THE USE OF WESTERN BLOTTING TO INVESTIGATE A MEMBRANE-ASSOCIATED GLYCOPROTEIN

A DISSERTATION

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INTRODUCTION

Translocation of Secretory and Membrane Proteins

All cells produce proteins which must interact with cellular membranes. Some of these immediately pass through the membrane while others remain associated with it, either permanently or for some time prior to their secretion. Whether these proteins are secreted into the external environment or remain membrane bound, they seem to share several properties which suggest a common mechanism for their translocation across the membrane. These properties are as follows: the presence of an amino-terminal signal sequence; transfer of at least part of the polypeptide across a lipid barrier; sensitivity to ribosomally directed antibiotics (Wickner, 1980).

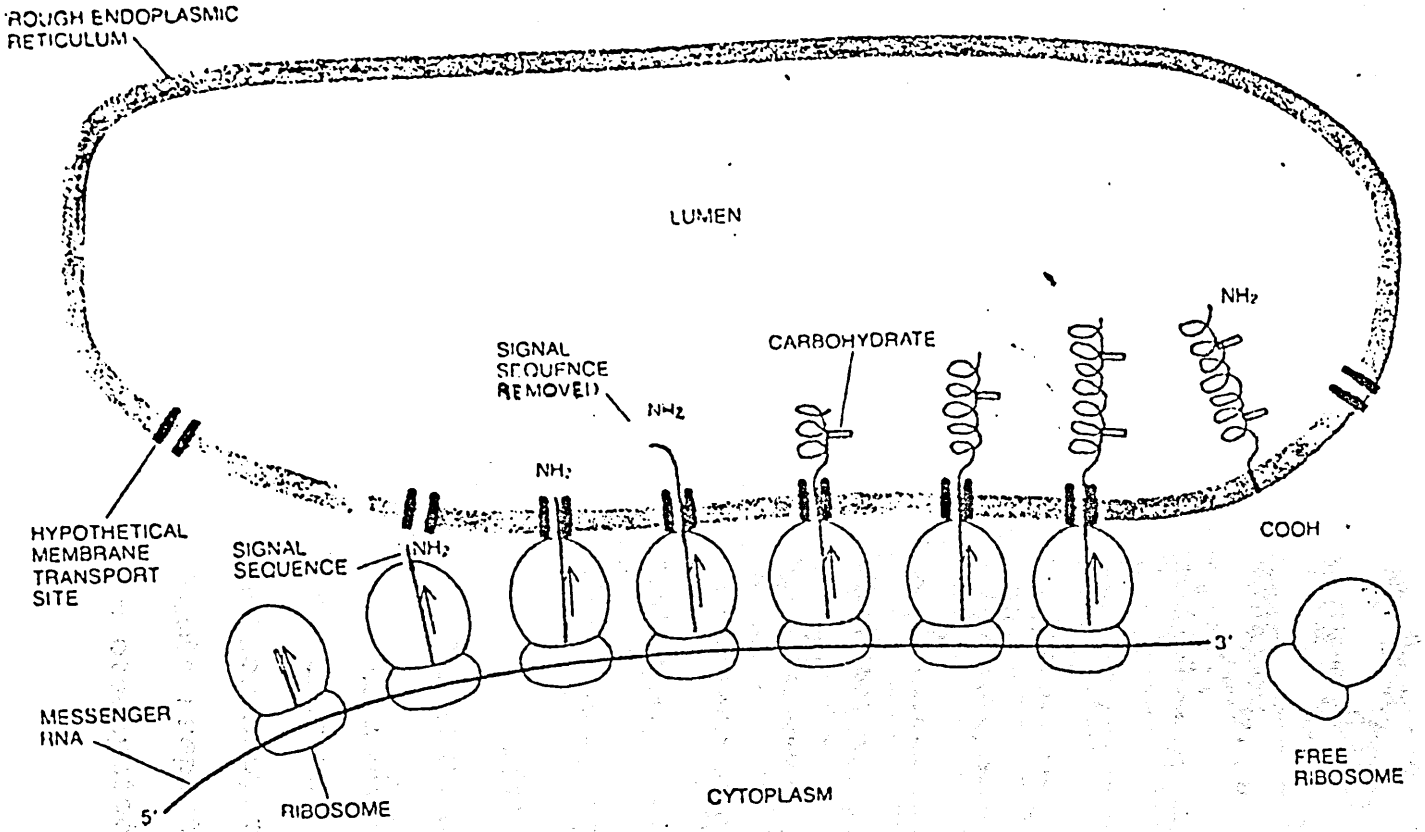
In trying to elucidate the mechanism of translocation, it has been found that some secretory proteins are synthesized on polysomes attached to the rough endoplasmic reticulum (Palade, 1975). These proteins, whether they are destined to become membrane bound or to be secreted, contain a sequence of 15 to 30 hydrophobic amino acid residues, usually at their amino termini, which allows them to pass entirely or partially through the hydrophobic

lipid bilayer and enter or extend into the lumen of the endoplasmic reticulum (Marx, 1980; Blobel, 1980; Blobel and Dobberstein, 1975a). These residues have been termed the signal sequence. As proteins pass through the membrane this sequence is cleaved by a protease located on the luminal side of the endoplasmic reticulum (Palade, 1975).

Microsomal membranes have been shown to be incapable of translocating a secretory protein if, after trypsin treatment of these membranes, the soluble fraction is removed (Walter, et al., 1979). When this fraction is added back to the trypsinized microsomes, normal translocation can occur. This suggests that the membrane contains a transmembrane protein at least part of which is exposed on the cytoplasmic side of the membrane and which is responsible for the recognition of the signal sequence and/or the ribosome. Walter et al. also propose that the domain remaining with the membrane forms an environment favorable to the passage of a secretory protein through the lipid bilayer.

According to Blobel's signal hypothesis (Figure 1), translocation across the membrane begins soon after protein synthesis is initiated. Thus, in an in vitro protein synthesizing system containing microsomes, if membranes are added after protein synthesis has progressed too far,

Figure 1. Signal hypothesis for the translocation of a protein across a membrane.


ROUGH ENDOPLASMIC RETICULUM
LUMEN
SIGNAL SEQUENCE REMOVED
NH2
CARBOHYDRATE
NH2
NH2
HYPOTHETICAL MEMBRANE TRANSPORT SITE
SIGNAL SEQUENCE
NH2
COOH
3'
MESSENGER RNA
5'
RIBOSOME
CYTOPLASM
FREE RIBOSOME

the secretory protein will not be found sequestered within the membrane vesicle. Instead, the protein plus its hydrophobic signal sequence will be found outside the vesicle (Blobel and Dobberstein, 1975b).

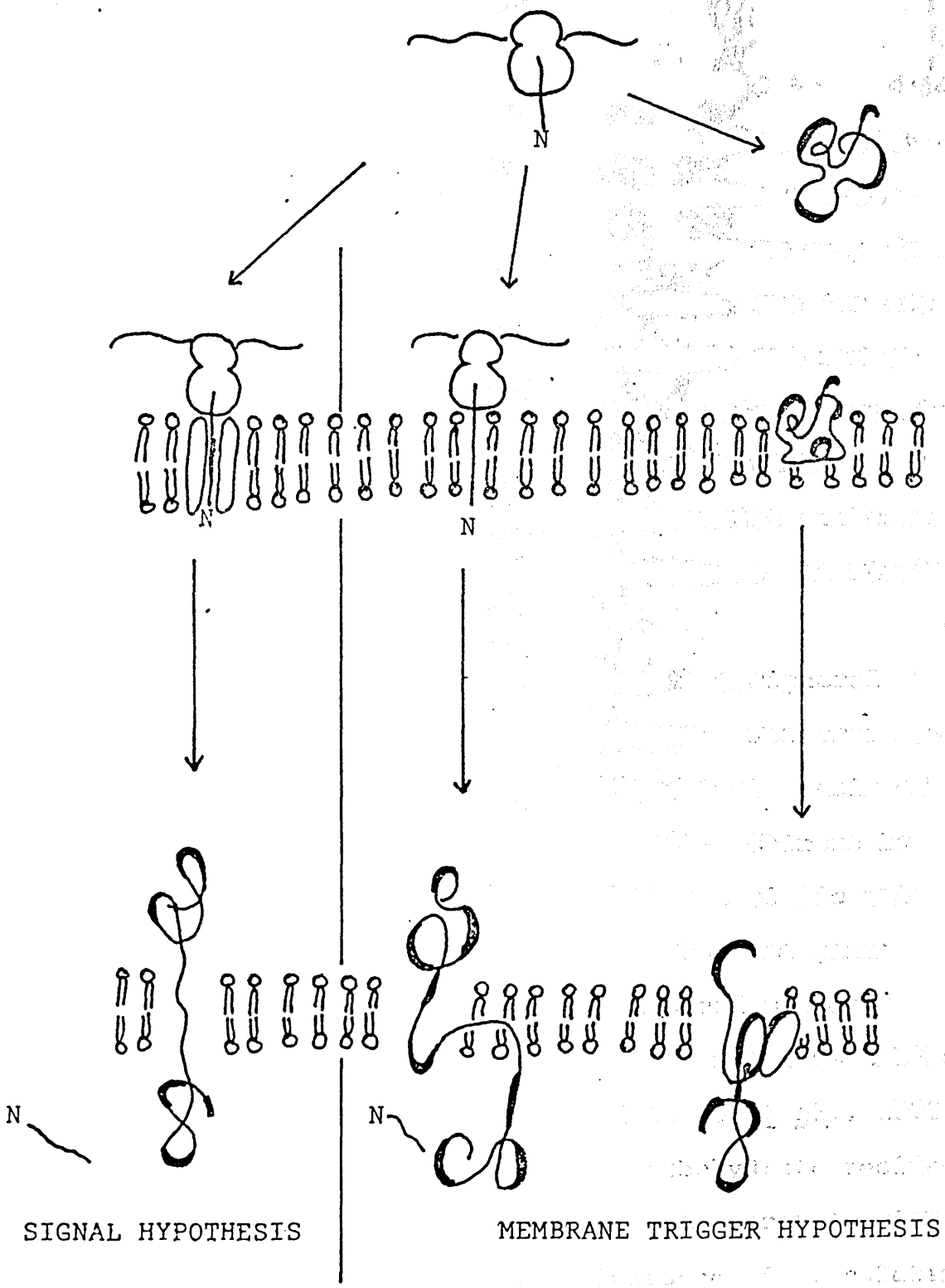
Wickner (1979) proposes that the signal sequence may not be required for translocation, or that it may play an alternative role. He suggests that an amino terminal signal sequence induces a conformation which enables the protein to interact favorably with the aqueous environment of the cytoplasm before being inserted into the membrane. As folding of the protein continues, hydrophobic residues are exposed to the lipid bilayer enabling the polypeptide to be inserted into and eventually cross the membrane. Removal of the amino-terminal leader sequence, if one is present, renders the process irreversible. This proposal would explain the secretion of such proteins as β-lactamase and maltose-binding protein of Escherichia coli which are produced as cytoplasmic proteins before they are secreted; and the intracellular secretion of ribulosebisphosphate carboxylase which exists as a cytoplasmic preprotein before entering the chloroplast where it is proteolytically processed (Wickner, 1980).

