasma membrane may play a role in changing the precursor into the product.

DEDICATION

For their guidance in my childhood, tolerance in my adolescence and friendship in my adulthood, I dedicate my dissertation to my dear parents.

ACKNOWLEDGMENTS

I am extremely grateful to Dr. Morton W. Miller for his continuous support and encouragement throughout my graduate studies in Biology. My sincere appreciation goes to my sisters, friends, and especially Nuha Yacoub for their humor, patience, and understanding during the laborious and at times exhausting hours of research. I take this opportunity to thank my major professor, Dr. Michael J. Rudick, for teaching me how to think constructively and then put the constructive thought into action efficiently. I also wish to express thanks to my committee members for their valuable suggestions, and last but not least Susan Allen for excellent typing.

TABLE OF CONTENTS

TABLE OF CONTENTS CONTINUED

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES CONTINUED

LIST OF FIGURES CONTINUED

INTRODUCTION

Glycoproteins have been a matter of considerable interest for almost a decade. One of the important reasons for such interest is that glycoproteins are widely distributed in plants as well as in animals and they participate in a number of biological functions (Spiro, 1970). The role(s) of covalently bound carbohydrate are not certain yet but they are thought to include such processes as intercellular adhesion, cellular recognition, and cellular secretion.

Researchers agree that in mammalian cells the polypeptide backbone of these glycoproteins is synthesized according to the well known pathway of protein synthesis. However, as far as the glycosylation of protein and the completion of oligosaccharide side chains are concerned, two major hypotheses exist. One school of thought initiated by Neutra and Leblond (1966) is called the single site hypothesis, according to which the carbohydrate chain is built up in the Golgi cisternae of mammalian cells by a multienzyme complex of carbohydrate transferases, which is located in the membrane of this organelle. This hypothesis is based upon the observation that when rats were injected with [^{3}H] glucose or [^{3}H] galactose,

macromolecules other than glycogen were labeled only in the Golgi region. Similar results were reported by Wagner and Cynkin (1969) where [^{3}H] glcosamine and [^{14}C] mannose were incorporated into the proteins of a smooth microsomal membrane fraction rich in Golgi fragments, but not into the rough microsomal fractions of rabbit and rat liver. Bosmann et al. (1968) reported similar results using HeLa cells.

In the second, or multisite, hypothesis the glycosylation of protein is initiated on the ribosomes during the growth of the polypeptide chain, but the remainder of the oligosaccharide chain is completed within the cisternae of the rough and smooth endoplasmic reticulum, and perhaps the Golgi body in rat liver (Molnar et al, 1975), and in sheep thyroid (Bouchilloux et al., 1973).

Recent developments in glycoprotein research show not only the site of glycosylation, but also the mechanics of sugar attachment. It appears that binding of the sugars to the polypeptide chain one at a time is unlikely. Several investigators (Heifetz and Elbein, 1977; Struck and Lennarz, 1977; Duskin and Bornstein, 1977; Herscovics, et al., 1977) have shown that at first a core of oligo-saccaride is synthesized as a lipid intermediate. According to Keily and Schimke (1976) initiation of

attachment of the core of oligosaccharide takes place while nascent polypeptide chains of ovalbumin are still bound to the ribosomes. The completion of glycosylation may occur in rough and smooth endoplasmic reticulum (Eagon and Heath, 1977). In light of these reports, glycosylation appears to take place in more than one organelle.

Aspergillus niger produces a number of extracellular enzymes, such as glucosidases, galactosidases, mannosidases, all of which are glycoproteins containing covalently bound mannose and glucosamine. This fungus represents a unique system to study glycosylation of protein, because, first of all, its nutritional requirement for growth is simple. It needs a few common salts and a source of energy. Secondly, it can be grown in large quantities in about 48 hours at 30°C. Thirdly, all of the glycoproteins secreted by A. niger contain only two sugars, namely mannose and glucosamine. In short A. niger is easy to grow and its secretory glycoproteins are simple to study. Several secretory glycoproteins of this fungus have been isolated and purified. So far almost no work has been done to locate intracellular site(s) of glycosylation of protein in this system. The purpose of this research is to find the intracellular location(s)

of protein glycosylation and to some extent elucidate the pathway of transport of secretory glycoproteins inside the cells of A. niger.

MATERIALS AND METHODS

Growth Conditions

Aspergillus niger (A. niger) formerly known as Aspergillus fumigatus by Rudick and Elbein (1973, 1974, 1975) until reclassified by Wadley Institute as of November 1975 was grown in a liquid medium containing (grams per liter) : KH_2PO_4 (2), $(NH_4)_2SO_4$ (1.4), Urea (0.3), $MgSO_4$ (0.3), $CaCl_2$ (0.3), mannose (0.1), Difco yeast extract (0.05) and guar flour (5).

A one-liter Erlenmeyer flask containing 500 ml of medium was inoculated with a loop of conidia. After 48 hr of growth on a rotary shaker (120 cycle per minute) at 30°C, mycelia were collected by filteration through 4 layers of cheese cloth. The collected mycelia were washed with distilled water and then used for experimentation.

Preparation of Subcellular Fractions

All operation were done at 0 - 4°C. Five grams of mycelia were distrupted with a Virtis homogenizer at high speed for 5 minutes in 30 ml of 0.25 M sucrose buffered with 1 mM Tris-HCl, Ph 7.4 (ST buffer) with 5 g of glass beads. The homogenate was centrifuged at 2000 rpm (Beckman Model J21B refrigerated centrifuge) or 140 xg to remove glass

beads, cell wall and cell debris. The supernatant was centrifuged at 14,000 rpm or 7,000 xg for 20 min and the mitochondrial pellet resulting from this centrifugation was suspended in 6 ml of ST buffer and stored in the cold. The post mitochondrial supernatant (PMS) was centrifuged through different types of sucrose gradients to get the best separation between the smooth endoplasmic reticulum (SER) and the rough endoplasmic reticulum (RER).

Sucrose Gradient Centrifugation

Method #1

Adelman et al. (1973) designed a method which also required a two-step sucrose gradient, with 2 ml of 1.5 M sucrose in 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$ in the top layer and 2 ml 2 M sucrose same buffer on the bottom layer. The PMS was carefully layered on the top of the gradient, a supernatant on the top of the gradient, two bands in the gradient and a pellet were obtained after centrifuging at 27,000 rpm or 139,000 xg in the SW 27 rotor for 22 hr.

Method #2

Bouchilloux and co-workers reported in 1973 a two-step sucrose gradient which had 2 ml of 2 M sucrose in 20 mM Tris-HCl pH 7.6, 25 mM KCl, 5 mM $MgCl_2$ on the

bottom layer and 1.35 M sucrose in the same buffer in the top layer. After layering the PMS on the top of the gradient, centrifugation was at 27,000 rpm in the SW 27 rotor for 5 hr. A supernatant on the top of the gradient, two bands in the gradient and pellet resulted from this centrifugation.

Method #3

The PMS was carefully layered on 8 ml of a 6 - 60% continuous sucrose gradient buffered with 20 mM Tris-HCl, pH 7.6, 25 mM KCl, 5 mM $MgCl_2$. The tubes containing these gradient were centrifuged at 25,000 rpm or 130,000 xg for 2.5 hr in the SW 27 rotor.

Density markers (Beckman Instruments) were put into two sets of sucrose gradients and then the PMS was layered on the top. One set of gradients was spun for 2.5 hr and the other set for 24 hr. The positions of the markers in both sest were compared.

Analytical Methods

(1) RNA Determination

The purity of the cell fractions was checked as a function of their RNA content using the Orcinol test of Schneider (1960). Since the Orcinol reagent interacts with sucrose, the cell fraction was first precipitated

with 10% trichloroacetic acid (TCA); the precipitates were washed twice with 5% TCA and hydrolyzed with 0.2 ml of 2 M KOH at 37°C for 18 hr and the hydrolysate was neutralized with 2 M Na_2HPO_4. Two-tenth ml of supernatant from each of the neutralized cell fractions was diluted with 1.5 ml of water and 1.5 ml of orcinol reagent was added. Controls were set up containing 0.2 ml of 10% TCA and 0.2 ml of 2 M Na_2HPO_4 neutralized with 2 M KOH. A standard curve was constructed consisting of 0, 25, 50, 75 and 100 nmoles of ribose also treated the same way. The tubes were kept in a boiling water bath for 20 min and their absorbance was determined at 660 nm using a Beckman model 25 spectrophotometer.

RNA content of cell fractions was also assayed by means of the Cerriotti (1951) method. All the fractions were deproteinated by treating them with an equal volume of concentrated perchloric acid, the absorbance of the supernatant was measured at 260 nm. A standard curve of yeast tRNA ranging from 25 to 125 µg was prepared.

(2) Enzyme Assays

(a) Cytochrome c Oxidase

Cytochrome c oxidase was assayed essentially according to Smith (1955). A solution of 1.7×10^{-5} M cytochrome c from horse heart (Nutritional Biochemical

Corporation) in 0.03 M potassium phosphate buffer, pH 7.4 was reduced with 1.2 M $Na_2S_2O_4$ until the ratio of absorbance at 550 nm to 565 nm was greater than 6. The reduced cytochrome c was placed into serum bottles, aerated with nitrogen gas for 10 min, and stored at 4^oC. The activity of cytochrome c oxidase was measured by placing a suitable dilution of cell fraction and 1.7×10^{-5} M of the reduced cytochrome c, into 1.0 ml quartz cuvettes, keeping the final volume of reaction mixture equal to 0.5 ml. Two sets of controls were prepared at room temperature: one contained reduced cytochrome and no cell fractions; the other contained cell fractions without reduced cytochrome c. The decrease in absorbance at 550 nm was recorded for 10 min for each tube and was linear with time.

(b) Glucose-6-phosphatase

This assay was adopted from Beaufay et al. (1974). Two-tenth ml of a suitable dilution of cell fraction incubated with 0.8 ml of a solution of 0.04 M glucose-6-phosphate, 0.007 M histidine, HCl pH 6.5, 0.001 M EDTA at 37^oC for 30 min and then 1 ml of 10% TCA was added to each of the tubes to stop the reaction. The amount of liberated inorganic phosphate was measured by using the method of Chen et al. (1950), as follows. One volume of 6 N H_2SO_4 was mixed with 2 volumes of distilled water,

1 volume of 2.5% ammonium molybdate and 1 volume of freshly prepared 10% ascorbic acid. Two ml of this mixture were added to the tubes containing incubated cell fractions and the absorbance was determined at 700 nm. Two types of controls were used: One contained no glucose-6-phosphate, the other no cell fraction. A standard curve was also prepared using 50-200 mmoles of K_2HPO_4.

(c) NADPH - Dependent Cytochrome c Reductase

At room temperature, according to Beaufay et al. (1974) 0.20 ml of 0.5 M potassium phosphate buffer, pH 7.6, was mixed with 0.10 ml of 0.82 M nicotinamide, the volume of reaction mixture was brought up to 3 ml with distilled water. To this 0.30 ml of 0.001 M NaCN and 0.20 ml of a suitable dilution of cell fraction were added, followed immediately by 0.40 ml of 1.9×10^{-4} M of cytochrome c and 0.1 ml of 2.25 M of NADPH the increase in absorbance at 550 nm was measured for 10 min. Two types of controls were prepared: one with no cytochrome c and the other with no cell fraction.

(d) Glycosyl Transferase

This assay was also adopted from Beaufay et al. (1974), where 0.05 µCi of GDP [^{14}C] mannose (210 mCi/m M: New England Nuclear) with 1 mM Tris-HCl pH 7.4 and 5 mM $MnCl_2$ was incubated with a suitable dilution of cell

fraction at 30 °C in a final volume of 0.2 ml. The reaction was stopped by adding an equal volume of 0.2 ml. The reaction was stopped by adding an equal volume of 10% TCA. The precipitates were collected on filters (0.45 μ pores), after keeping the tubes overnight in the cold. The filters were then dried and counted by using scintillation fluid containing 4 g PPO and 50 mg POPOP in 1.0 ℓ of toluene in a Beckman Liquid Scintillation Counter. Similar types of experiments were performed using UDP-N-Acetyl-[^{3}H] glucosamine (0.6 Ci/mM; New England Nuclear Corporation) as glycosyl donor.

(3) Protein

Protein was determined by using the method of Lowry et al. (1952) with bovine serum albomen as standard.

In Vivo Labeling Experiments

The mycelia were grown, collected and weighed, as described previously. Two-tenth of gram of mycelia was placed into each of seven 20-ml flasks and 2.5 g of mycelia were added to seven 250-ml flasks. Two μCi of [^{3}H] mannose (sp act 13.2 Ci /mM; New England Nuclear Corporation) were added to each of the seven 20-ml flasks. All 14 flasks were placed in the rotary shaker (120 cycle/min) at 30°C. At 0, 10, 20, 30, 40, 60, and 120 min intervals one 20-ml flask and one 250-ml flask were removed from the shaker. The mycelia from both flasks were filtered through cheese

cloth and the mycelia-free medium from the 20-ml flask was stored at 4°C. The filtered mycelia were mixed, homogenized and separated into various subcellular fractions. Aliquots of 0.5 ml from each fraction as well as from the mycelia-free media of the 20-ml flasks were precipitated with equal volumes of cold 10% TCA overnight at 4 C. The precipitates were filtered through millipore filters (0.45 μ pores); the filters were then dried and counted.

Similar types of experiments were done using [$^{-3}$H] glucosamine (13.4 Ci/mM, Amersham/Searle). In some cases cycloheximide was also added to a final concentration of 10 μg/ml.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gels (7.5% SDS) were prepared in the following way using 2.5 ml of bisacrylamide solution containing 30% acrylamide and 0.8% bisacrylamide, 1 ml of 0.01 M Na_2HPO_4 was added. To this mixture 0.1 ml of 1% SDS and 0.005 ml tetramethylethylenediamine (TEMED) were added and the volume was brought up to 9.95 ml by adding 6.35 ml of water. To this 0.05 ml of 10% ammonium persulphate was added. Three ml of this mixture were added to each glass cylinder (7 cm long and 0.2 cm in inner diameter). The bottoms of these cylinders had been plugged

by rubber stoppers. Using a micropipet, 50 µl of water was layered onto the top of the gel mixture. The gels were allowed to solidify at room temperature. Protein was dissociated by adding an equal volume of dissociating solution containing 1% SDS, 0.01 M Na_2HPO_4 buffer, 1% (v/v) mercaptoethanol and 10% glycerol to protein dissolved in water. The mixture was heated at 100°C for 1 min. A drop of 0.1% bromophenol blue was added to this mixture. After the gels had solidified, the water from the top was removed and the cylinders containing gels were placed in electrolyte buffer containing 0.001 M Na_2HPO_4 and 0.1% SDS. Two hundred µg of dissociated protein were carefully layered onto top of each of the gels and then 8 amps per gel were applied. When the tracking dye reached the bottom of the cylinders, the current was turned off, The gels were removed from the cylinder and stained either with 0.1% Coomassie brilliant blue dissolved in methanol: Acetic acid: water (5:1:5) or with fuchisin-sulphite stain specific for carbohydrates using the technique of Zacharias et al. (1969). The gels stained for at least 12 hrs. by Coomassie brilliant blue stain were destained electrophoretically in acetic: methanol: water (7:5:88).

Smooth ER Membrane Solubilization

The mycelia were grown, harvested, homogenized and separated into various subcellular fractions. The membrane of the SER fraction was solubilized using the technique of Blackburn and Kasper (1976).

Immune Precipitation

To 0, 10, 25, 50, 100 and 200 µg of antigen of specific activity 20,000 cpm per 100 µg of protein dissolved in 0.5 ml of 0.9% saline solution 0.5 ml of rabbit anti-extracellular α-glucosidase was added. The antiserum was prepared by injecting a rabbit intramuscularly with 0.5 mg of extracellular α-glucosidase purified as by Rudick and Elbein (1973) one every week for 3 weeks, after which the rabbit was bled from its ear vein. The blood was allowed to clot for a few min at room temperature and then was centrifuged at 5,000 rpm for 20 min. The pellet was discarded and the serum was frozen. The specificity of the serum was checked using the immunodiffusion technique, with serum diluted ten-fold. The tubes containing antigen and antiserum were incubated at 37°C for an hour and then kept at 4°C for 18 to 24 hr. The precipitates were obtained by centrifuging at 5,000 rpm for 20 min, and were counted in a Beckman Liquid Scintillation

Counter using Bray's Scintillation fluid (100 ml methanol, 20 ml ethlene glycol, 60 g napthalene, 4 g PPO, 0.2 g POPOP which was brought upto 1.0 ℓ with dioxane). To each of the supernatants was then added 0.5 ml of rabbit anti-extracellular β-glucosidase. After incubation at 37°C for 1 hr and then at 4°C for 18 to 24 hr, the precipitates were obtained and counted. The level of non specific precipitation was checked in the following way. The results of the immune precipitation using anti-extracellular α-glucosidase were plotted as amount of radioactivity in the precipitates as a function of amount of antigen. A point near the plateau of the curve was selected and the corresponding amount of antigen was dissolved in 0.5 ml of 0.9% saline solution to which was then added 0.5 ml of rabbit anti-ovalbumin (a generous gift of Dr. John Nishimura). Then 50 μg of ovalbumin were added and the immune precipitates were obtained and counted as above. To this supernatant 0.5 ml of rabbit anti-extracellular α-glucosidase was added and again precipitates were obtained and counted.

RESULTS

Preparation of Subcellular Fractions

Mycelia of Aspergillus niger (A. niger) were grown, harvested and homogenized as described in 'Materials and Methods'. Cell wall and cell debris, were removed from the homogenate by centrifuging at low speed, the resultant supernatant was centrifuged at 14,000 rpm or 7,000 xg to obtain the post mitochondrial supernatant and the mitochondrial pellet. The post-mitochondrial supernatant (PMS) was centrifuged through different types of sucrose gradients to get the best separation between the smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER) and ribosomes. The purity of these fractions was checked as a function of their RNA content. The membrane of the SER fraction should not have any RNA associated with it, while that of the RER fraction and ribosomes should have a considerable amount of RNA associated with them.

Method #1

According to the method of Adelman et al. (1973), the PMS was layered onto a two-step sucrose gradient. A supernatant on the top of the gradient, two bands in the gradient and a pellet were obtained after centrifuging

at 27,000 rpm in the SW 27 rotor for 22 hrs. Figures 1a and 1b, respectively, show the centrifuge tube with gradient and the PMS before and after centrifugation. Table 1 shows the destribution of RNA in those fractions.

Band I is supposed to be the SER fraction, band II to be the RER fraction and the pellet the ribosomal fraction. There should be a significant difference in RNA content between the SER and RER fractions. Here a very little difference is found in the distribution of RNA in each fraction.

Method #2

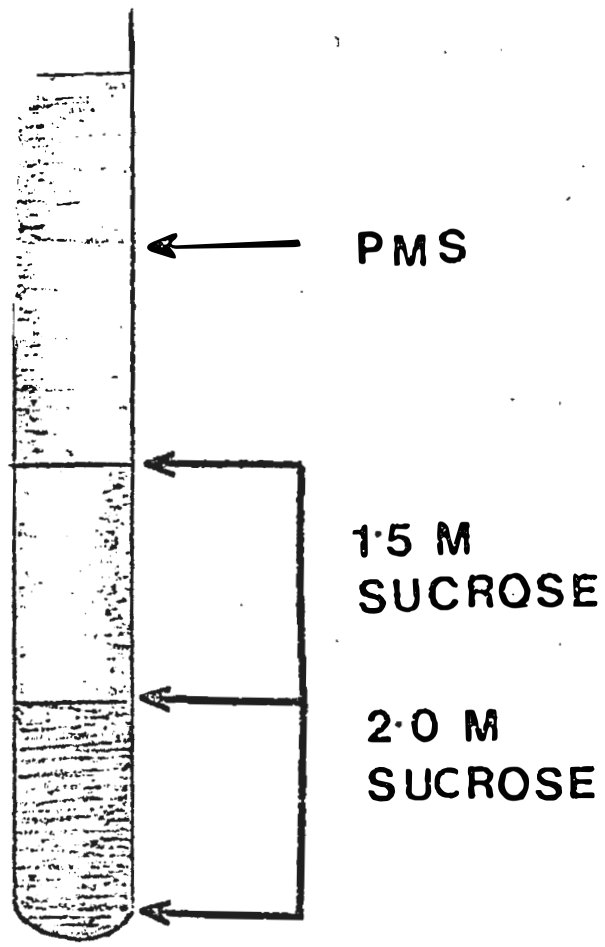
According to Bouchilloux et al. (1973), after layering the PMS on the two-step sucrose gradient prepared as explained in 'Materials and Methods', centrifugation was at 27,000 rpm in the SW 27 rotor for 5 hrs. Figures 2a and 2b illustrate the gradient tube before and after centrifugation. After the centrifugation a pellet, two bands in the gradient and a supernatant on the top of the gradient were obtained. Table II shows the RNA distribution in these fractions.

Here also a small difference is found in the RNA content between the high speed supernatant (HSS), band I, band II and the pellet. It was found through repeated

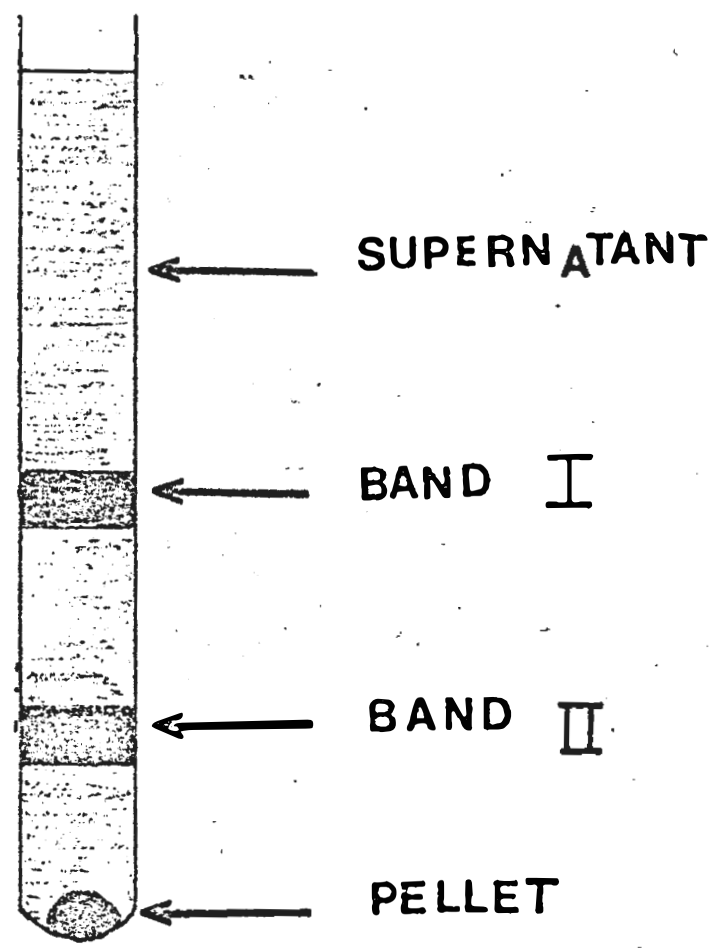
Figure 1. Sucrose gradient centrifugation of PMS according to the method of Adelman et al. (1973).

Figure 1a. The centrifuge tube prior to centrifugation containing 2 M sucrose on the bottom layer and 1.5 M sucrose in the top layer. PMS was layered on the top of the gradient.

Figure 1b. Centrifuge tube after centrifugation for 22 hr at 27,000 rpm. This centrifugation resulted into a pellet, two bands in the gradient and a supernatant on the top of the gradient.


PMS
1·5 M
SUCROSE
2·0 M
SUCROSE

a


SUPERNATANT
BAND I
BAND II
PELLET

b

TABLE I

Distribution of RNA in various subcellular fractions using the technique of Adelman et al. (1973).

Subcellular fractions	RNA content*	
	Orcinol	Absorbance at 260 nm
High speed supernatant	100	85
Band I	150	123
Band II	167	140
Ribosomes	200	167

* n moles

Figure 2. Sucrose gradient centrifugation of PMS according to the method of Bouchilloux et al. (1973).

Figure 2a. The centrifuge tube prior to centrifugation containing a two-step gradient; 1.5 M sucrose in the top and 2.0 M sucrose on the bottom layer. PMS was layered on the top of the gradient.

Figure 2b. Centrifuge tube after centrifugation for 5 hr at 27,000 rpm. This centrifugation resulted into a pellet, two bands in the gradient and a supernatant on the top of the gradient.

TABLE II

Distribution of RNA in various subcellular fractions using the technique of Bouchilloux et al. (1973).

Subcellular fractions	RNA content*	
	Orcinol	Absorbance at 260 nm
High speed supernatant	110	100
Band I	158	150
Band II	200	177
Ribosomes	186	164

* n moles

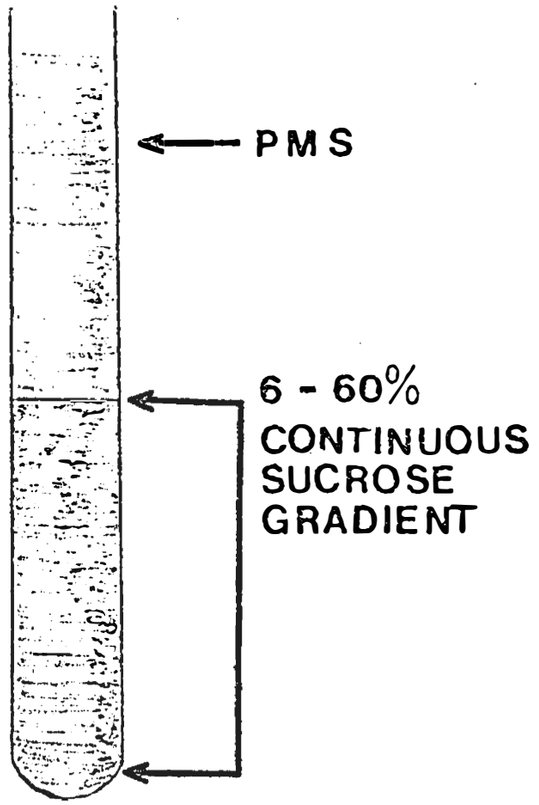
discontinuous sucrose gradient centrifugation that some membraneous material always gets trapped at the interface between sucrose solution of two different densities. Thus band I and band II may not represent the SER and RER fractions, respectively. It is also likely that some free ribosomes, too, get trapped at the same interface and perhaps that is why almost equal amounts of RNA seem to be distributed all through the fractions.