Regardless of the role of the initial sequence, once the growing polypeptide is directed into the

intracisternal space it is glycosylated by membrane-bound glycosyl transferases. These enzymes transfer the core oligosaccharide to an asparagine residue of the nascent protein from an oligosaccharide pyrophosphoryldolichol intermediate (Forsee and Elbein, 1975; Li et al., 1978; Waechter and Lennarz, 1975).

According to Blobel (1978), if the protein is to remain membrane bound, it will contain a sequence of hydrophobic amino acids which stops translocation and allows the protein to remain embedded in the lipid bilayer. In contrast, Wickner proposes that sequential hydrophobic residues are not required. Instead the protein's conformation in the membrane would be such that the hydrophobic residues are exposed to the lipid bilayer while the hydrophilic residues are sequestered inside the molecule. On the other hand, if a protein is to be secreted it contains no such hydrophobic series of amino acids or exposed hydrophobicity. Figure 2 shows a comparison of the signal and membrane trigger hypothesis (Wickner, 1979). It is interesting to note, however, that some secretory proteins, such as a penicillinase from Bacillus licheniformis (Yamamoto and Lampen, 1975) appear to remain associated with the membrane for some time before being released.

Figure 2. Comparison of the signal hypothesis and the membrane trigger hypothesis. The thin lines represent hydrophobic amino acid residues and the thicker lines represent hydrophilic residues.


N
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N
N
N
SIGNAL HYPOTHESIS
MEMBRANE TRIGGER HYPOTHESIS

Peripheral Proteins

The cell membrane is made up of a phospholipid bilayer with the lipids aranged such that the nonpolar fatty acid chains are interior to the membrane away from contact with an aqueous environment. Some proteins are held to this membrane by ionic interactions and may be dissociated from the membrane *in vitro* by simply exposing them to high ionic strength. Examples of peripheral proteins include cytochrome c in mitochondrial membranes and spectrin in erythrocyte membranes (Singer and Nicolson, 1972; Marchesi and Steers, 1968).

There are several means by which a peripheral protein may be associated with a membrane. For example, a glycoprotein's carbohydrate portion can be the basis of its association with a membrane-bound protein. Chinese hamster ovary cells which are deficient in membrane sialic acid are resistant to the cytotoxic effects of wheat germ agglutinin, a lectin which binds sialic acid residues (Briles, *et al.*, 1977) and which has been shown to inhibit amino acid transport in lymphocytes (Greene *et al.*, 1975).

Another example of a specific carbohydrate residue playing a role in the association of a glycoprotein with a membrane receptor occurs during clearance of circulating glycoproteins from the blood by liver. Ashwell and Morell

(1974) demonstrated that when terminal sialic acid residues were removed from ceruloplasmin, leaving galactose exposed, the survival time of the glycoprotein in the circulation dropped from 56 h to approximately 15 min. Concomitantly, the neuraminidase-treated ceruloplasmin rapidly accumulated in liver parenchymal cells. For binding of this asialo-ceruloplasmin to the hepatic receptor to occur, the receptor itself had to contain sialic acid. Therefore, the presence of a specific sugar residue is not only necessary on the circulating glycoprotein, but on the receptor glycoprotein as well.

There are other cases which may be cited to demonstrate the role of the carbohydrate in recognition. For example a mannose-specific lectin present on the surfaces of E. coli has been shown to be responsible for the adhesion of the bacteria to epithelial cells, since α-methylmannoside will inhibit the adherence of the bacteria to the cells (Eshdat, et al., 1978). The association of lysosomal proteins with a specific membrane component seems to require a modified sugar residue. Mannose-6-phosphate has been identified on lysosomal enzymes from normal human cells (Hasilik and Neufeld, 1980b; Tabas and Kornfeld, 1980). Cultures of cells from patients with I-cell disease, a lysosomal storage disease, contained lysosomal enzymes

that not only were defective in their degree of phosphorylation, but also were located outside the cell indicating that mannose-6-phosphate is a necessary recognition marker for proper intracellular localization of the enzyme (Hasilik and Neufeld, 1980a).

A different means by which peripheral membrane proteins are sometimes associated with the membrane is through their interaction with other membrane proteins. One such example is the IgE binding glycoprotein isolated from rat basophilic leukemia cells. Crosslinking studies have shown that the IgE binding protein becomes crosslinked in a 1:1 ratio with another membrane protein. When the two are separated, the IgE binding protein can still interact with IgE normally, indicating that the other membrane component plays no role in the interaction of the IgE binding protein with IgE (Kanellopoulos et al., 1980).

Integral Proteins

The integral membrane proteins may be removed from the membrane only by harsh methods, such as treatment with detergent. This indicates that portions of the protein are actually embedded in the lipid bilayer. The integral proteins have a hydrophobic region which interacts with the lipids composing the membrane and, upon removal, tend

to form aggregates in an aqueous environment (Singer and Nicolson, 1972).

One example of an integral membrane protein is the exopenicillinase of B. licheniformis. This enzyme is found in both a membrane-bound and secretory form. Trypsin treatment of the membrane-bound form of the enzyme results in a hydrophilic penicillinase that is very similar to the exoenzyme and a 3,000 dalton hydrophobic peptide derived from the carboxy terminus (Yamamoto and Lampen, 1975).

Another example of mature secreted forms which are derived from higher molecular weight membrane-bound precursors is produced by rabbit glandular epithelial cells. The mature secretory enzyme in this case represents a proteolytic fragment of the transmembrane precursor form (Mostov et al., 1980).

α-glucosidase of Aspergillus niger

The fungus Aspergillus niger synthesizes several secretory enzymes including α-glucosidase, β-glucosidase, α-galactosidase, and β-mannosidase. Each of these glycosidases is a glycoprotein with a single asparagine-linked, high mannose type oligosaccharide chain (Rudick and Elbein, 1973; 1974; 1975; Adya and Elbein, 1977; Elbein et al., 1977; Rudick, 1979).

One of the secretory proteins of A. niger is a 63,000 dalton α-glucosidase (Rudick and Elbein, 1974). It is synthesized on membrane-bound ribosomes and glycosylation takes place on the nascent polypeptide chain (Long and Rudick, 1979). After its production, the enzyme appears to remain attached to the membrane for some time before it is released into the medium. This attachment is not affected by treatment with α-methylmannoside, an analogue of the nonreducing end of the oligosaccharide chain, which seems to rule out attachment by its carbohydrate portion (Fitzgerald, 1979). High salt treatment releases some of the enzyme from the membrane suggesting that, at most, only part of the α-glucosidase present is held to the membrane by ionic attractions. The studies reported here are the result of an investigation of the association of α-glucosidase with the membranes of A. niger. These results have been incorporated into an interpretive model. Also, a technique is described which allows the determination of the molecular weight of a specific unpurified protein.

MATERIALS AND METHODS

Materials

The following were obtained from Sigma Chemical Co., St. Louis, Mo.: bovine serum albumin (BSA); p-nitrophenyl-α-D-glucopyranoside; Coomassie Brilliant Blue R-250 (CBB R-250); diethylaminoethyl (DEAE)-cellulose; the protease inhibitors phenylmethyl sulfonyl fluoride (PMSF), p-amino-benzamidine, and trypsin inhibitor; Triton X-100; triethanol-amine (TEA); dimethyl suberimidate (DMS); 3,3'-diamino-benzidine and mannose-6-phosphate. Tritiated-leucine (52 Ci/mM) was purchased from ICN, Irvine, Cal. Coomassie Brilliant Blue G-250 (CBB G-250), X-Omat R film and High Contrast Copy film were from Eastman Kodak Co., Rochester, N. Y. Hydroxyapatite, N,N'-diallyltartardiamide and Bio-Gel A-5m were products of BioRad Laboratories, Richmond, Cal. Sephacryl S-200 was purchased from Pharmacia Fine Chemicals, Inc., Uppsala, Sweden, and nitrocellulose sheets from Millipore Corp., Bedford, Mass. EN^{3}HANCE, EN^{3}HANCE spray and Aquasol were obtained from New England Nuclear, Boston, Mass. Fluram was a product of Hoffmann-LaRoche, Inc., Nutley, N. J. The protease inhibitors N-α-tosyl-L-lysylchloromethane hydrochloride (TLCK),

tosyl-L-phenylalanylchloromethane (TPCK), and p-toluene sulphonyl-L-arginine methyl ester hydrochloride (TAME) were from Calbiochem-Behring, San Diego, Cal. Bacterial alkaline phosphatase was a gift of Dr. Myron Jacobson, North Texas State University and [^{14}C]-lysine (50 mCi/mmole) was kindly provided by Dr. Ben Harris, North Texas State University. All other chemicals were reagent grade or the highest purity available.

Growth of Fungus

Aspergillus niger was grown for 48 h at 30 C as previously described (Rudick and Elbein, 1973). Mycelia were harvested by filtration through 4 layers of cheesecloth and washed with distilled water before homogenization.

Analytical Methods

Protein. Prior to protein analysis a sample of membrane was brought to 50 µl by the addition of water. To this, 50 µl of 88% formic acid was added and the mixture was allowed to stand at room temperature for 10 min. Five ml of Coomassie reagent were added and the absorbance at 595 nm determined (Bradford, 1976).

α-glucosidase. The substrate for the assay of α-glucosidase was p-nitrophenyl-α-D-glucopyranoside. The reaction mix for the determination of glucosidase activity

contained the following: 0.1 ml substrate (5mM), 0.1 ml sodium acetate buffer (1 M, pH 5), 0.2 ml water, and 0.1 ml enzyme. Following incubation at 37 C for 90 min, 0.5 ml glycine buffer (0.4 M, pH 10,5) was added and the absorbance at 405 nm determined (Rudick and Elbein, 1973).

Determination of radioactivity. Five ml of Aquasol were added to radioactive samples which were then counted in a Beckman LS 9000 Liquid Scintillation Counter.