A new approach was taken to fractionate the PMS in which a continuous sucrose gradient was used for the reasons given above and the fact that electron micrographs of A. niger grown in aerobic conditions show very little rough ER (Fitzgerald and Rudick, unpublished results). According to Damsky et al. (1976), yeast cells grown aerobically do not have an appreciable quantity of rough ER. Hence with the continuous sucrose gradient attempts were made to obtain simply smooth ER along with ribosomes and high speed supernatant from the PMS. A 6 to 60% continuous gradient was prepared, as described before, and the PMS was layered onto it. The tubes were centrifuged at 25,000 rpm for 2.5 hr. Figures 3a and 3b show, respectively, the centrifuge tube before and after the centrifugation.

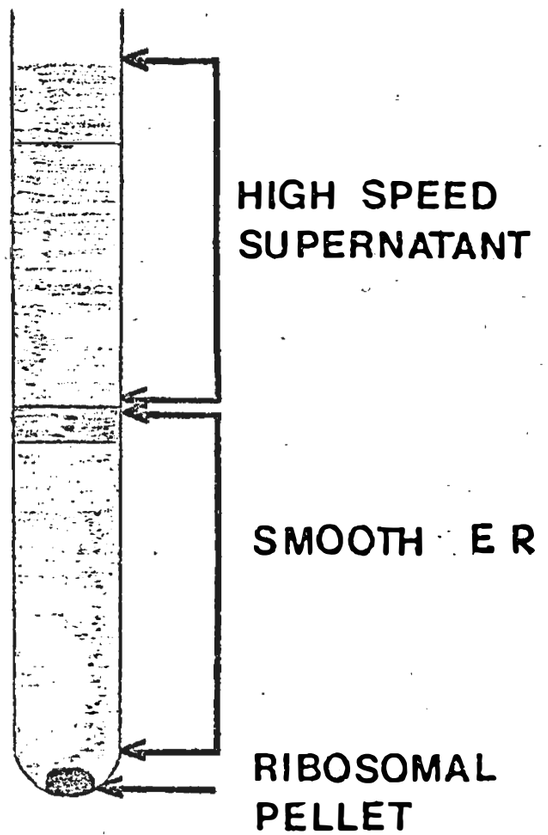
Figure 3. Continuous sucrose gradient centrifugation of PMS.

Figure 3a. Centrifuge tube prior to centrifugation containing 6 - 60% continuous sucrose gradient, with the PMS on the top of the gradient.

Figure 3b. Centrifuge tube after centrifugation for 24 hr at 25,000 rpm. This centrifugation resulted into a pellet, milky white SER fraction in the gradient and a supernatant on the top of the gradient.


PMS
6 - 60%
CONTINUOUS
SUCROSE
GRADIENT

a


HIGH SPEED
SUPERNATANT
SMOOTH E R
RIBOSOMAL
PELLET

b

Purity of Subcellular Fractions

Instead of using RNA content as a marker for various fractions, several enzyme assays were used to check the homogeneity of the subcellular fractions. DeDuve et al. (1965) and Beaufay et al. (1974) reported that cytochrome c oxidase is a marker enzyme for the mitochondrial fraction while NADPH-dependent cytochrome c reductase and glucose-6-phosphatase are marker enzymes for the microsomal fraction including SER and RER of rat liver cells. Hogeboom (1949) and Donaldson et al. (1972) have reported NADPH dependent cytochrome c reductase as an important marker enzyme for the microsomal fraction of plant cells. Table III shows the results of the subcellular fractionation of A. niger using marker enzyme activities. The distribution of protein in these fractions was also determined and tabulated.

Only the mitochondrial fraction showed cytochrome c oxidase activity and was inhibited by 0.001 M NaCN. This result suggested that other subcellular fractions are not contaminated with the mitochondria.

Glucose-6-phosphatase activity is found in almost equal amounts in the high speed supernatant and the SER fraction. NADPH-dependent cytochrome c reductase activity is found to be almost 50 times higher in the SER

TABLE III

Distribution of enzyme activities and protein in various subcellular fractions.

Subcellular fractions	Enzymes				
	Cytochrome-c-oxidase[1]	Glucose-6-phosphatase[2]	NADPH dependent cytochrome-c-reductase[3]	Total protein (mg)	Percent protein
Pre-mitochondrial supernatant	—	—	—	76.0	100.00
Mitochondria	0.36	0.2	0.10	13.5	17.76
Post-mitochondrial supernatant	—	—	—	57.6	75.79
High speed supernatant	0.0	2.65	0.25	33.0	43.42
Smooth ER fractions	0.0	2.50	11.50	30.6	42.26
Ribosomes	0.0	0.65	0.65	1.5	1.97

1. μ moles cytochrome-c- oxidized per min per mg of protein.
2. μ moles of inorganic phosphate liberated per mg of protein per 30 min.
3. μ moles of cytochorme-c- reduced per min per mg of protein.

fraction than in the HSS fraction and a little more than a 100 times higher than that in the mitochondrial fraction. These results indicate that the other fractions have not been appreciably contaminated with the SER fraction.

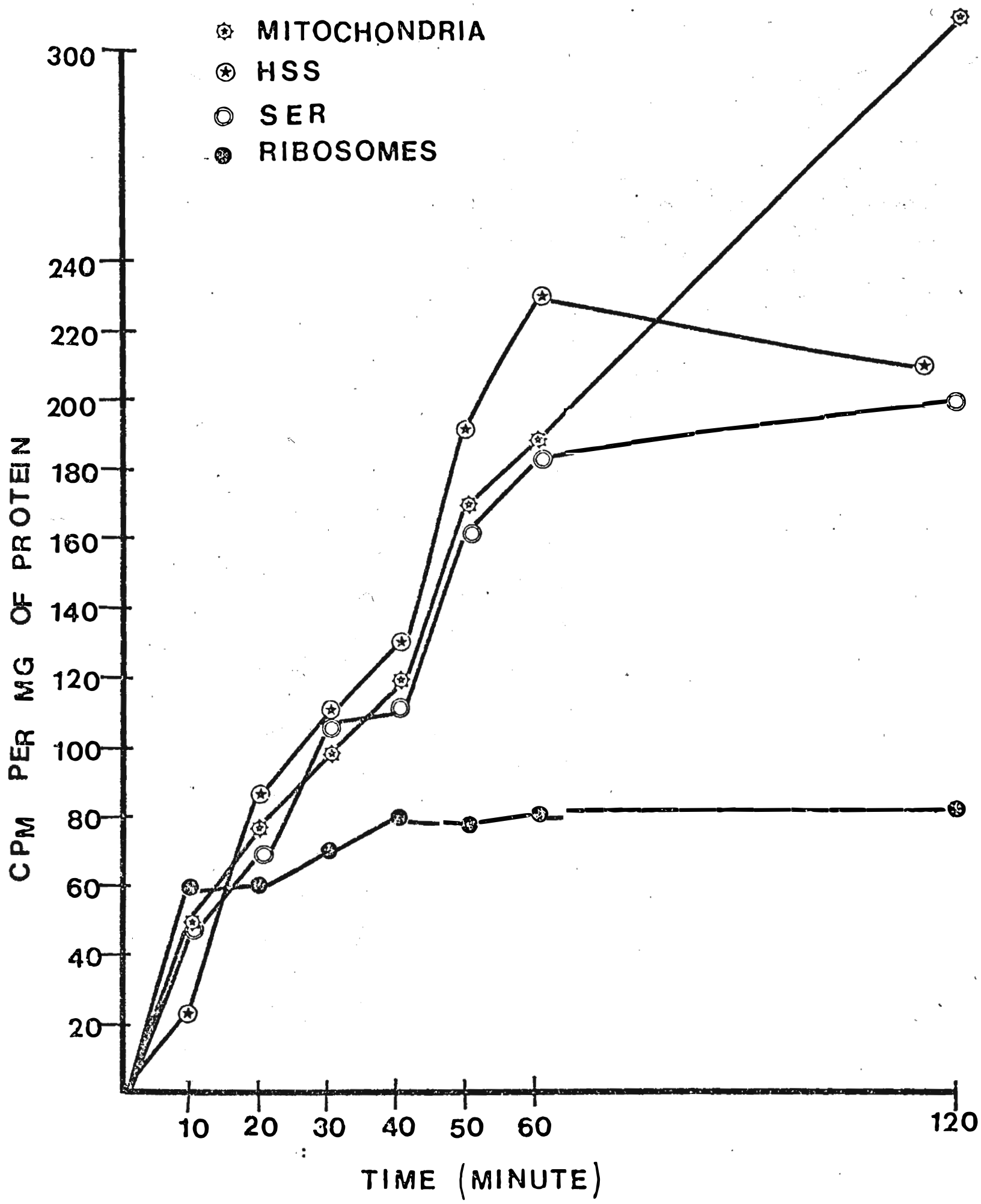
The amount of protein in the mitochondrial fraction and that in the PMS adds up to the amount of protein in the pre-mitochondrial supernatant. Also the sum of the amounts of protein in the mitochondrial fraction, high speed supernatant, smooth ER and ribosomal fraction is in good agreement with the protein of the pre-mitochondrial supernatant.

In Vivo Labeling Experiments

After establishing the homogeneity of the sub-cellular fractions, glycosylation of these fractions as a function of time was examined in the following way. The mycelia were labeled with [^{3}H] mannose for 10, 20, 30, 40, 60 and 120 min, and the TCA precipitable radioactivity of the subcellular fractions was plotted as a function of time (Figure 4). The following observations were made:

1. Within the first 10 min radioactivity is found almost equally distributed in all the fraction except the HSS which have half the amount of the rest of the fractions.

Figure 4. Radioactive TCA precipitates per mg of protein plotted as a function of time. The mycelia were incubated with [^{3}H] mannose for 0, 10, 20, 30, 40, 60 and 120 min, radioactive TCA precipitates were obtained from various sub-cellular fractions.


MITOCHONDRIA
HSS
SER
RIBOSOMES
300
240
220
200
180
160
140
120
100
80
60
40
20
CPM PER MG OF PROTEIN
10
20
30
40
50
60
120
TIME (MINUTE)

2. Between 20 and 60 min, there is an increase in the radioactivity of the TCA precipitates in all the subcellular fractions except the ribosomal fraction where the radioactivity levels off from 10 min on up to 120 min.

3. Between 60 and 120 min, radioactivity levels off in the SER and HSS fractions. However, in the mitochondrial fraction, radioactivity keeps on increasing.

Similar experiments with labeled glucosamine were not continued because not enough glucosamine was incorporated into the protein. Several conditions were changed to attempt to incorporate [^{3}H] glucosamine into protein: for example, the incubation time of mycelia in [^{3}H] glucosamine was changed up to 12 hrs without success. The concentration of [^{3}H] glucosamine was changed from 2 µCi/20 ml to 10 µCi/20 ml with little success. Finally an attempt was made to see if the SER fraction contains glucosaminyl transferase which would transfer [^{3}H] glucosamine from its donor, UDP-N-acetyl [^{3}H] glucosamine, to an endogenous acceptor protein, again without success.

From the first observation concerning Figure 4, it could not be said where glycosylation takes place. However, in the light of the first 2 observations it may be that initiation of glycosylation takes place in the ribosomal fraction, where the specific activity of the

radioactive TCA precipitate does not change after the first 10 min and further glycosylation may take place elsewhere. To determine if further glycosylation might take place elsewhere an experiment was designed to detect the presence of mannosyl transferase in the subcellular fractions. This enzyme transfers mannose from a donor guanosine-diphosphomannose (GDPM) to a protein acceptor in the presence of Mn^{++}. Two hundred µg of protein from each of the subcellular fractions were incubated with GDP [^{14}C] mannose in the presence of Mn^{++} for various periods of time and then the reactions were stopped by addition of equal volumes of 10% TCA and labeled TCA precipitates were collected on millipore filters (Figure 5). The greatest amount of [^{14}C] mannose bound to endogenous acceptors was found in the smooth ER. When increasing amounts of protein from the smooth ER fraction were used in the mannosyl transferase assay (Figure 6), there was a corresponding increase in incorporated [^{14}C] mannose labeled TCA precipitates up to 200 µg of protein. These results suggest some stage of glycosylation occurs in the SER fraction.

To see if this radioactivity is still in the mannose residue, the radioactive high speed supernatant obtained as explained in the 'Material and Methods' was

Figure 5. Mannosyl transferase activities of subcellular fractions. Protein (200 µg) from various subcellular fractions were incubated with GDP [^{14}C] mannose in the presence of Mn^{++} for various periods of time and radioactive TCA precipitates were obtained.