Fluorography. After electrophoresis, the slab gel was fixed overnight in a solution containing 10% TCA, 10% acetic acid and 30% methanol. After the radioactive proteins were fixed in the gel, the gel was incubated with EN^{3}HANCE for 1 h followed by incubation in cold water for 1 h. The gel was than dried on a Savant SGD-200 slab gel dryer. Nitrocellulose (NC) sheets, to which had been transferred radiolabelled proteins, were lightly sprayed with EN^{3}HANCE spray 3 times in preparation for fluorography. After treatment, the presence of radioactive bands on both the gels and NC were detected by placing Kodak X-Omat R film over the gel or NC sheet and allowing exposure for 10 days at -80 C. The film was then developed using Kodak X-ray developer.

Determination of molecular weight. Five mg BSA and 6 mg DMS were placed into 1 ml 0.2 M TEA, pH 9. After

incubation at room temperature for 90 min, an equal volume of 2% SDS and 2% β-mercaptoethanol in 0.2 M TEA (pH 9) was added. This was frozen in dry ice-acetone and stored for 18 h at -10 C. The BSA sample was then incubated at 37 C for 2 h and the resultant oligomers used as molecular weight markers on polyacrylamide gels (Carpenter and Harrington, 1972).

Photographic Methods

Gels were photographed using Kodak High Contrast Copy film. Fluorescent bands on NC sheets were photographed using Kodak High Contrast Copy film and an orange filter under an ultraviolet black light with exposure times ranging from 5 to 12 min.

Preparation of Membranes

Isolation of total membrane. Mycelia were suspended in 50 mM Tris-HCl, pH 7.2, containing 10 mM $MgCl_2$ and homogenized with an equal weight of glass beads for 8 min in a Waring blendor. Homogenate was filtered through 3 layers of cheesecloth and the filtrate was centrifuged for 10 min at 2,000 x g_{av} in a Beckman J-21B centrifuge to remove nuclei and cell walls. The resultant supernatant was centrifuged at 7,800 x g_{av} for 20 min to remove mitochondria, and the post-mitochondrial supernatant was

centrifuged for 2 h at 72,893 x g_{av} in a Beckman L5-65 ultracentrifuge. Supernatant was discarded and the white, uppermost layer of the pellet suspended in buffer appropriate for the ensuing experiment.

Treatment with protease inhibitors. To prevent proteolysis, the following concentrations of protease inhibitors were used during the last 2 h of growth of the mycelia and/or during membrane isolation:

1. 2 mM PMSF (Takeda et al., 1975; James, 1978).
2. 6 mM p-aminobenzamidine (Cox et al., 1978).
3. 250 µg/ml TLCK (Schmeckpeper et al., 1975).
4. 100 µg/ml TPCK (Schmeckpeper et al., 1975).
5. 875 µg/ml TAME (Schmeckpeper et al., 1975).
6. 10 µg/ml trypsin inhibitor.

To detect membrane-bound species of secretory α-glucosidase (α-glu_E) protease inhibitor treated membranes were electrophoresed on 10% sodium dodecyl sulfate (SDS)-polyacrylamide slab gels, transferred to NC sheets and incubated with Fluram-labeled rabbit anti-α-glucosidase IgG (anti-α-glu_E IgG).

Gel filtration chromatography was also used to detect species of α-glucosidase in protease inhibitor-treated membranes. Membranes which had been isolated in the presence of 2 mM PMSF and solubilized with 0.5% Triton

X-100 in 50 mM Tris-HCl buffer, pH 7.2, containing 10 mM $MgCl_2$ were fractionated on a Sephacryl S-200 column equilibrated with the same buffer containing 0.5% Triton X-100. Fractions were assayed for α-glucosidase activity and the point of elution of the membrane-bound α-glucosidase (α-glu_M) compared with that of α-glu_E.

Crosslinking of membrane proteins. To determine an appropriate concentration of crosslinker, IgG (40 µg) was crosslinked at 20 C for 15 min in 0.25 M TEA buffer, pH 9, with concentrations of DMS ranging from 0.4 to 2.4 mg/ml. The reaction was stopped by addition of excess sample buffer and boiling for 2 min. Samples were placed onto 7.5% SDS-polyacrylamide slab gels and electrophoresed until the tracking dye reached the bottom of the gel. The gel was stained for protein.

Membrane (48 and 64 µg protein) isolated in the presence of 2 mM PMSF was crosslinked for 15 min at 20 C with from 0.2 to 1.0 mg/ml DMS in 0.25 M TEA, pH 9. The reaction was stopped by the addition of 100 µl of dissociation solution and boiling for 2 min. Following SDS-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to NC sheets and the presence of α-glucosidase detected using anti-α-glu_E IgG.

Polyacrylamide Gel Electrophoresis

SDS gels. SDS gels were prepared using varying amounts of acrylamide solution (22.2 g acrylamide and 0.5 g bisacrylamide brought to 100 ml with water) depending on the final concentration of acrylamide desired. In addition to acrylamide solution, the resolving gel contained 15 ml lower gel buffer (1.5 M Tris-HCl, pH 8.8), 0.3 ml ammonium persulfate (40 mg/ml), 0.03 ml TEMED, 0.3 ml SDS (10%), and water to a final volume of 30 ml. The stacking gel contained 6 ml water, 2.5 ml upper gel buffer (0.5 M Tris-HCl, pH 6.8), 1.35 ml acrylamide solution, 0.1 ml ammonium persulfate (40 mg/ml), 0.1 ml SDS (10%) and 0.01 ml TEMED. Samples, in deionized water, were diluted 2- to 4-fold with sample buffer (0.2 M Tris-HCl, pH 6.8, 2% SDS and 2% β-mercaptoethanol). The samples were layered on the gels and electrophoresed at 10 mA during stacking and then at 30 mA until the tracking dye reached the bottom of the gel (Weber and Osborn, 1969).

Staining. Tube gels were stained overnight with 0.1% CBB R-250 prepared in methanol:water:acetic acid in the ratio of 4.5:4.5:1 (Chrambach et al., 1967). They were destained electrophoretically in a solution containing 7 parts acetic acid, 5 parts methanol and 88 parts water. Slab gels were fixed overnight in 50% TCA and then stained

for 1 h at 37 C with 0.1% CBB R-250 made in 50% TCA. Destaining was performed by repeated washing in 7% acetic acid (Laemmli, 1970).

Immunological Methods

Isolation of α-glucosidase. α-glucosidase was isolated from 72-h cultures using the following steps: (1) ammonium sulfate precipitation, (2) acetone precipitation, (3) DEAE-cellulose anion exchange chromatography, (4) hydroxyapatite chromatography and (5) gel filtration chromatography using Sephacryl S-200. The first 3 steps were performed as described by Rudick and Elbein (1974). For step 4, lyophilized protein from step 3 was dissolved in 0.01 M phosphate buffer, pH 7.5. This was applied to a hydroxyapatite column which had been prepared and equilibrated with the same buffer. After application of the protein, the column was washed with 1 column volume of 0.01 M phosphate buffer, pH 7.5. The protein was then eluted with a linear gradient (0.01 M to 0.1 M phosphate buffer, pH 7.5). The fractions containing α-glucosidase activity were pooled, dialyzed against water and lyophilized. The protein was dissolved in a small volume of 0.05 M potassium phosphate buffer, pH 7.6, containing 14 mM β-mercaptoethanol and applied to a Sephacryl S-200 column equilibrated with

the same buffer. Fractions containing α-glucosidase activity were pooled, dialyzed against water and lyophilized.

Preparation of antibody. The α-glucosidase was dissolved in PBS, pH 7.5, and weekly intramuscular injections administered to rabbits. After 4 weeks, blood was withdrawn from the ear vein, allowed to clot, and the serum collected. The serum was placed into the center well and purified $\alpha\text{-glu}_E$ into the outer wells of double immunodiffusion plates and these plates allowed to stand at room temperature. Precipitin lines indicated the presence of anti-$\alpha\text{-glu}_E$ IgG in the serum (Ouchterlony, 1968).

Immunoglobulin was isolated from the serum by 3 sodium sulfate precipitation steps. All precipitations were carried out at room temperature and were followed by centrifugation at 7,000 x g_{av} for 15 min. Following the final precipitation, the IgG was dissolved and stored in PBS, pH 8, until use (Kawomura, 1969).

Preparation of double diffusion plates. Double diffusion was carried out overnight at room temperature in petri dishes containing 35 ml of 1% agar. For membrane samples, the plates were prepared containing 0.5% Triton X-100 or 1% SDS.

Immunochemical assay of isolated membranes.

Membranes were assayed for the presence of α-glu_M by incubating isolated membranes for 30 min at 4 C with anti-α-glu_E IgG which had been coupled to horseradish peroxidase (Fitzgerald, 1979). The membrane was than passed through a Bio-Gel A-5m column, using PBS (pH 8) as the eluate, to separate unreacted IgG-peroxidase from the membrane-IgG-peroxidase complexes. Fractions were collected and the presence of protein determined by absorbance at 220 nm. Peroxidase activity was assayed as follows: To 0.5 ml of sample was added 0.5 ml of 0.1% diaminobenzidine in 0.1% hydrogen peroxide. The absorbance at 340 nm was determined after a 10-min incubation at room temperature.

Membranes were subjected to various treatments in an attempt to determine the nature of the attachment of α-glucosidase to the membrane. In these cases the Bio-Gel A-5m column was equilibrated with the appropriate reagents before application of the treated sample to the column. The membrane treatments were as follows:

1. 0.5 M NaCl for 30 min.

2. 0.2% dithiothreitol for 30 min followed by centrifugation at 150,315 x g_{av} for 1 h. The membrane pellet was then resuspended in PBS, pH 8.

3. 1 mM mannose-6-phosphate for 30 min.

4. 1 unit/ml bacterial alkaline phosphatase in 0.15 M NaCl and 0.01 M Tris-HCl, pH 8 for 1 h at 37 C.

After treatment, the membranes were chromatographed on a Bio-Gel A-5m column and the presence of protein and peroxidase activity assayed, as described above.

Transfer of Proteins to Diazobenzyloxymethyl (DBM)-paper Electrophoresis. Polyacrylamide/agarose gels were prepared from stock solutions containing, in a total volume of 100 ml, 28.5 g acrylamide and 1.5 g N,N'-diallyltartardiimide (DATD). To prepare the gel, 6 ml of buffer (1.88 M Tris-HCl, pH 8.8), 11.5 ml of water and 0.3 g of agarose were heated to 50 C. To this, 12 ml of stock solution, 0.3 ml ammonium persulfate (10%) and 0.03 ml TEMED were added. Following electrophoresis at room temperature, the gels were incubated twice, for 30 min each, with 2% periodic acid to cleave the DATD crosslinker and thereby facilitate diffusion of the proteins out of the gel (Renart et al., 1979).