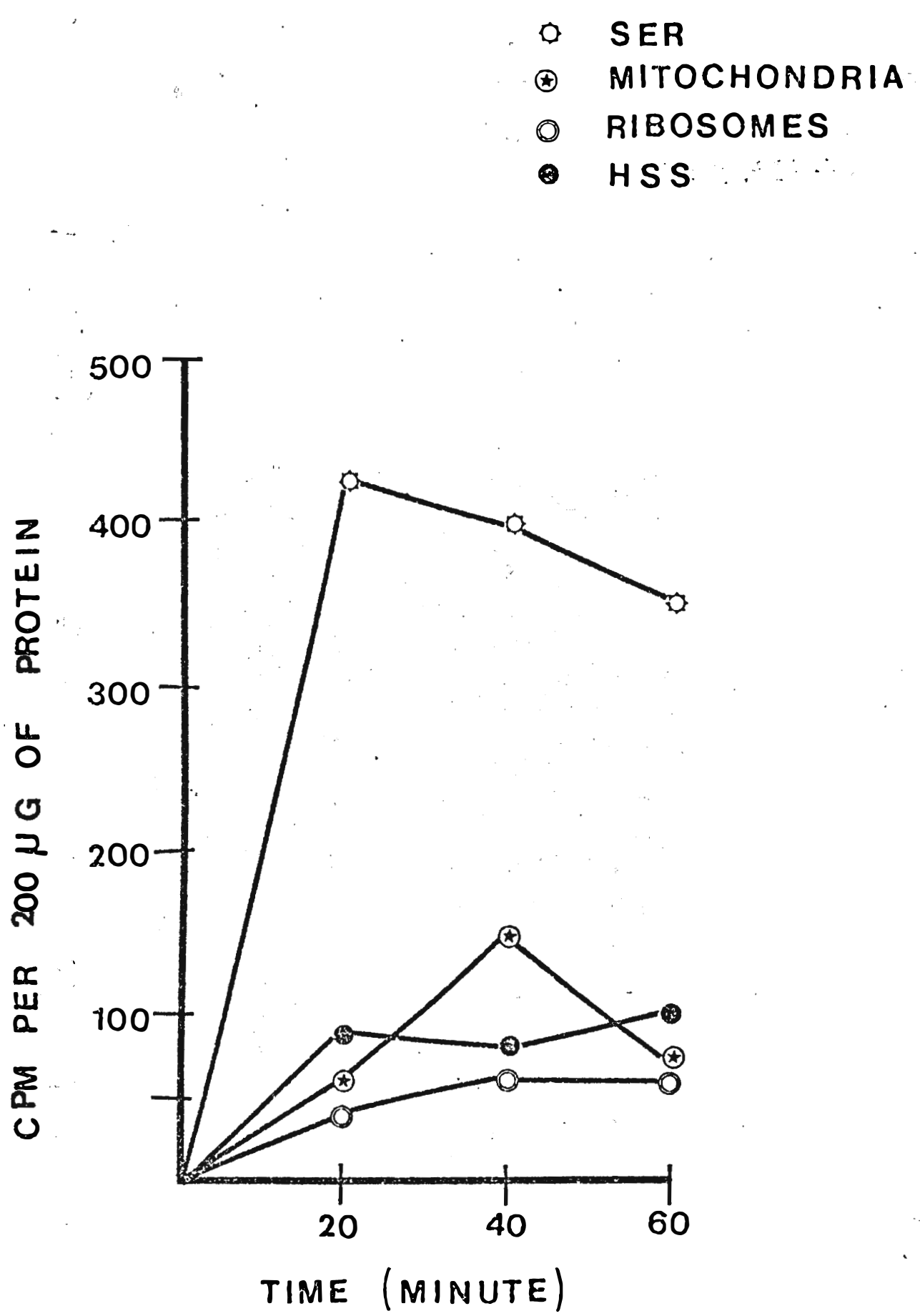
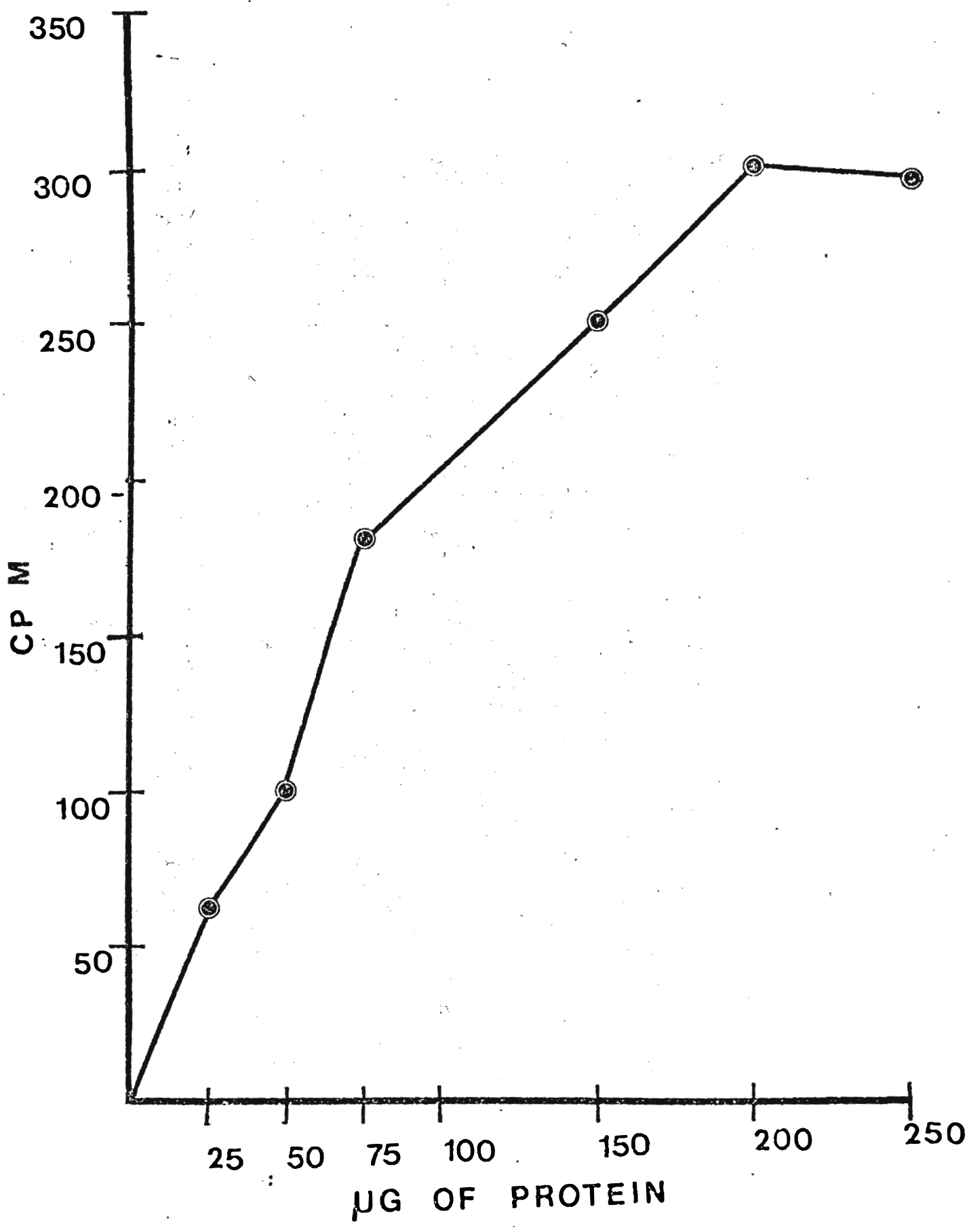

SER
MITOCHONDRIA
RIBOSOMES
HSS
500
400
300
200
100
CPM PER 200 µG OF PROTEIN
20
40
60
TIME (MINUTE)

Figure 6. Dependence of mannosyl transferase activity on the amount of smooth ER. Various amounts of protein from the SER fraction were incubated with a constant amount of GDP [^{14}C] mannose for 30 min and the radioactive TCA precipitates were obtained.


350
300
250
200
150
100
50
CP M
25
50
75
100
150
200
250
µG OF PROTEIN

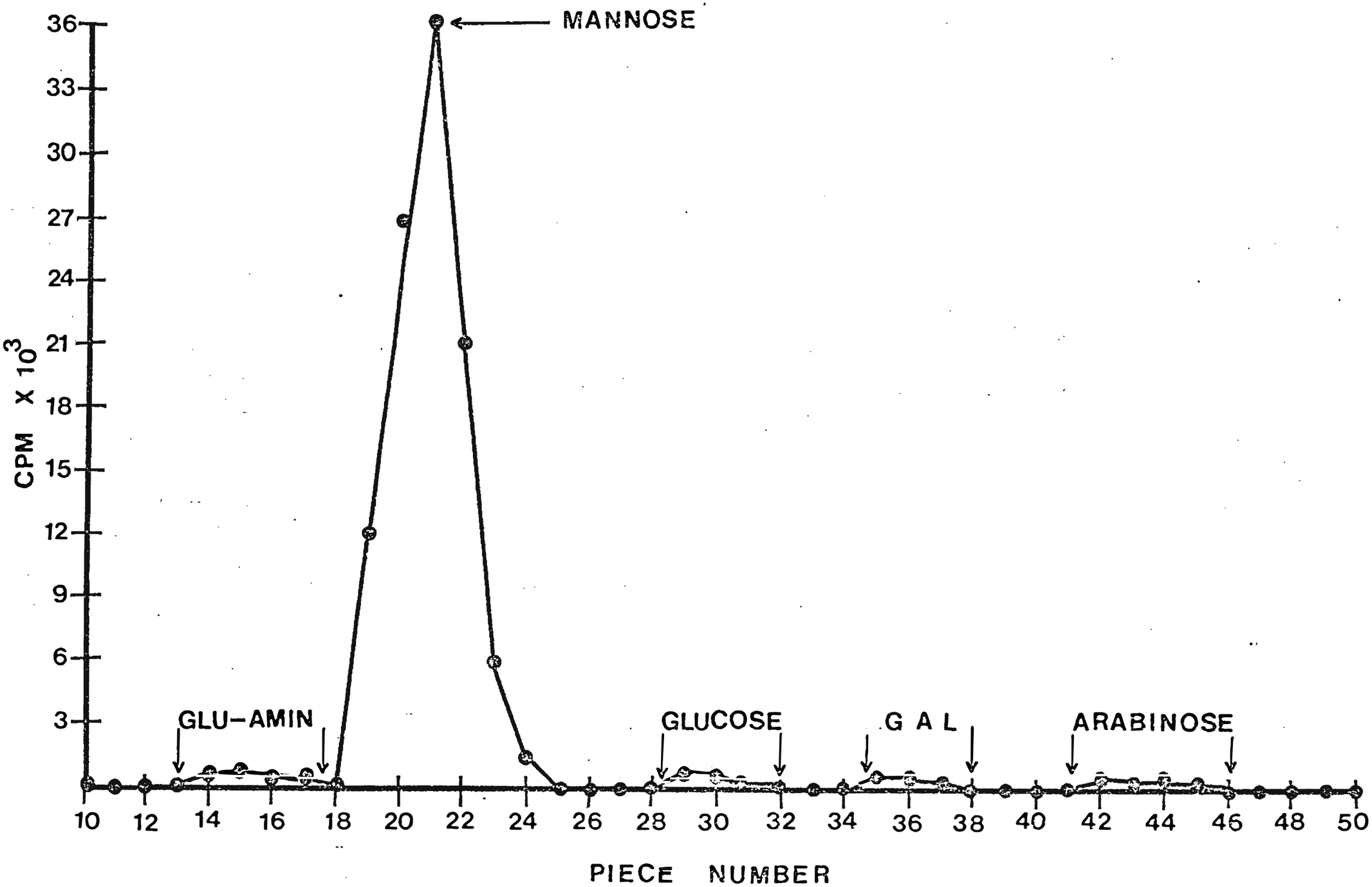
precipitated in the cold overnight with the addition of an equal volume of cold 10% TCA, and then the precipitate was obtained by centrifuging at 5,000 rpm for 20 min. The supernatant was discarded and the precipitate was dissolved in 2 ml of water. Trifloracetic acid (TFA) was added to a final concentration of 2 N and the precipitate was hydrolyzed at 120°C for 90 min, dried on a rotary evaporator, and passed through a Dowex 50 [H+] column. The column was first washed with water to obtain neutral sugars and then washed with 1.5 M NH_4OH to obtain amino sugar (glucosamine). Both fractions were dried on a rotary evaporator. These samples were applied to Whatman 3 MM chromatography paper and were chromatographed (butanol/pyridine/water, 6/4/3). After about 36 hrs, the paper was dried and the strips of the paper containing various sugar standards and each of the samples were cut. The standards were detected by periodate-permanganate spray. The strips containing each sample were cut into pieces 1 cm wide and numbered. Each piece of paper was counted in toluene scintillation fluid. Table IV and Figure 7 show the distribution of radioactivity obtained at various stages. Almost 85% of the radio-activity of the TCA precipitate was found to be in mannose.

TABLE IV

Distribution of radioactivity at various stages in preparation of acid hydrolysis of TCA precipitate of the high speed supernatant.

Fraction	Total Radioactivity (cpm)
TCA precipitate of HSS	56,090
TFA digest	55,225
Washing of Dowex 50 [H^+] column with water	42,020
Washing of Dowex 50 [H^+] column with 1.5 M NH_4OH	———
Mannose spot	36,620
Glucosamine spot	500
Rest of the sugar spots combined	1,042

Figure 7. Distribution of radioactivity on the chromatography paper.


MANNOSE
CPM X 10^3
36
33
30
27
24
21
18
15
12
9
6
3
GLU-AMIN
GLUCOSE
G A L
ARABINOSE
10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
PIECE NUMBER

Protein Synthesis Inhibition

To understand the relationship between protein synthesis and glycosylation an experiment was designed where mycelia were incubated with [^{3}H] mannose for 30 min and then half of them were treated with cycloheximide for 10, 20 and 30 min in the presence of [^{3}H] mannose. The radioactive precipitates were obtained from each of the subcellular fractions (Figure 8). The amount of radioactivity did not change much in each subcellular fraction, whether or not the fraction was obtained from the mycelia treated with cycloheximide for 10, 20 or 30 min.

An identical experiment was done where mycelia were incubated with [^{3}H] leucine for 30 min (Figure 9). Here too, the amount of radioactivity did not change much if the mycelia were treated with cycloheximide for 10, 20, or 30 min, indicating that there was no protein synthesis or degradation during the period of inhibition. Figure 4 shows that radioactivity leveled off between 60 and 120 min in all subcellular fractions except in the mitochondria. To know if the leveling off of glycosylation is related to protein synthesis or not, the mycelia were labeled with [^{3}H] mannose for 60 min and then half of them were treated with cycloheximide for 10, 20, or 30 min

Figure 8. Effect of cycloheximide on the incorporation of [^{3}H] mannose into subcellular fractions. Histograms of [^{3}H] mannose per mg of protein obtained from various subcellular fractions. The mycelia were incubated for 30 min with [^{3}H] mannose and 10, 20, and 30 min with cycloheximide.

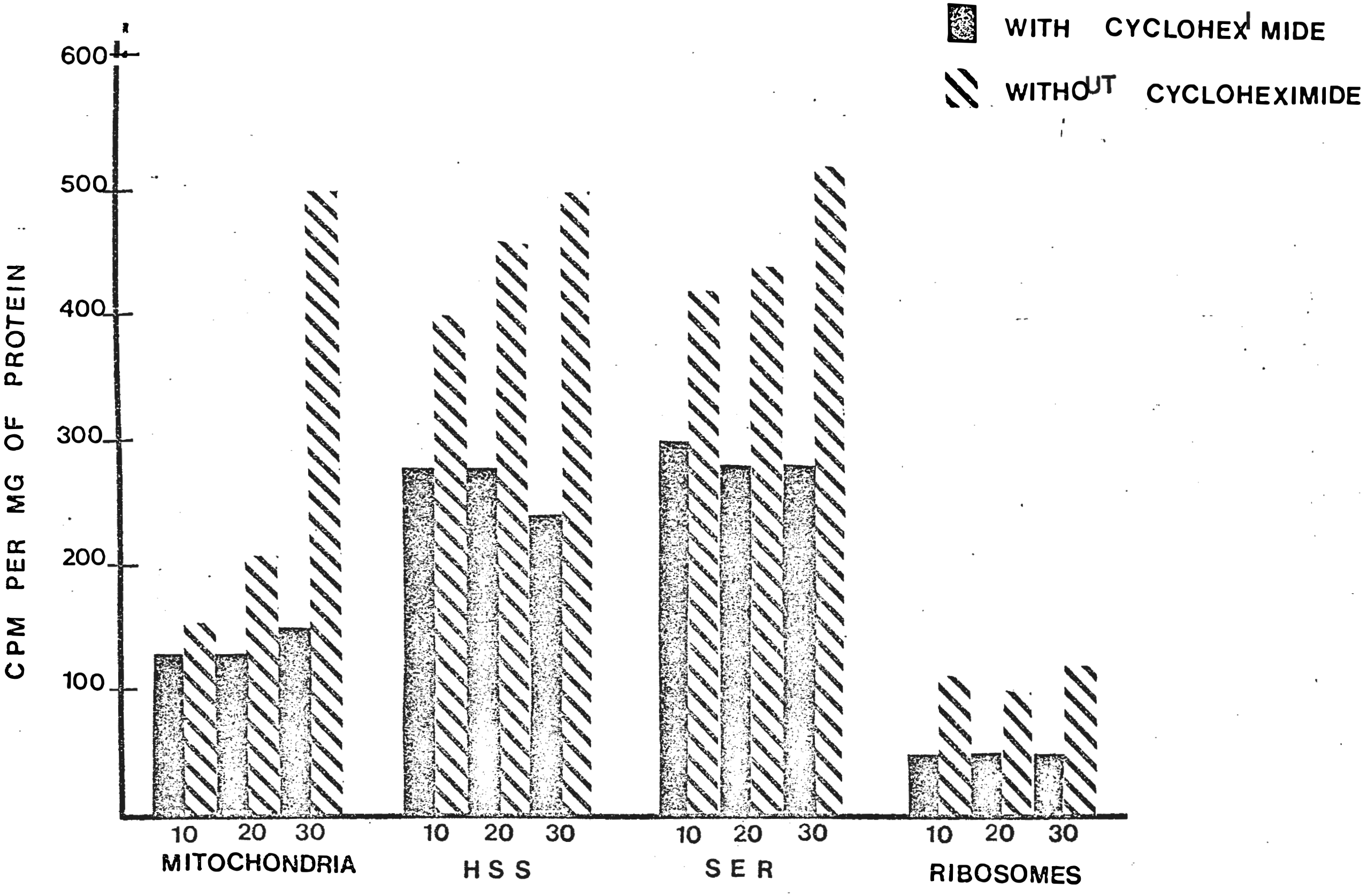
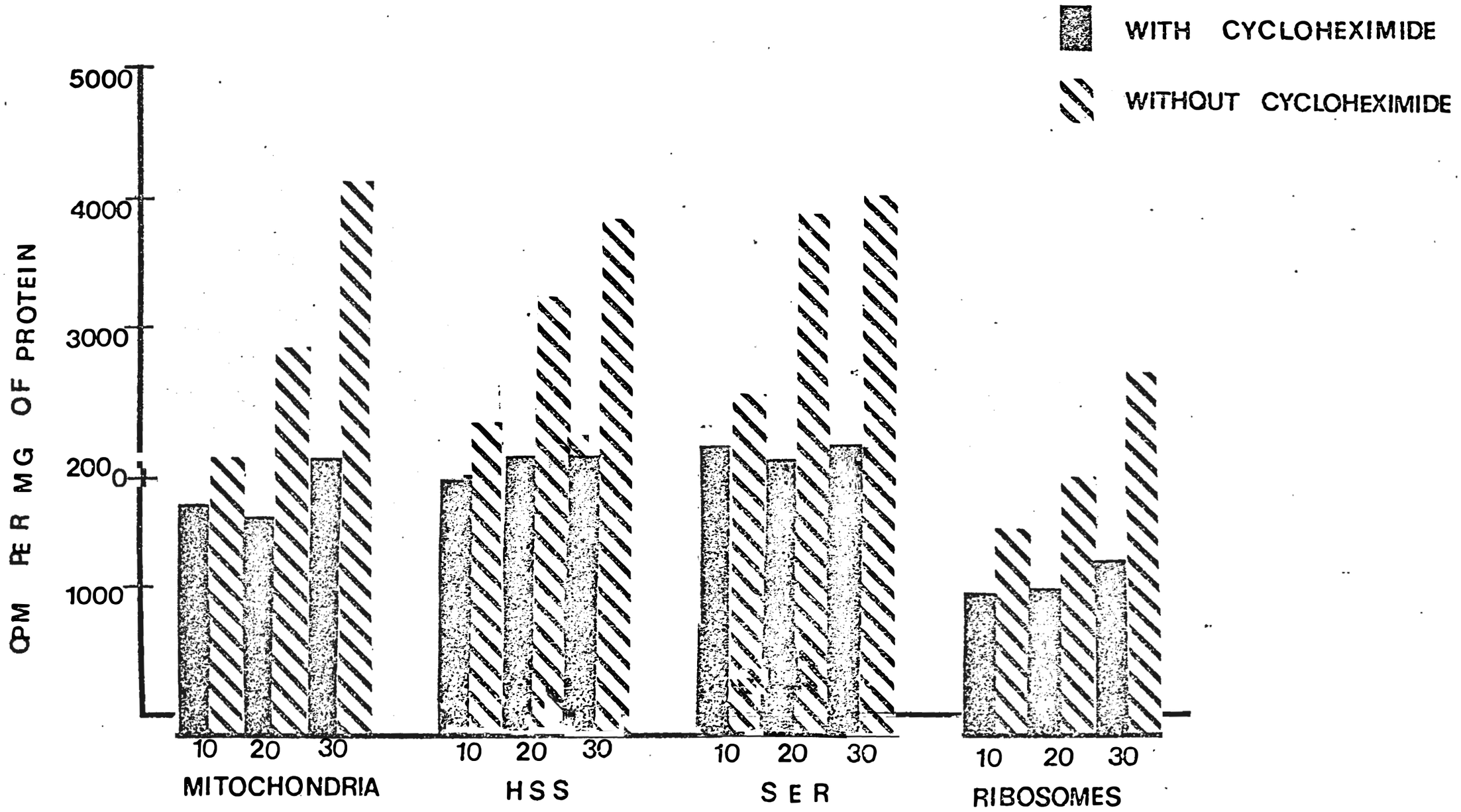

WITH CYCLOHEXIMIDE
WITHOUT CYCLOHEXIMIDE
CPM PER MG OF PROTEIN
600
500
400
300
200
100
10 20 30
MITOCHONDRIA
10 20 30
HSS
10 20 30
SER
10 20 30
RIBOSOMES

Figure 9. Effect of cycloheximide on the incorporation of [^{3}H] leucine into subcellular fractions. Histograms of [^{3}H] leucine per mg of protein obtained from various subcellular fractions. The mycelia were incubated for 30 min with [^{3}H] leucine and 10, 20, and 30 min with cycloheximide.


WITH CYCLOHEXIMIDE
WITHOUT CYCLOHEXIMIDE
CPM PER MG OF PROTEIN
5000
4000
3000
2000
1000
0
10 20 30
MITOCHONDRIA
10 20 30
HSS
10 20 30
SER
10 20 30
RIBOSOMES

(Figure 10). In each subcellular fraction, except the mitochondrial fraction, the amount of radioactive TCA precipitate obtained from cycloheximide treated samples did not change from those obtained from the untreated samples, regardless of the time for which the mycelia were treated. It appears that protein synthesis stops in the mycelia grown in these conditions after 60 min. Again an identical experiment was done where mycelia were incubated with [^{3}H] leucine for 60 min and 120 min (Figure 11). The 60 min batch was treated with cycloheximide, as described before. The radioactivity of TCA precipitate of any fraction except the mitochondrial fraction between 60 and 120 min remains the same.

SER Solubilization

Figure 4 showed a considerable amount of TCA precipitable [^{3}H] mannose labeled activity was found in the SER fraction. To know what kinds of glycoproteins exist in the SER fraction, in particular if secretory glycoproteins are found in the SER fraction, the membrane of the SER fraction was partially solubilized with urea using the technique of Blackburn and Kasper (1976). Urea soluble and insoluble fractions were applied to 7.5% SDS gels, which were stained with Coomassie brilliant blue.

Figure 10. Effects of cycloheximide on the incorporation of [^{3}H] mannose into subcellular fractions. Histograms of [^{3}H] mannose per mg of protein obtained from various subcellular fractions. The mycelia were incubated for 60 min with [^{3}H] mannose and 10, 20, and 30 min with cycloheximide.