Preparation of DBM-paper. 1-[(m-nitrobenzyloxy) methyl] pyridinium chloride (NBPC) was prepared by bubbling dry HCl into a solution containing 158 g of paraformaldehyde and 200 g of m-nitrobenzyl alcohol in 1 l of benzene at room temperature. After stirring overnight the organic

phase was removed and dried with anhydrous Na_2SO_4 and the benzene removed using a Buchler flash-evaporator. The remaining liquid was distilled and the fraction boiling between 150 and 154 C was collected.

A sheet of Whatman 540 paper (14 x 25 cm) was soaked with 10 ml of solution containing 0.8 g NBPC and 0.25 g sodium acetate. The sheet was dried and heated for 35 min at 130-135 C. The dried sheet was then washed twice with water for 20 min each, dried, and washed twice with benzene for 20 min each. After drying, the sheet was treated with 20% sodium dithionite for 30 min at 60 C, washed for 20 min with water, washed for 20 min with 30% acetic acid, washed again with water and, after drying, stored dessicated at 4 C.

Immediately before use, the paper was treated for 30 min at 4 C with a solution containing 40 ml of water, 80 ml of 1.8 M HCl and 3.2 ml $NaNO_2$ (10 mg/ml). The paper was then wash 5 times, for 5 min each, with cold water followed by 2 washes, for 10 min each, with ice cold 50 mM sodium borate buffer, pH 8 (Alwine et al., 1977).

Southern blotting. DBM-paper was placed onto the periodic acid-treated gel. This was overlayered with several sheets of filter paper. The gel was placed into a shallow layer of 50 mM sodium phosphate buffer, pH 7.5,

and the proteins transferred from the gel to the paper by blotting overnight at room temperature (Southern, 1975).

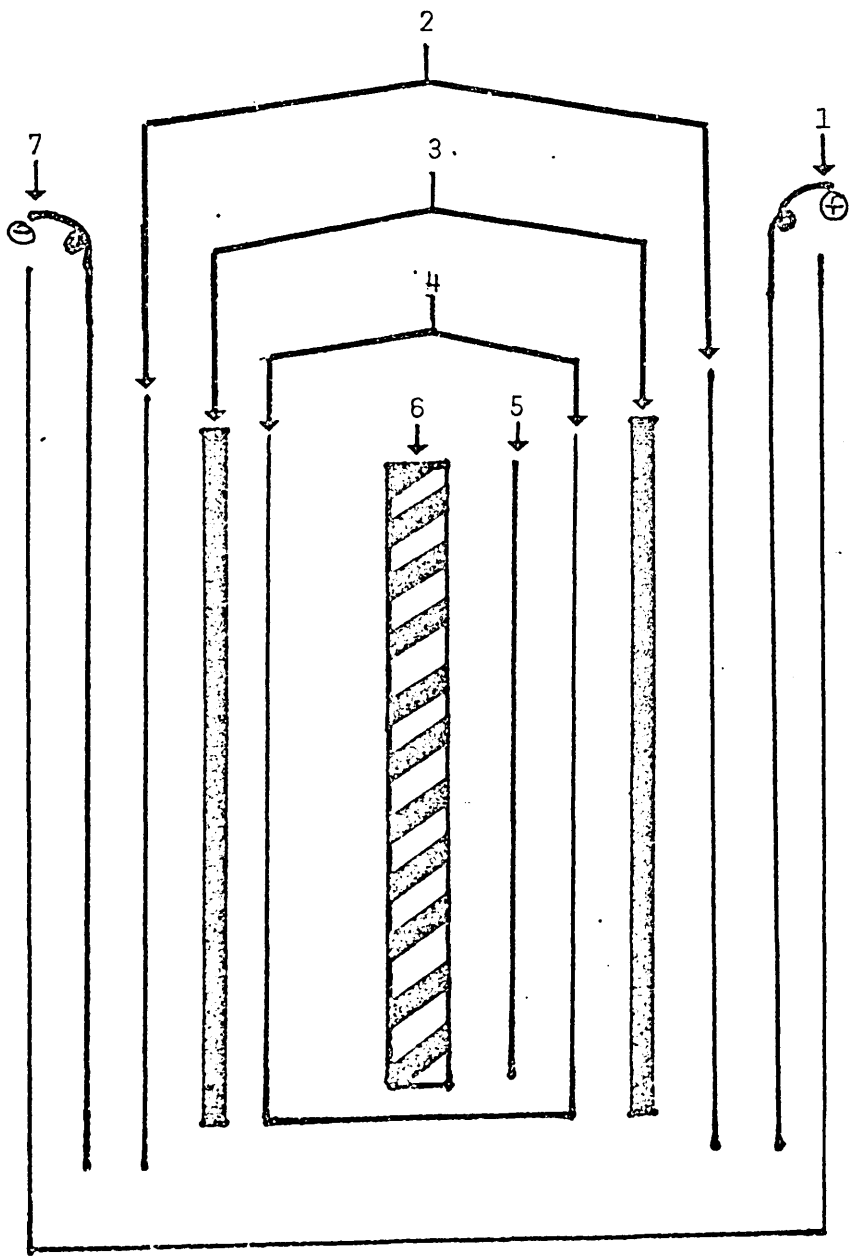
Electrophoretic Transfer of Proteins to Nitrocellulose Sheets

Southern blotting requires an overnight incubation for diffusion of the proteins from gel to paper. Therefore, an electrophoretic transfer method for proteins (Western blotting) was used to increase the rate of transfer of proteins from gel to paper.

Western blotting was carried out by placing the gel and paper into a chamber containing 25 mM sodium phosphate buffer, pH 7.5 (transfer buffer), and applying an electrical current (12 V), as shown in Figure 3 (Finkelstein, personal communication).

To determine whether NC sheets could be substituted for DBM-paper, ovalbumin was labeled with Fluram as follows: Approximately 0.1 to 100 nanomoles/ml of protein were placed into 1.5 ml of 0.2 M sodium borate buffer, pH 9. While vortexing, 0.5 ml of a Fluram solution in acetone was added (15 to 30 mg Fluram/100 ml). Following SDS-PAGE, the gel and NC were placed into transfer buffer and an electrical current (12 V) applied. After transfer, the fluorescent protein could be visualized using long wave length uv light.

Figure 3. Apparatus used for electrophoretic transfer of proteins from polyacrylamide slab gels to DBM-paper or nitrocellulose sheets. (1) anode, (2) plastic support, (3) Scotch-Brite pads, (4) Whatman 3 MM paper, (5) DBM-paper or nitrocellulose sheet, (6) polyacrylamide gel, and (7) cathode.


2
7
3
1
4
6
5

After Western blotting, α-glucosidase could be detected on the NC sheets by incubating the sheets with Fluram-labeled anti-α-glu_E IgG at 37 C, with shaking, for from 4.5 to 12 h. Before incubation with the antibody the sheets were incubated at 4 C for 30 min with BSA to prevent nonspecific binding of Fluram-labeled anti-α-glu_E IgG to the NC.

RESULTS

Immunochemical Assay of Membranes

Using an IgG-horseradish peroxidase (IgG-HRPO) complex as a probe, it has been shown previously that the association of α-glucosidase with the membranes of *A. niger* is not affected by treatment with α-methylmannoside (Fitzgerald, 1979). This compound would be expected to compete with the nonreducing end of the oligosaccharide chain and displace the glycoprotein if it was held to the membrane by its carbohydrate. Some, but not all of the α-glucosidase activity can be released from the membrane by treatment with 0.5 M NaCl indicating that α-glucosidase is not exclusively a peripheral membrane protein (Figure 4).

In order for lysosomal enzymes to reach their correct and final destination, a phosphorylated mannose residue must be present on the oligosaccharide chain (Hasilik and Neufeld, 1980a). To determine whether a phosphorylated mannose residue plays a role in the membrane-association of α-glucosidase, membranes were incubated with 1 mM mannose-6-phosphate followed by chromatography on a Bio-Gel A-5m column. Figure 5 shows that the addition of mannose-6-phosphate to the membranes had no effect on

Figure 4. Fractionation of high salt-treated membranes on Bio-Gel A-5m. Membranes were incubated for 30 min. with 0.5 M NaCl, followed by incubation for 30 min. with IgG-HRPO. Treated (dashed line) and untreated (solid line) membranes were chromatographed on a Bio-Gel A-5m column to determine the effect of treatment on α-glucosidase binding. V_o indicates the void volume and IgG-HRPO indicates the point of elution of the IgG-HRPO complex.