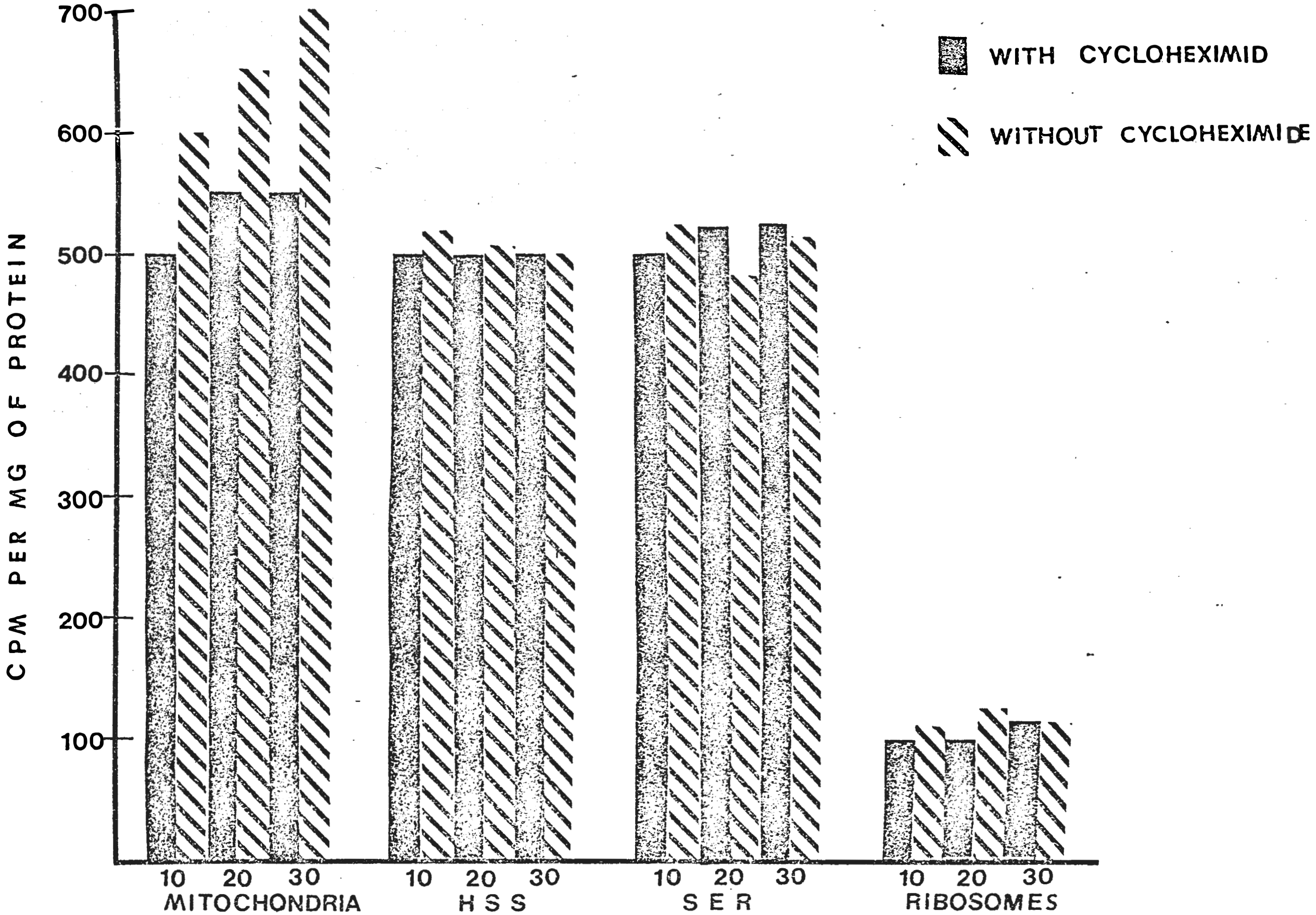
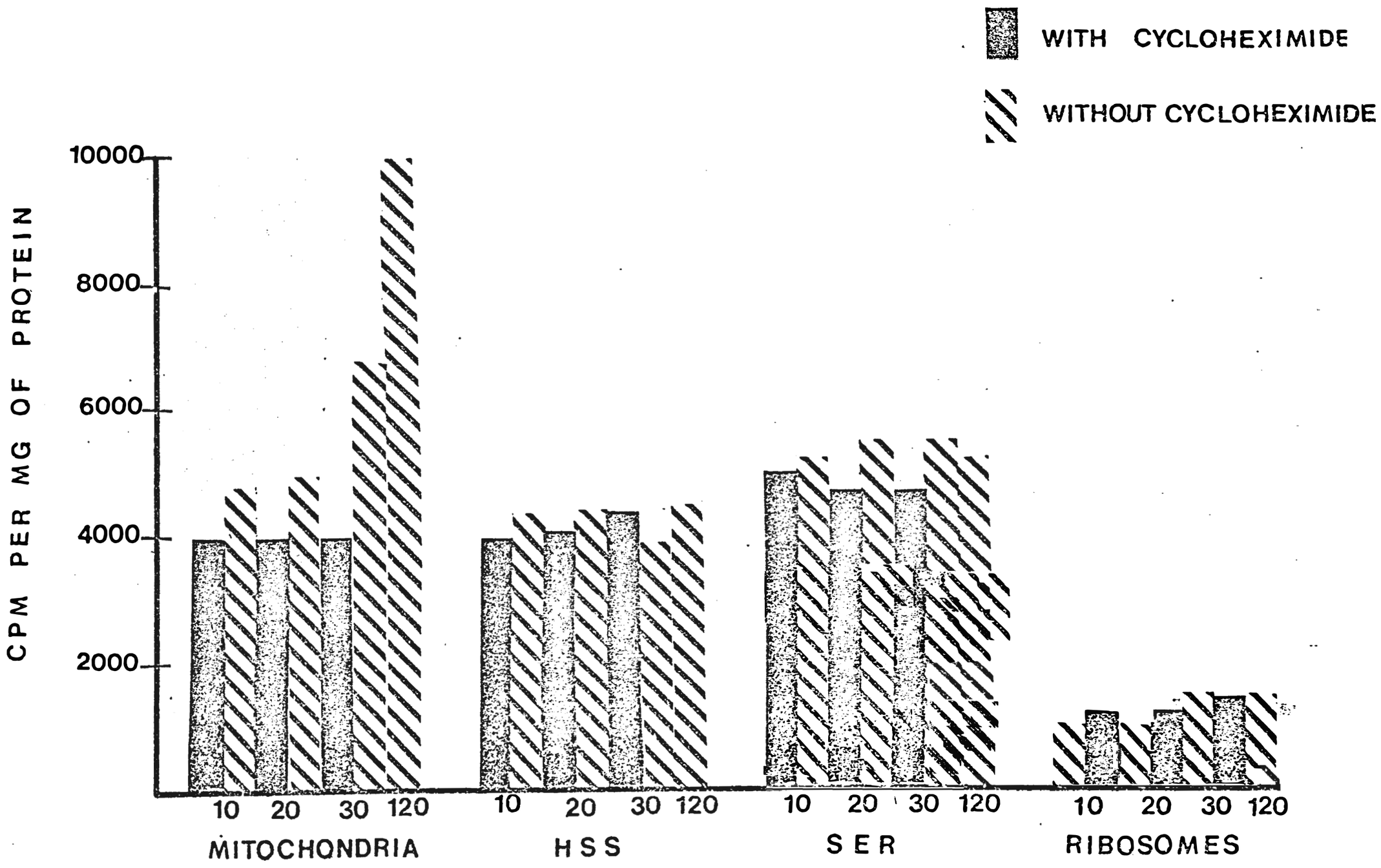

CPM PER MG OF PROTEIN
700
600
500
400
300
200
100
WITH CYCLOHEXIMID
WITHOUT CYCLOHEXIMIDE
10 20 30
MITOCHONDRIA
10 20 30
H S S
10 20 30
S E R
10 20 30
RIBOSOMES

Figure 11. Effect of cycloheximide on the incorporation of [^{3}H] leucine into subcellular fractions. Histograms of [^{3}H] leucine per mg of protein obtained from various subcellular fractions. The mycelia were incubated with [^{3}H] leucine for 60 min and then with cycloheximide for 10, 20, and 30 min.


WITH CYCLOHEXIMIDE
WITHOUT CYCLOHEXIMIDE
CPM PER MG OF PROTEIN
10000
8000
6000
4000
2000
10 20 30 120
MITOCHONDRIA
HSS
SER
RIBOSOMES

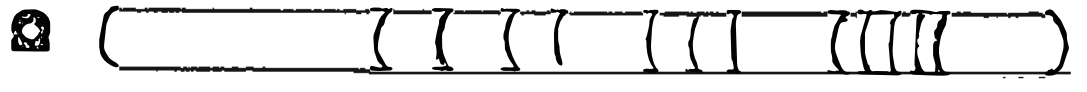
A set of duplicate gels was stained with Fuchsin-sulphite to detect carbohydrate (Figure 12). Fourteen different bands were stained by Cóomassie brilliant blue on gels containing urea soluble material, of which only the first two bands were stained by Fuchsin-sulphite, indicating the presence of only two membrane-bound glycoproteins. The gels containing urea insoluble material had six bands stained by Coomassie brilliant blue, but none of these bands was stained by Fuchsin-sulphite, indicating that the urea insoluble fraction, probably contained no glycoproteins. To confirm these results, the mycelia grown 100 ml of medium in a 250-ml flask were labeled with 500 µCi of [^{3}H] mannose, the SER fraction was obtained and treated with urea. The specific activity of urea soluble material was approximately 25,000 cpm per 100 µg of protein, while that of urea insoluble material was only 900 cpm per mg of protein. Two hundred µg of urea soluble material were applied to 7.5% SDS gels. Half the gels were stained by Coomassie brilliant blue and the other half were kept unstained. From the unstained gels bands corresponding to the stained gels were cut, dissolved in 0.2 ml of 3% hydrogen peroxide by incubation at 37^{o}C overnight, dissolved in 0.2 ml of soluene (Packard

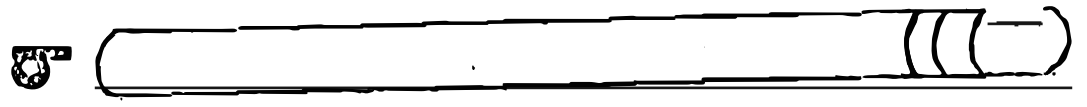
Figure 12. SDS polyacrylamide gel electrophoresis of urea treated SER fraction.

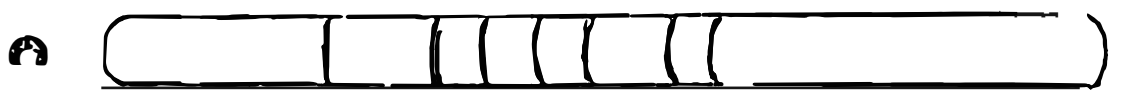
Figure 12a. Gel containing urea soluble SER fraction stained by Coomassie brilliant blue.

Figure 12b. Gel containing urea soluble SER fraction stained by Fuchsin-sulphite.

Figure 12c. Gel containing urea insoluble SER fraction stained by Coomassie brilliant blue.







Chemicals), and counted in Bray's scintillation fluid Table V. Only the first two bands of the gels containing urea soluble material had radioactivity.

Partial Purification of Glycoproteins from the High Speed Supernatant

A considerable amount of radioactivity was present in the high speed supernatant (Figure 4). According to Palade (1975) in mammalian systems secretory glycoproteins are not found in the cytoplasm of the cell. This is presumably due to the susceptibility of secretory glycoproteins in the cytoplasm to attack by proteolytic enzymes. Therefore, the secretory glycoproteins travel in a membranous environment. If Palade's hypothesis is valid for A. niger, then glycoproteins found in the highspeed supernatant would be degraded and thus be of low molecular weight. To check this, the method of Rudick et al. (1977) was used to partially purify intracellular enzymes. The mycelia were grown in a 250-ml flask for 48 hr under the usual conditions, and were then incubated with 25 μCi of [^{3}H] mannose for 1 hr. They were harvested, homogenized and centrifuged to obtain the PMS, which was spun at 29,000 rpm or 105,000 xg for 2.5 hr with the Ti 30 rotor in a Beckman ultracentrifuge. The resultant supernatant

TABLE V

Distribution of radioactivity in the bands of gels containing soluble SER membrane.

Bands	Radioactivity (cpm)
1	17,089
2	15,055
3	425
4	329
5	446
6	269
7	300
8	349
9	336
10	546
11	448
12	469
13	402
14	419
H_2O_2 + soluene	423

was dialyzed at 4°C for 24 hr against 0.1 M sodium acetate buffer, pH 5.0, with at least one change of buffer, this way all proteins whose isoelectric pH is 5.0, which does not include secretory glycoproteins of A. niger, are precipitated. The precipitate in the dialysate was discarded after centrifuging for 30 min at 5,000 rpm. To the supernatant, 2 volumes of ice cold acetone were added dropwise in constant stirring. The resulting acetone precipitate was collected by centrifuging at 5,000 rpm for 30 min. The precipitate was dissolved in 2 ml of water and stored frozen. Table VI shows the amount of radioactivity measured at various stages in this procedure. Approximately 66% of the total protein-bound radioactivity from the high speed supernatant is recovered in the acetone precipitate.

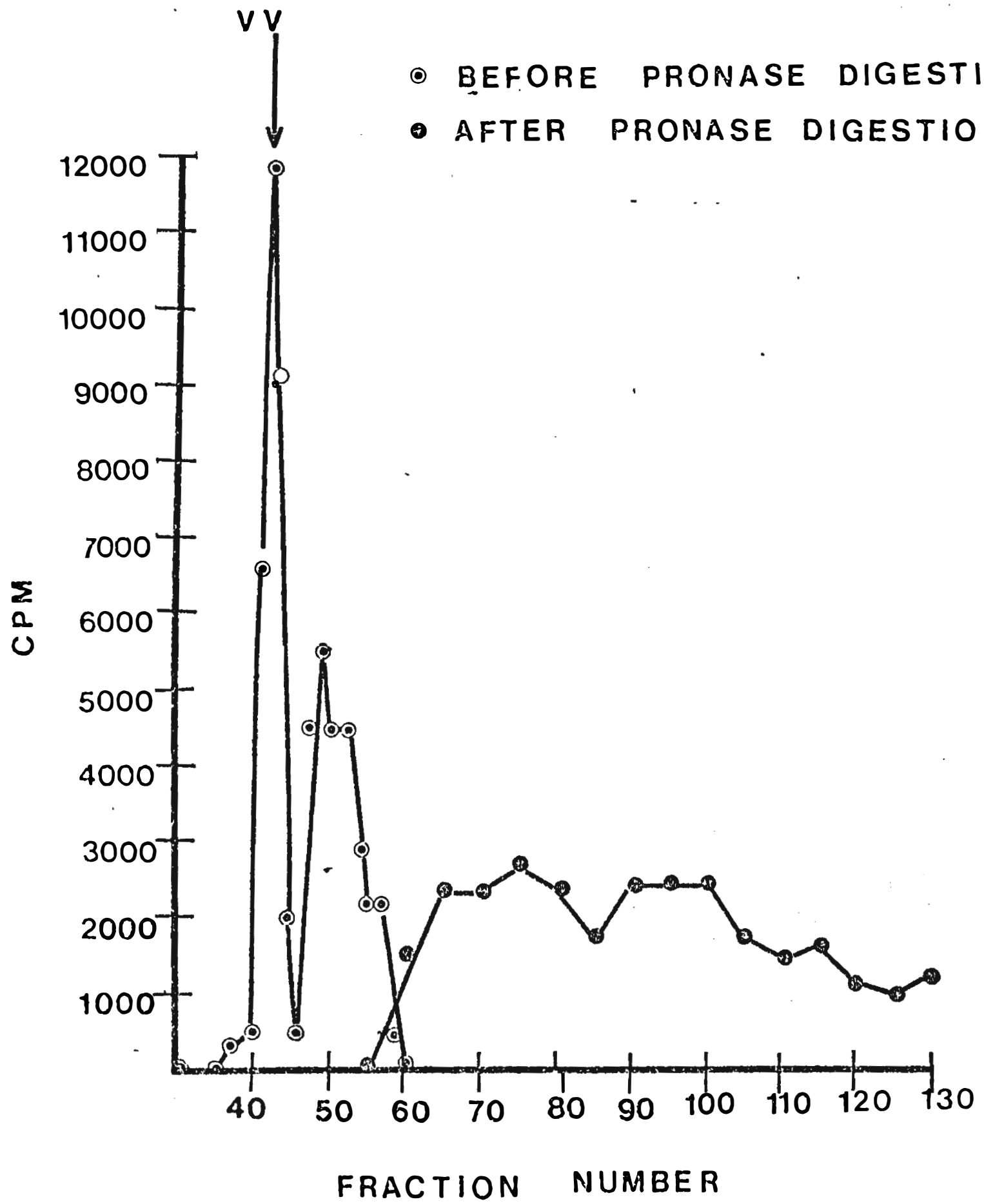
The second step was to see if the radioactive mannose bound to protein obtained from the high speed supernatant is of high molecular weight or not. The radioactive acetone precipitate was passed through a sephadex G-50 column equilibrated and eluted with 1.0 mM acetic acid (Figure 13). Approximately 56% of the total radioactivity was recovered at the void volume and the rest eluted just after the void volume peak. These

TABLE VI

Distribution of radioactivity at various stages in preparation of the acetone precipitate of dialyzed high speed supernatant.

Fraction	Total Radioactivity (cpm)
High speed supernatant	58,049
Precipitates after dialysis	6,000
Supernatant after dialysis	51,290
Acetone precipitate	40,000
Acetone supernatant	7,432

Figure 13. Column chromatography of [^{3}H] acetone precipitate before and after pronase digestion. Radioactive acetone precipitate was applied to a Sephadex G-50 column. The arrow indicates the void volume. The elute was digested with pronase and applied to the same column.


V V
BEFORE PRONASE DIGESTION
AFTER PRONASE DIGESTION
CPM
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
40
50
60
70
80
90
100
110
120
130
FRACTION NUMBER

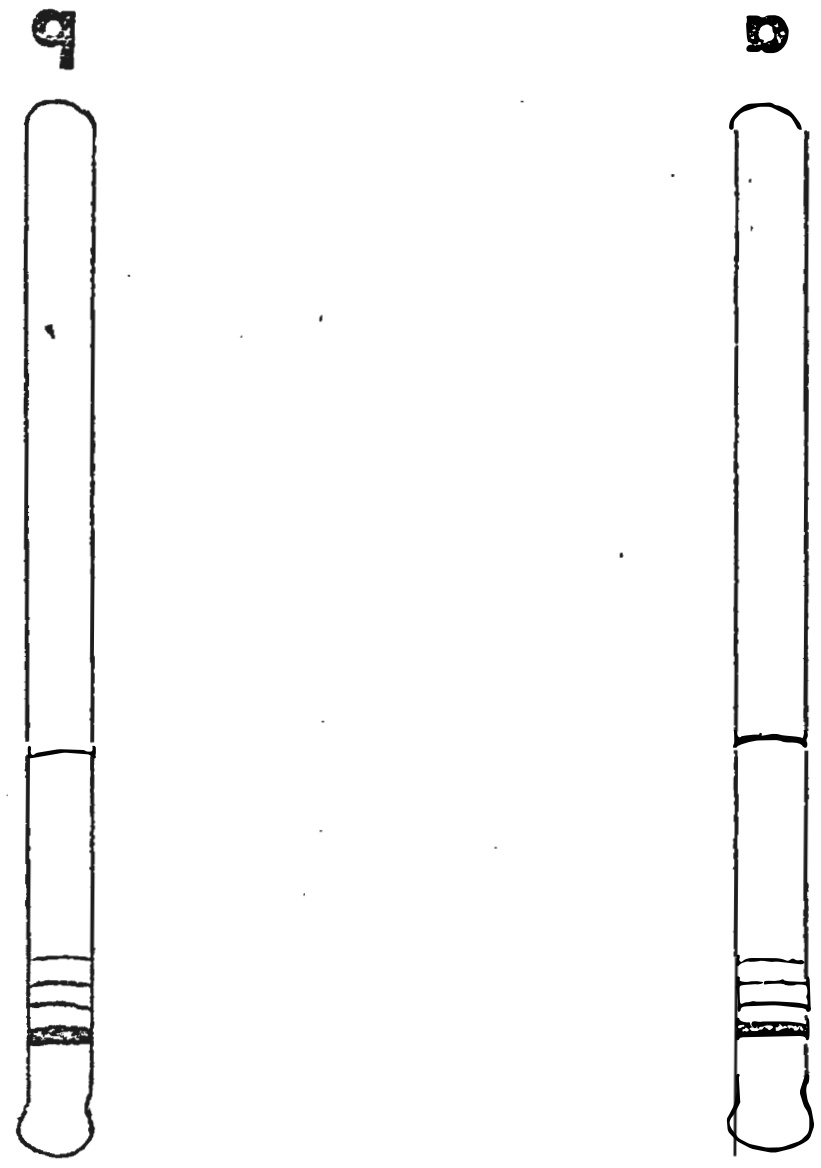
results suggested that all of the proteins have molecular weights equal of greater than about 40,000 daltons. The elute of this column was subjected to pronase digestion by dissolving 10 mg of it in 2 ml of a solution of 0.1 M Tris-HCl pH 7.9, 2 mM $CaCl_2$ containing 1 mg of pronase. A few drops of toluene were also added as a bacteriostatic agent. The reaction mixture was incubated at 37°C for 24 hr after which the same amount of pronase was added and the reaction was carried on for another 24 hr. The contents were lyophilized, dissolved in 1 ml of 1.0 mM acetic acid and passed through the same Sephadex G-50 column (Figure 13). It is clear that the high molecular weight material is susceptible to pronase digestion, indicating that the [^{3}H] mannose labeled acetone precipitate obtained from the high speed supernatant contains glycoproteins of high molecular weight.

The acetone precipitate was also applied to 7.5% SDS gels, half of which were stained by Coomassie brilliant blue and the other half with Fuchsin-sulphite (Figure 14). Five bands were stained in both gels, indicating the existance of at least 5 different glycoproteins in the acetone precipitate. Interestingly, the first 2 bands in these gels corresponded to the first 2 bands of the gels containing urea soluble SER proteins.

Figure 14. Acetone precipitates applied to 7.5% SDS gels.

Figure 14a. Stained with Coomassie brilliant blue.

Figure 14b. Stained with Fuchsin-sulphite specific for carbohydrates.

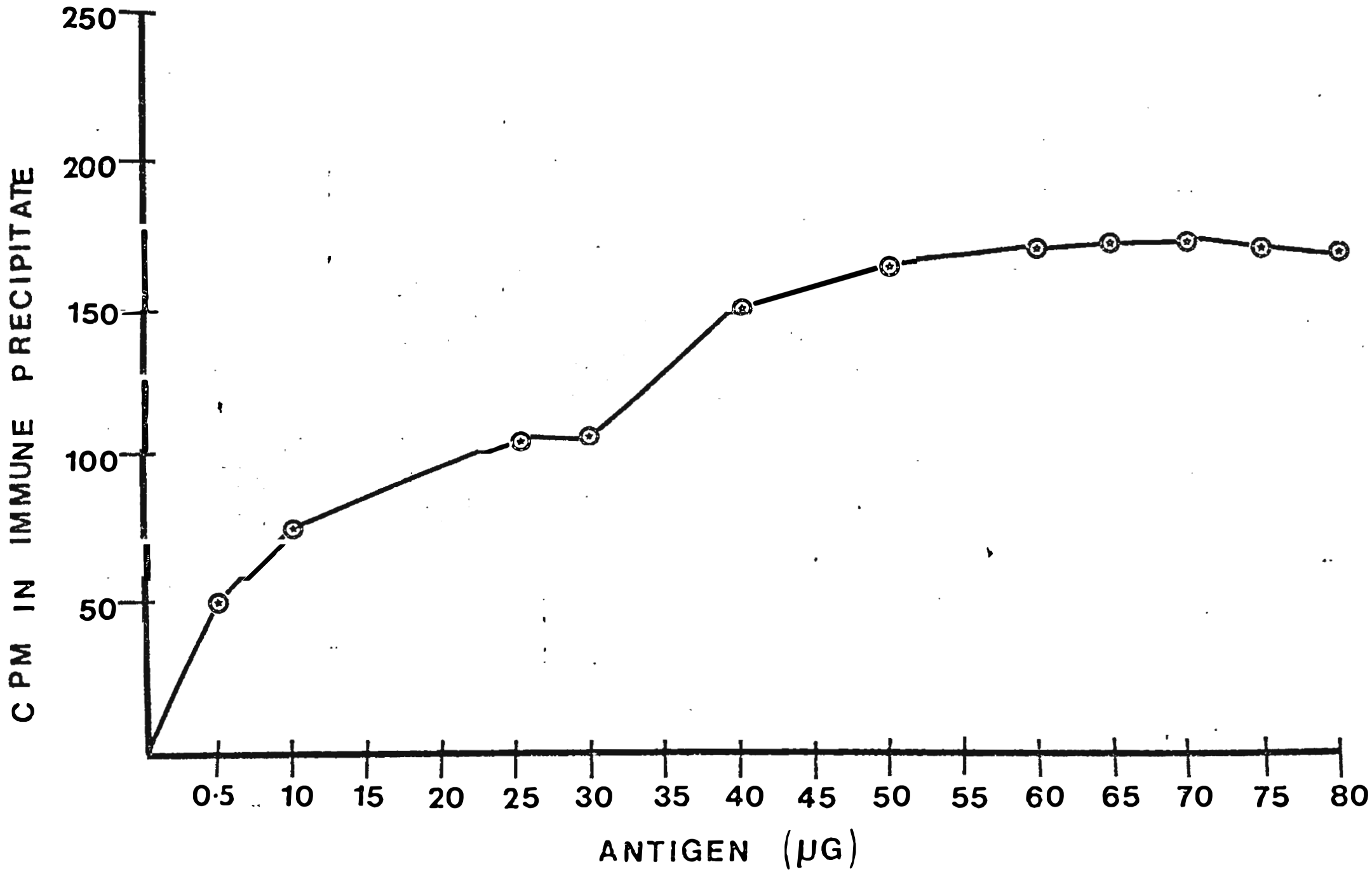

a
b

Isolation of Secretory Glycoproteins from the HSS Fraction

An attempt was made to see if the radioactive acetone precipitate contains any of the secretory glycoproteins isolated earlier (Rudick, et al., 1973, 1974, 1975, 1977). The rabbit antisera against extracellular α-glucosidase (α-gluE) and extracellular β-glucosidase (β-gluE) were prepared and used as described in 'Materials and Methods'. Anti-gluE (0.5 ml) was incubated with 0, 10, 25, 50, 75, 80 µg of dissolved radioactive acetone precipitate of specific activity 20,000 cpm per 100 µg of protein. The final volume this reaction mixture was 1 ml. The immune precipitate was collected by centrifuging at 5,000 rpm for 20 min, washed twice with 0.9% saline, and was counted in Beckman scintillation counter (Figure 15). Up to 40 µg of antigen, the radioactivity of the corresponding precipitate increased, after which it reached a plateau at about 175 cpm suggesting that any α-gluE present may represent only about 2.5% of the glycoprotein in the acetone precipitate. This calculation assumes that the specific activity of each labeled glycoprotein is the same.

Since so little of α-gluE was found to be present, an attempt was made to check the level of non-specific precipitation. The antigen dissolved in saline was incubated with 10 µg of rabbit anti-ovalbumin and 10 µg of

Figure 15. Immune precipitation of [^{3}H] acetone precipitate. Radioactive acetone precipitate of various concentration in 0.5 ml of 0.9% saline were treated with 0.5 ml of rabbit anti-α-gluE.


CPM IN IMMUNE PRECIPITATE
250
200
150
100
50
0·5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
ANTIGEN (µG)

ovalbumine, the precipitate was obtained and counted, and to this supernatant rabbit anti-α-gluE (0.5 ml) was added. The resultant second immune precipitate was counted and to this second supernatant rabbit anti-β-gluE (0.5 ml) was added yielding a third immune precipitate which was counted (Table VII). The total radioactivity of the antigen was about 10,000 cpm, out of which 4% was precipitated with anti-α-gluE and about 10% was precipitated with anti-β-gluE. According to Rudick and Elbein (1973, 1974, 1975) in the extracellular environment, α-glucosidase is found in much larger proportion than β-glucosidase. But when one tries to precipitate α- and β-glucosidases from the high speed supernatant; that is the intracellular environment, the outcome is different.

The immune precipitates corresponding to α- and β-glucosidases were applied to SDS gels. From rabbit anti-chicken serum (Pentex Biochemicals) IgG was prepared according to the method of Kabat (1970). One ml of serum was diluted with 1 ml of PBS and bringing this to 50% saturation with the addition of an equal volume of saturated $(NH_4)_2SO_4$. The mixture was stirred in the cold for 30 min and then was centrifuged at 9,000 rpm for 15 min. The supernatant was discarded and the precipitate was dissolved in twice the original volume of PBS. To this

TABLE VII

Distribution of radioactivity in immune precipitation experiments.

	Fraction	Total Radioactivity (cpm)
	Acetone precipitate (APPT)	10,000
Expt 1	APPT precipitated with anti-α-gluE	250
	Supernatant (after precipitation with anti-α-gluE)	9,620
Expt 2	APPT treated with anti-ovalbumin	125
	Supernatant II (after treatment with anti-ovalbumin)	9,805
	Supernatant II treated with anti-α-gluE	395
	Supernatant III (after treatment with anti-α-gluE)	9,201
	Supernatant III treated with anti-β-gluE	1,000
	Supernatant IV (after treatment with anti-β-gluE)	7,899

1. 10,000 cpm represents 50 µg of acetone precipitated protein.

saturated $(NH_4)_2SO_4$ was added to 50% saturation, and the mixture was centrifuged at 9,000 rpm for 15 min. The pellet was dissolved in 0.2 ml of PBS and dialyzed against water for 12 hr. The dialysate was lyophilized and applied to SDS gels (Figure 16a, b, c). The molecular weight of α-gluE is 63,000 daltons (Rudick and Elbein, 1973), that of β-gluE is 40,000 daltons (Rudick and Elbein, 1974), that of the heavy chain of IgG is 57,000 daltons and 19,000 is that of light chain of IgG. These results show that α-gluE and β-gluE are obtained from the intracellular environment. Interestingly enough these bands do match the bands on gels containing acetone precipitate, but the bands corresponding to α-gluE and β-gluE on gels containing acetone precipitate are not the pronounced bands. This too, indicates that α-gluE and β-gluE are not the major glycoproteins of the intracellular environment. A possible reason for this is that there might be a precursor of α-gluE in the intracellular environment (α-gluI) Rudick et al. (1977).