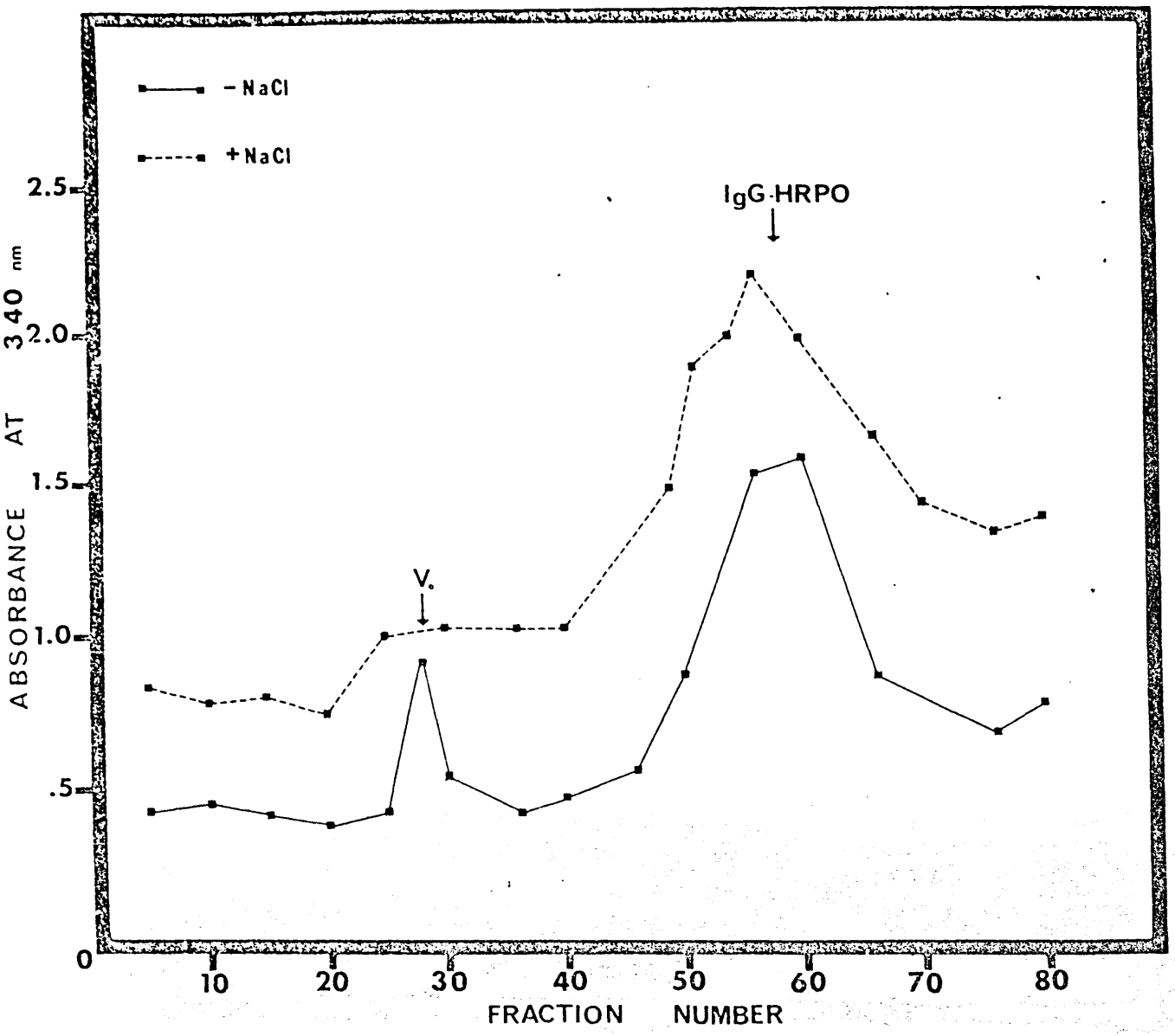
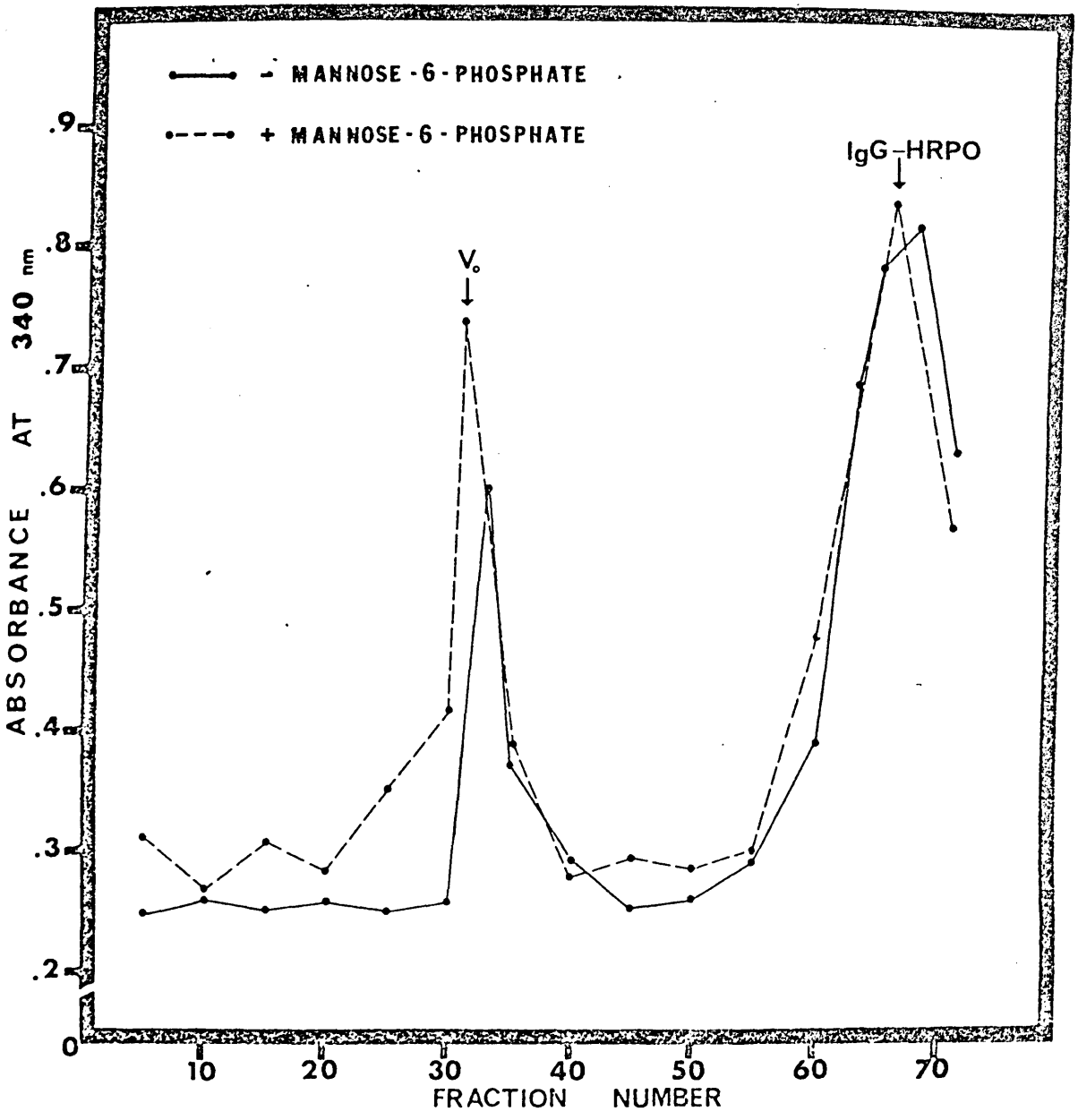

– NaCl
+ NaCl
IgG-HRPO
V.
ABSORBANCE AT 340 nm
2.5
2.0
1.5
1.0
.5
0
10
20
30
40
50
60
70
80
FRACTION NUMBER

Figure 5. Fractionation of mannose-6-phosphate treated membranes on Bio-Gel A-5m. Membranes were incubated for 30 min with 1 mM mannose-6-phosphate, followed by incubation for 30 min with IgG-HRPO. Treated (dashed line) and untreated (solid line) membranes were chromatographed on a Bio-Gel A-5m column to determine the effect of treatment on α-glucosidase binding. V_o indicates the void volume and IgG-HRPO indicates the point of elution of the IgG-HRPO complex.


- MANNOSE-6-PHOSPHATE
+ MANNOSE-6-PHOSPHATE
IgG-HRPO
V_o
ABSORBANCE AT 340 nm
.9
.8
.7
.6
.5
.4
.3
.2
0
10
20
30
40
50
60
70
FRACTION NUMBER

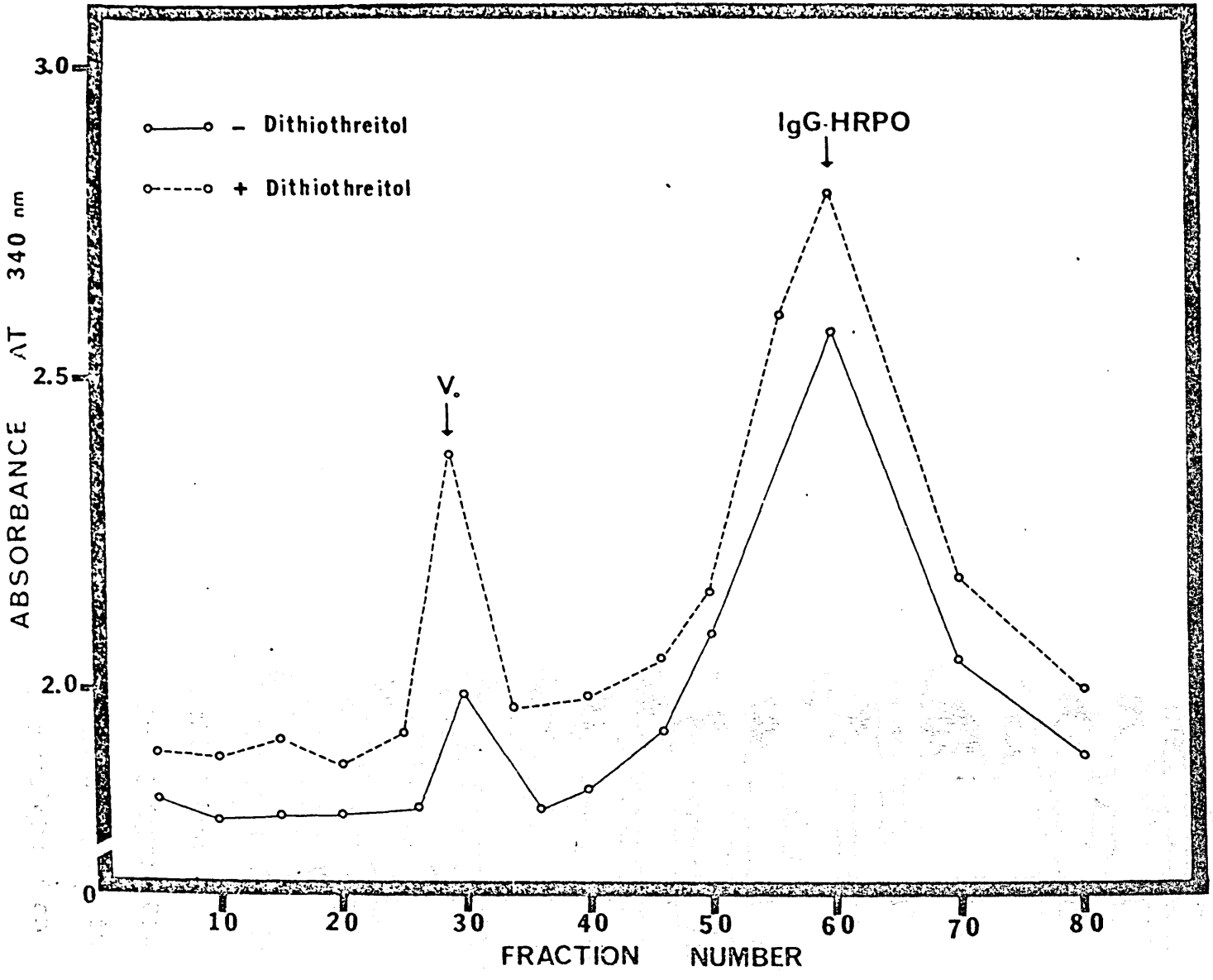
the association of this enzyme. In addition, the elution pattern of membranes treated with 1 unit/ml bacterial alkaline phosphatase was identical to that of control membranes.