Antibody against α-gluI was prepared and dissolved radioactive acetone precipitate was incubated with 0.5 ml of anti-α-gluI. The precipitates were obtained and counted (Figure 17). A sharp increase in radioactivity was obtained in 125 µg. Since specific activity of the

Figure 16a. Immune precipitates obtained by precipitating acetone precipitates with rabbit-anti extracellular α-glucosidase, applied to 7.5% SDS gels.

Figure 16b. Immune precipitates obtained by precipitating acetone precipitates with rabbit-anti extracellular β-glucosidase, applied to 7.5% SDS gels.

Figure 16c. IgG applied to 7.5% SDS gels.

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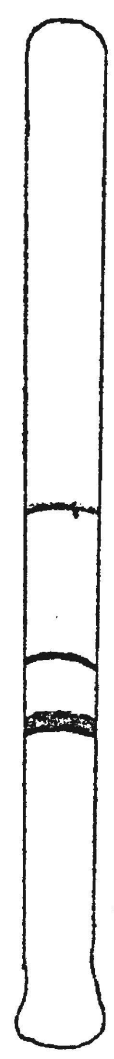

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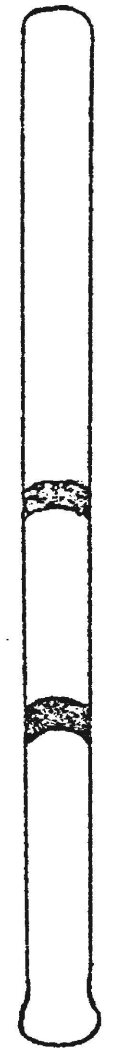

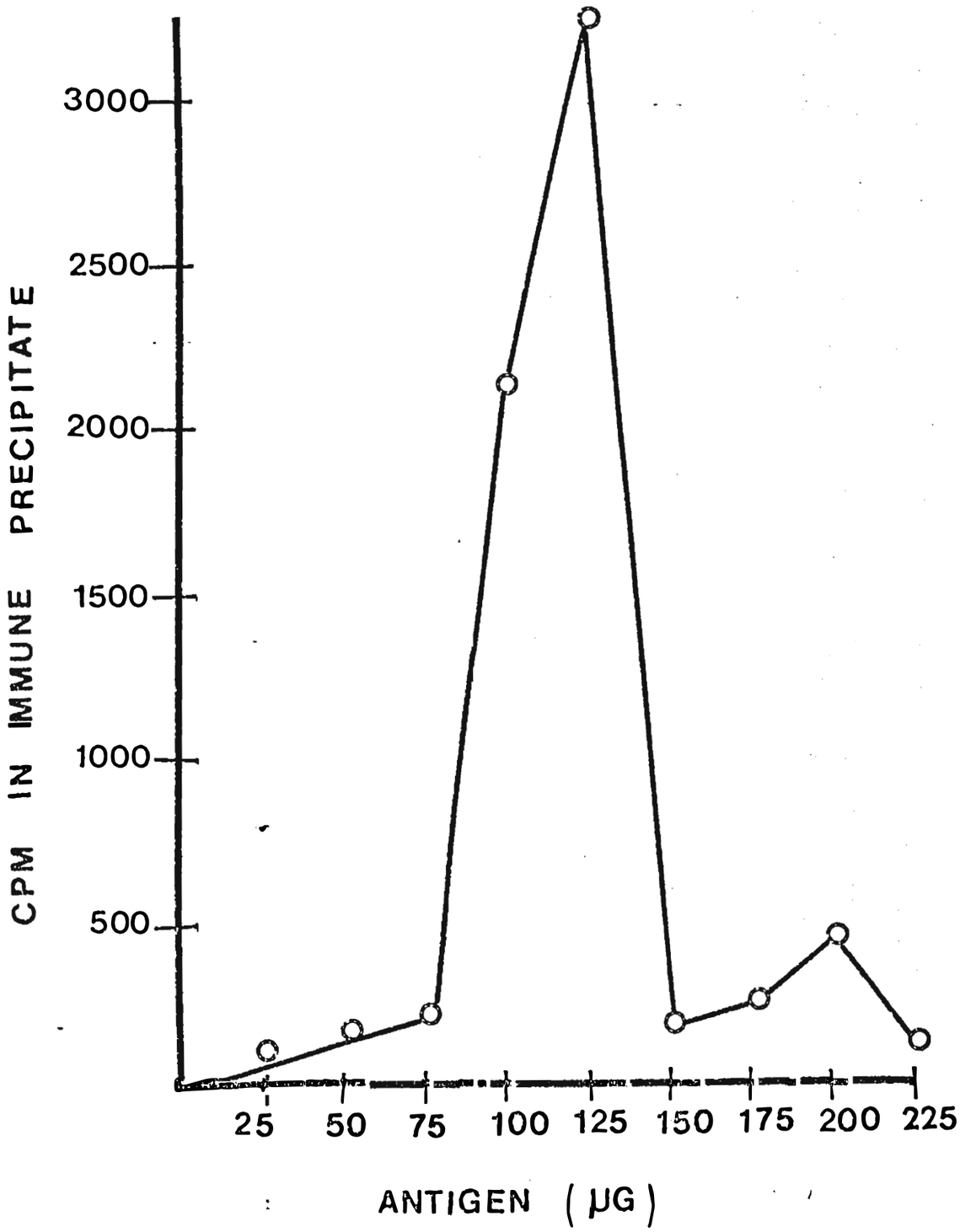
CPM IN IMMUNE PRECIPITATE
3000
2500
2000
1500
1000
500
25
50
75
100
125
150
175
200
225
ANTIGEN (µG)

Figure 17. Radioactive immune precipitates as a function of concentration of acetone precipitates. Radioactive acetone precipitates of various concentration was precipitated with 0.5 ml of rabbit anti-intracellular α-glucosidase.

acetone precipitate was 12,600 cpm per μg of protein. Approximately 30% of glycoproteins in the acetone precipitate may be represeñted by α-gluI, using the same assumption mentioned above.

DISCUSSION

In in vivo labeling experiments where mycelia were exposed to [^{3}H] mannose for various periods of time, the radioactivity of all subcellular fractions, except the ribosomal one, increases after the first 10 min of incubation. This may be because initiation of glycosylation of proteins takes place while polypeptide-chains are still bound to the ribosomes. Since mannosyl transferase activity was found associated with only the SER fraction, it is likely that polypeptides bound to ribosomes come into contact with the SER at some stage during glycoprotein synthesis. From the isolation of various subcellular fractions and electron micrographs of A. niger (Rudick, et al., 1977), it was concluded that a very small amount of membrane-bound ribosomes exists in this system. Molnar (1975) reported that sugar is attached to the elongating nascent polypeptide chain in rat liver and rat ascites cells. However, he did not say what kinds of proteins are glycosylated at the ribosomal site. Experiments of Keily and Schimke (1976) specifically showed that initiation of ovalbumin glycosylation occurs while nascent polypeptide chains are still attached to the ribosomes.

Although secretory glycoproteins of A. niger contain two sugars, namely mannose and glucosamine (Rudick and Elbein, 1974; 1975; 1976), attempts to incorporate radioactive glucosamine into the proteins were unsuccessful. The major component of the cell wall of A. niger is chitin which is a polymer of N-acetylglucosamine (Chu and Rudick, unpublished results). It is very possible that the intracellular pool of glucosamine is sufficiently large that any radioactive glucosamine added to the system is too diluted to be detected. However, the reason for lack of glucosamine incorporation is unknown.

In Figure 4, the mitochondria showed interesting behavior in two respects. First of all, within the first 10 min. of incubation with radioactive mannose, the amount of labeled protein was almost the same as in the other fractions such as ribosomes and SER, which are involved in mannosylating proteins. It is known from the research of several investigators that the mitochondria of Tetrahymena pyriformis (Crugy et al., 1974), Neurospora crassa (Kurigyama et al., 1973) and rat liver (Vries et al., 1973) contain membrane bound 55S ribosomes other than cytoplasmic ribosomes which synthesize some of the mitochondrial protein. Research of Bosmann et al. (1974) and Ibrahim and Beattie (1976) went one step further and showed that

pure mitochondrial preparations isolated form chick embryo fibroblasts contain several glycosyl transferases and thus glycosylate some of their own protein. In the mitochondria of A. ~~niger~~ a low level of mannosyl transferase activity was found. Perhaps that is why within the first 10 min the level of radioactive protein in the mitochondria was the same as in those organelles known to glycosylate the protein.

Secondly, the level of radioactive protein after 60 min was much higher in the mitochondria than in any other organelle. Electron micrographs of A. niger have shown that SER is connected with the outer membrane of the mitochondria (Fitzgerald and Rudick, unpublished results), so that perhaps via this connection the mitochondria may also receive some membrane glycoproteins from the SER. Thus the mitochondria in addition to mannosylating their own proteins receive protein synthesized and mannosylated by the SER. That is why perhaps it has such a high level of radioactive protein after 60 min.

Since the mannosyl transferase activity and a considerable amount of radioactivity were found in the SER fraction, there was reason to believe that completion of glycosylation may also take place in the SER and many different glycoproteins may be found there. However,

upon solubilization of the SER fraction, only two glycoproteins were found after SDS polyacrylamide gel electrophoresis, as determined both by staining with carbohydrate specific stain and labeling with radioactive mannose. If secretory and membrane-bound glycoproteins are both mannosylated in the SER, then this result suggests that binding of secretory glycoproteins with the membrane of the SER is a transient one and once this glycosylation is completed, they leave the SER and thus their presence cannot be detected. The glycoproteins which are destined to bind to the membranes of the other organelles of the cells may also leave the membrane of SER as soon as their glycosylation is completed.

To see the relationship between protein synthesis and glycosylation, cycloheximide (CH), and inhibitor of cytoplasmic protein synthesis, was employed. It appeared that within 10 min of administration of CH, the incorporation of labeled leucine and mannose into the proteins of ribosomal, SER and HSS fractions was stopped. This result suggests that there exists a one to one relationship between protein synthesis and mannosylation. Regardless of the duration of treatment with CH, the amount of labeled protein in the above mentioned fractions did not decrease, implying that secretion of glycoproteins

seems to be halted, because if secretion of glycoprotein from the cell to the medium had taken place, then the amount of radioactive protein in the HSS would have decreased. This is in agreement with the work of Ikehara et al. (1971) in which CH inhibited sugar incorporation into the ribosomal, SER and RER fractions of rat liver cells. However the reason for halting of secretion of glycoproteins under the influence of CH is unknown. Interestingly enough, the mitochondrial fraction was unaffected by the presence of cycloheximide.

As described earlier, the mitochondrial ribosomes are different from cytoplasmic ribosomes in that their sedimentation velocity is 55S and that of the cytoplasmic ribosomes is 80S. According to Ibrahim and Beattie (1976), CH blocks protein synthesis carried out by cytoplasmic ribosomes and not that carried out by mitochondrial ribosomes. This explains why levels of mitochondrial glycoproteins in A. niger were unaffected by the presence of CH.

As far as secretion is concerned, interesting results were obtained. A considerable amount of radioactivity was found in the HSS from in vivo labeling experiments. According to Palade (1975) it is highly unlikely to find intact glycoproteins in the cytoplasm

because of the presence of proteolytic enzymes. However, sephadex G-50 column chromatography showed that almost all [^{3}H] mannose labeled protein found in the HSS is of molecular weight greater than or equal to 40,000 daltons, suggesting that glycoproteins in the HSS do not seem to be subject to enzymatic attack. When the acetone precipitate obtained from the HSS was electrophoresed on SDS polyacrylamide gels, several glycoporteins were detected by staining with a carbohydrate specific stain. By immune precipitation a considerable amount of α-gluI and small amounts of α-gluE and β-gluE were detected. This suggests perhaps glycoproteins do travel through the cytosol. It appears from the research of Rudick et al. (1977) that α-gluI could be a precursor of α-gluE, and, if so, that the plasma membrane is involved in changing the precursor into the product (α-gluE), because α-gluI is found throughout the cytoplasm but α-gluE is found only on both sides of the plasma membrane and the outer cell wall of A. niger. Since only small amounts of α-gluE and β-gluE were found in the HSS, it is likely that the homogenization process stripped them from the plasma membrane.

A picture emerges from this research in which initiation of sugar attachment to the newly synthesizing

polypeptide chain takes place while nascent polypeptide chain is still bound to the (membrane bound) ribosomes. After the initiation, proteins destined to be secretory glycoproteins may transiently bind to the SER while those proteins destined to be membrane bound glycoproteins may enter the SER where further glycosylation is completed. There appears to be a one to one relationship between protein synthesis and glycosylation. It seems that once the glycosylation is completed, all the glycoproteins except those bound to the membrane of SER are immediately released from it. The mitochondria emerge as unique organelles in that they may recieve some of their glycoprotein from the SER, but they may also synthesize their own glycoprotein. Some of the glycoprotein released to the HSS (or cytosol) of the cell could be precursors of secretory glycoproteins, and the plasma membrane may play an important role in changing precursors into the products.

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