Disulfide bonds allow the associaiton of pieces of precursor forms of an enzyme after proteolysis has occurred. The chains of β-hexosaminidase synthesized in a mouse macrophage cell line are produced as larger precursor forms. When the mature and precursor forms are electrophoresed under nonreducing conditions, their mobility is identical. However, when the mature form is electrophoresed under reducing conditions two bands are seen; one corresponding to the mature enzyme and the other corresponding to the proteolytic fragment which results from cleavage of the precursor (Puchalski and Neufeld, 1981). Treatment with dithiothreitol did not cause the release of any membrane-bound α-glucosidase (Figure 6) indicating that no sulfhydryl-linkages are involved in the membrane association.

Immunological activity of Detergent-treated α-glucosidase

Since the disruption of membranes for examination of $\alpha\text{-glu}_M$ required the use of detergent, it was of interest to see what effect treatment with SDS would have on the immunological activity of the α-glucosidase associated with

Figure 6. Fractionation of dithiothreitol-treated membranes on Bio-Gel A-5m. Membranes were incubated for 30 min with 0.2% dithiothreitol followed by centrifugation at 150,315 x g_{av} for 1 h. The membranes, resuspended in PBS, were incubated for 30 min with IgG-HRPO. Treated (dashed line) and untreated (solid line) membranes were chromatographed on a Bio-Gel A-5m column to determine the effect on α-glucosidase binding. V_o indicates the void volume and IgG-HRPO indicates the point of elution of the IgG-HRPO complex.


3.0
2.5
2.0
0
ABSORBANCE AT 340 nm
– Dithiothreitol
+ Dithiothreitol
IgG-HRPO
V₀
10
20
30
40
50
60
70
80
FRACTION NUMBER

these membranes. Precipitin lines in Ouchterlony double diffusion plates prepared with 1% agar containing SDS show that α-glu$_M$, dissolved in buffer containing SDS, has the same antigenic activity as that of α-glu$_E$ which had also been dissolved in buffer containing SDS (Figure 7).

Analysis of α-glu$_M$ by Gel Filtration Chromatography

Membranes were isolated as described in Materials and Methods and solubilized in homogenization buffer containing 0.5% Triton X-100. Treated membranes were then chromatographed on a Sephacryl S-200 column equilibrated with the same buffer. Fractions were assayed for α-glucosidase activity and it was found that α-glu$_M$ eluted at the void volume (Figure 8). When fractions containing α-glucosidase activity were analyzed for their immunological activity using Ouchterlony double diffusion plates, it was found that these fractions at the void volume formed precipitin lines with rabbit anti-α-glucosidase IgG (Figure 9). On the gel filtration column, α-glu$_E$ which had been dissolved in buffer containing 0.5% Triton X-100 eluted 25 fractions (25 ml) later than α-glu$_M$ (Figure 8). These results indicate that α-glu$_M$ differs from α-glu$_E$ in that α-glu$_M$ seems to have a higher molecular weight than α-glu$_E$. However, it has been shown that polyethoxy-type

Figure 7. Antigenicity of α-glu$_M$ and α-glu$_E$. Rabbit anti-α-glucosidase IgG was placed into the center well of an Ouchterlony double diffusion plate prepared with SDS. α-glu$_E$ (A) and membrane (B) were solubilized in buffer containing SDS and placed into the side wells. The plate was incubated at room temperature overnight.

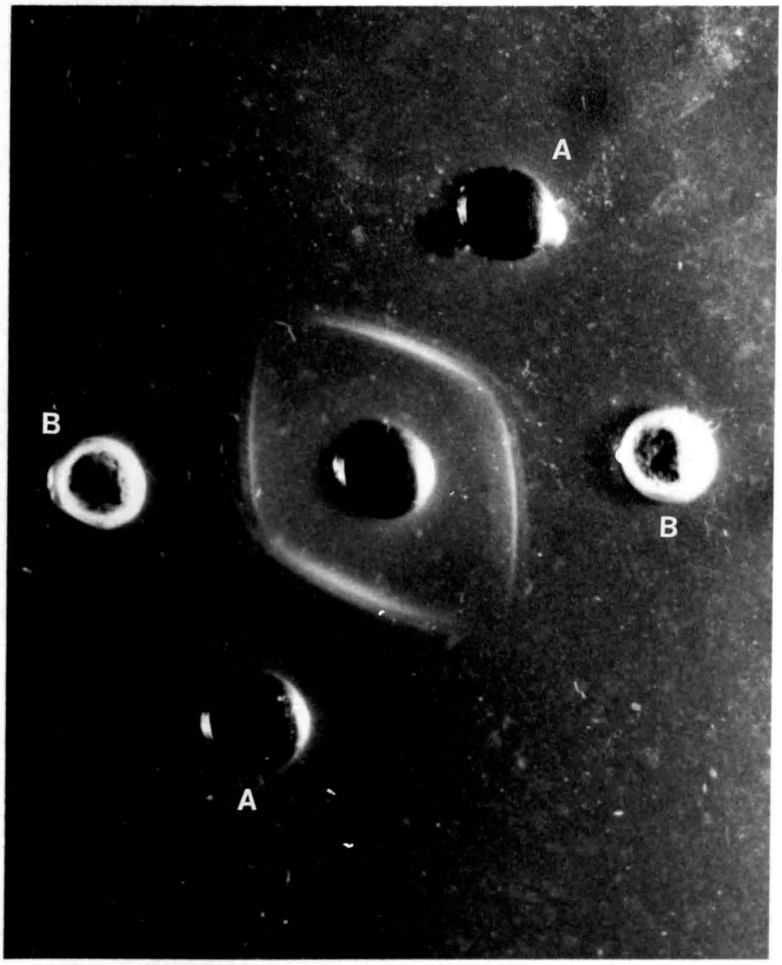

A
B
B
A

Figure 8. Fractionation of α-glu_M and α-glu_E on Sephacryl S-200. Isolated membranes were solubilized in homogenization buffer containing 0.5% Triton X-100 and chromatographed on a Sephacryl S-200 column equilibrated with the same buffer. Fractions were assayed for α-glucosidase activity (solid line). α-glu_E was dissolved in homogenization buffer containing Triton X-100 and applied to the Sephacryl S-200 column. Fractions were assayed for protein by reading the absorbance at 280 nm (dashed line). v_o indicates the void volume and α-glu_E indicates the point of elution of the secretory α-glucosidase.

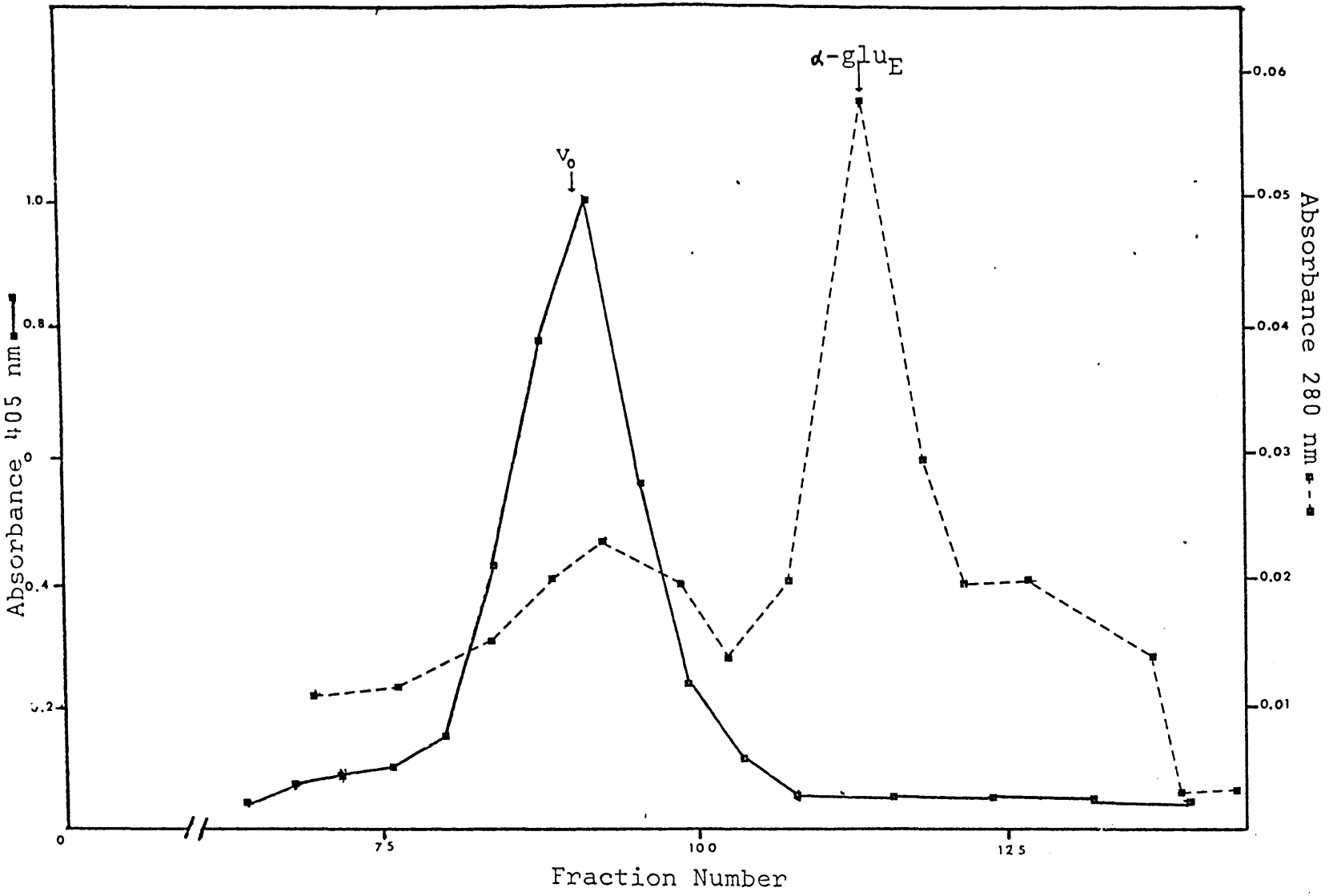
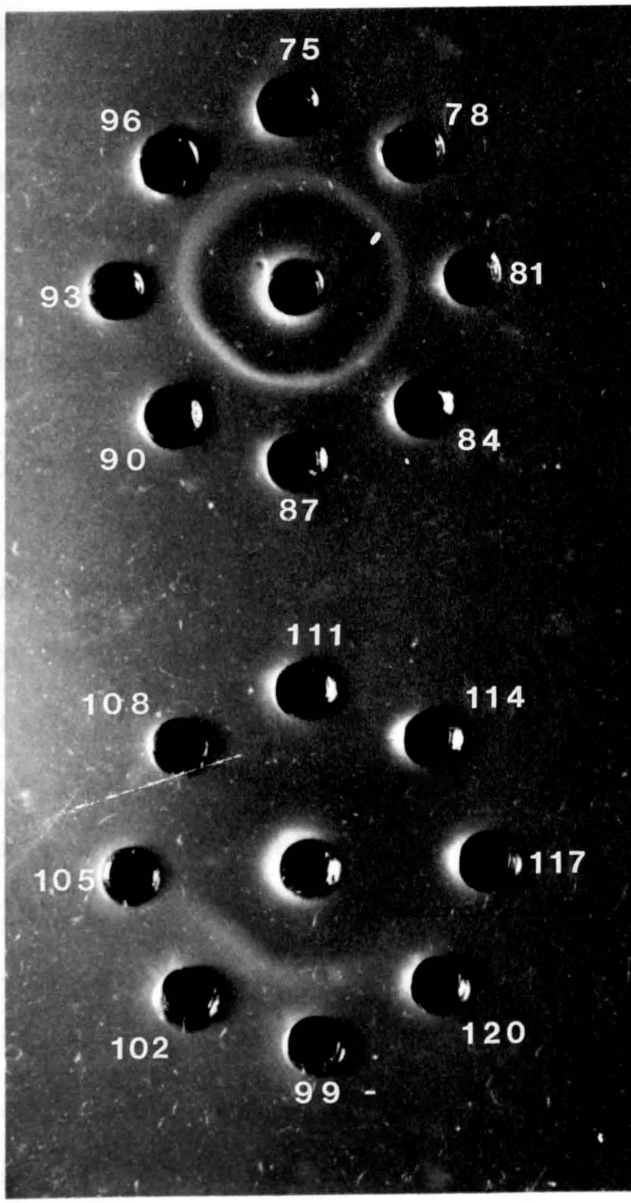

α-glu$_E$
V_o
Absorbance 405 nm
Absorbance 280 nm
Fraction Number
0
75
100
125
1.0
0.8
0.4
0.2
0.06
0.05
0.04
0.03
0.02
0.01

Figure 9. Antigenic properties of membrane fractions isolated by Sephacryl S-200 chromatography. Membrane solubilized in homogenization buffer containing 0.5% Triton X-100 was fractionated on a Sephacryl S-200 column equilibrated with the same buffer. A sample of every third fraction was placed into the outer wells of Ouchterlony double diffusion plates and rabbit anti-α-glu_E IgG placed into the center wells. The plates were incubated overnight at room temperature.


75
96
78
93
81
90
84
87
111
108
114
105
117
102
120
99

detergents, such as Triton X-100, are somtimes not efficient in breaking protein-protein interactions (Hjelmeland, 1980) and that these detergents have a tendency to form aggregates (Tanford and Reynolds, 1976). Therefore, the earlier elution of $\alpha\text{-glu}_M$ could result from either of these two possibilities as well. Membranes treated with 2 mM PMSF during isolation showed the same elution pattern as that of untreated membranes.

Electrophoretic Transfer of Proteins to Nitrocellulose Sheets

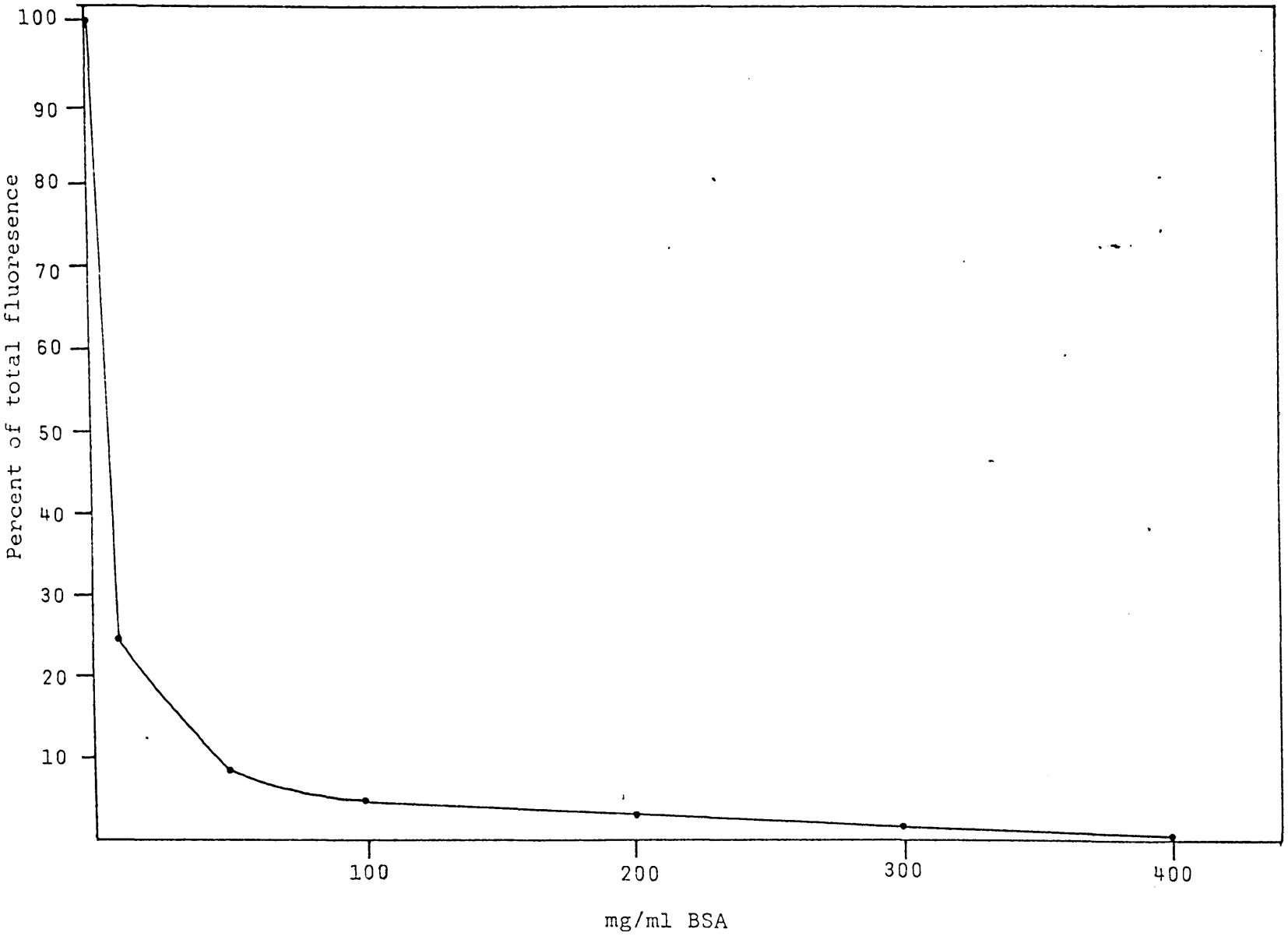
Initially, transfer of proteins was attempted from tube gels to DBM-paper using Southern blotting, a method originally developed to transfer DNA from gels to DBM-paper (Southern, 1975). Tritium-labeled proteins were electrophoresed on polyacrylamide/agarose gels prepared with DATD, a crosslinker susceptible to periodic acid cleavage (Renart et al., 1979). After gels were incubated overnight at room temperature or at 37 C the DBM paper was sliced, placed into a toluene-based scintillation cocktail and radioactivity determined. No radiolabeled proteins were detected on the paper. This may have been due to the fact that the tube gel was too thick for transfer or that the unstable binding sites of the DBM-paper did not remain active overnight.

Because of the problems with Southern blotting, an electrophoretic transfer method (Western blotting) was used to increase the rate of transfer of the proteins from the gel to paper. Ovalbumin was labeled with Fluram as described in Materials and Methods and electrophoretic transfer carried out. With time, the fluorescent protein was seen to move out of the gel. Since the Fluram-labeled protein could not be detected on the orange DBM-paper nitrocellulose (NC) sheets were substituted.

Using NC sheets to detect the location of a specific protein by incubating the sheet, after transfer, with labeled antibody to that protein, necessitated blocking the NC to prevent nonspecific binding of the labeled probe to the sheet. The NC was incubated with from 0 to 400 mg/ml BSA for 30 min at 4 C. After incubation, the sheets were washed with transfer buffer and incubated with 75 µg/ml Fluram-labeled ovalbumin for 6 h. This was followed by a wash with transfer buffer and the presence of Fluram-labeled protein bound to the NC was then detected using a Farrand Vis-UV chromatogram analyzer. More than 90% of nonspecific binding was prevented using 200 mg/ml BSA for blocking (Figure 10).

To determine the amount of anti-α-glu_E IgG necessary to detect α-glu_E on NC, a crude extract of secretory

Figure 10. Optimization of bovine serum albumin blocking of nitrocellulose sheets.


100
90
80
70
60
50
40
30
20
10
Percent of total fluoresence
100
200
300
400
mg/ml BSA

proteins from A. niger was spotted onto NC sheets which were incubated with 200 mg/ml BSA for 30 min at 4 C. They were then washed with transfer buffer and incubated with varying amounts of Fluram-labeled anti-α-glu_E IgG in transfer buffer at 37 C with gentle shaking for at least 5 h. After washing, the presence of bound anti-α-glu_E was detected using long wavelength uv light. It was found that 75 µg/ml of antibody provided a means to detect the presence of α-glucosidase on the filters. When ovalbumin or BSA alone were placed onto the filters, no labeled anti-α-glu_E IgG was found on the filter after incubation.

The following experiment was performed to determine the length of time necessary to transfer proteins from a polyacrylamide gel to NC. A 100-ml A. niger culture was labeled with 5 µCi $[^{14}C]$-lysine and 50 µCi $[^{3}H]$-leucine for 12 h prior to harvesting the mycelia. The procedure for total membrane isolation was followed except that 2 mM PMSF was used throughout the entire procedure. The high speed supernatant was dialyzed overnight against water and lyophilized. Sizty µg protein (10,000 cpm) were applied to lanes 2, 4, 6, and 8; and twice that amount applied to lanes 3, 5, 7, and 9 of a 7.5% SDS-polyacrylamide slab gel. After electrophoresis at 30 mA until the tracking dye reached the bottom of the gel, the gel was divided into 4 longitudinal

slices (each containing two lanes) and placed with NC sheets into the transfer chamber. The time of transfer for a given slice was 0, 30, 60 or 90 minutes. Following transfer for a specific time, a gel slice and NC sheet were removed from the chamber and prepared for fluorography as described in Materials and Methods. After treatment, the presence of radioactive bands was detected by placing Kodak X-Omat R film over the gels or NC and allowing exposure for 10 days at -80 C. The film was developed using Kodak X-ray developer. All the radioactivity appeared to be transferred from the gel to the paper after 90 min (Figure 11).

Detection of α-glucosidase in the Membranes of A. niger

Since the membranes isolated in the absence of protease inhibitors appeared to be highly susceptible to proteolysis (Figure 12), one or more protease inhibitors were used during the isolation of membranes.

Membranes isolated in the presence of 2 mM PMSF and 6 mM p-aminobenzamidine were treated with dissociating solution and proteins separated by SDS-PAGE. Proteins were transferred electrophoretically to NC and the position of $\alpha\text{-glu}_M$ determined by incubation with Fluram-labeled anti-$\alpha\text{-glu}_E$ IgG as described above. Figure 13 shows the presence of a higher molecular weight α-glucosidase in Lane 2. The band indicated by the arrow in Lane 1 has the

Figure 11. Kinetics of transfer of radiolabeled proteins from polyacrylamide gels to nitrocellulose. Labeled proteins were electrophoresed on 7.5% SDS-polyacrylamide gels and transferred for 30, 60, and 90 min to nitrocellulose sheets. The gels and nitrocellulose were then fluorographed.

7.5% SDS-Polyacrylamide Gel

Time of transfer (min.)

Nitrocellulose Sheet

Time of transfer (min.)

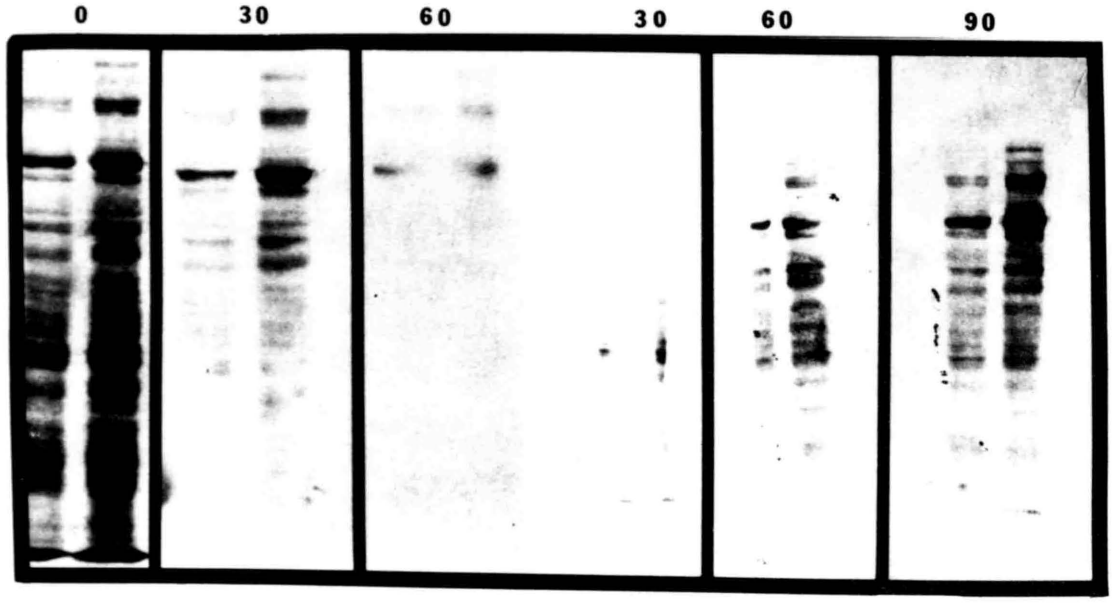


Figure 12. Western blot analysis of membranes isolated in the absence of protease inhibitors. Membranes were isolated in the absence of protease inhibitors, transferred to NC, and incubated with rabbit anti-α-glu_E IgG. Lane 1 contained uncrosslinked membrane (40 µg protein). Lanes 2 and 3 were membrane samples (40 µg and 60 µg protein respectively) which had been crosslinked with 1.0 mg/ml DMS for 15 min at 20 C.

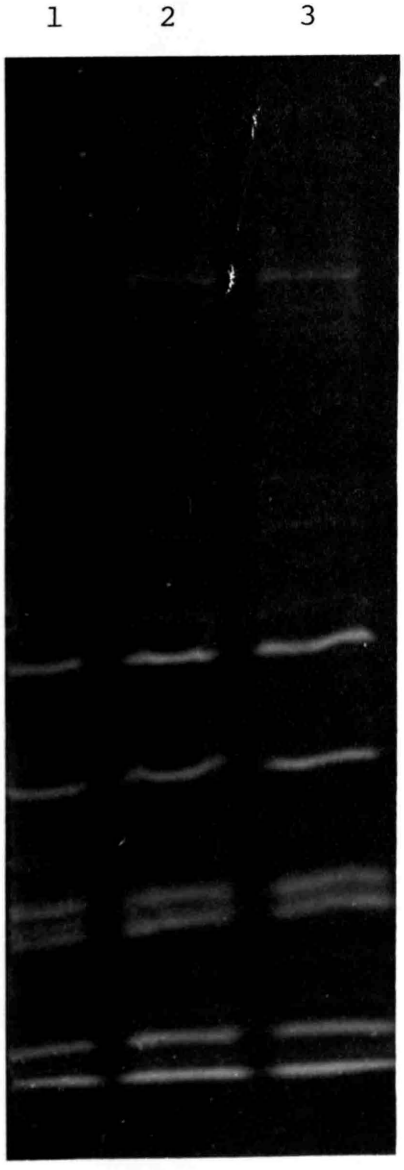
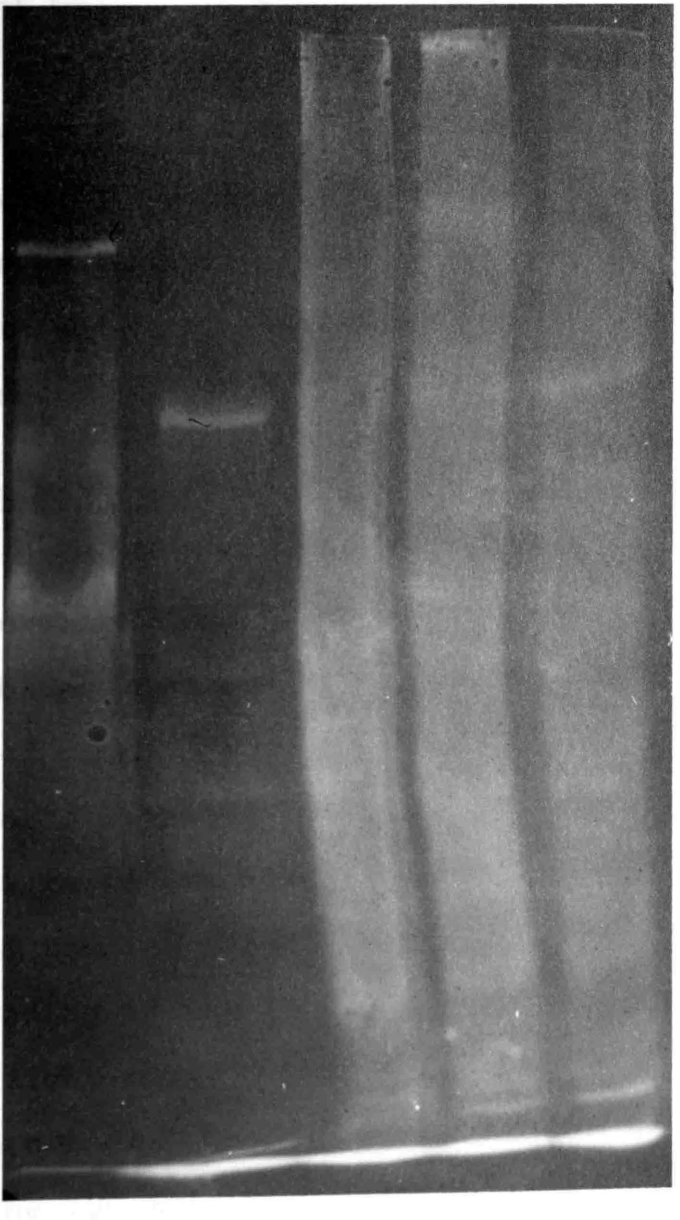

1
2
3

Figure 13. Western blot analysis of membranes isolated in the presence of protease inhibitors. α-glu_E (Lane 1) and membrane (Lane 2) were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose sheets. The position of α-glucosidase was detected by incubation with 75 µg/ml Fluram-labeled rabbit anti-α-glu_E IgG. The arrow in Lane 1 indicates the position of α-glucosidase. Membranes crosslinked with 0.4, 0.8, and 1.0 mg/ml DMS are shown in Lanes 3, 4, and 5. The arrow in Lane 5 indicates the position of an α-glucosidase containing component.


1
2
3
4
5

mobility of a 65,000 dalton protein which corresponds well to the molecular weight of α-glu_E (Rudick and Elbein, 1974). The band in Lane 2 has a molecular weight of 130,000 daltons. The molecular weights of these bands were determined by comparing their R_f's to those of crosslinked BSA (Figure 14). These results seem to indicate that the membrane-bound form of the enzyme is slightly over twice as large as the mature, secretory enzyme.

Crosslinking of Membranes with Dimethyl Suberimidate

DMS was used to crosslink membrane proteins in an attempt to determine whether the α-glu_M was associated with a specific membrane protein. To determine the appropriate concentration of crosslinker, IgG (40 µg) was crosslinked as described in Materials and Methods. It was found that from 0.8 to 1.2 mg/ml DMS would result in crosslinking of the subunits of the IgG molecule (Figure 15). Lane 2 shows bands corresponding to the 53,000 dalton heavy chain and 23,000 dalton light chain (Rose et al., 1979). Lanes 1 and 3 show the combination of products which result from crosslinking the IgG molecules with two different concentrations of DMS.

Figure 16 shows the results of crosslinking membrane with from 0.2 to 1.0 mg/ml DMS. As the concentration of

Figure 14. Molecular weight determination of α-glucosidase species. BSA and α-glucosidase were crosslinked as described in Materials and Methods. The R_f of each species was determined as follows:

$$R_f = \frac{\text{distance from origin of protein band}}{\text{distance from origin of tracking dye}}$$

Crosslinked BSA (●), α-glu_M (■), crosslinked α-glucosidase containing component (o).

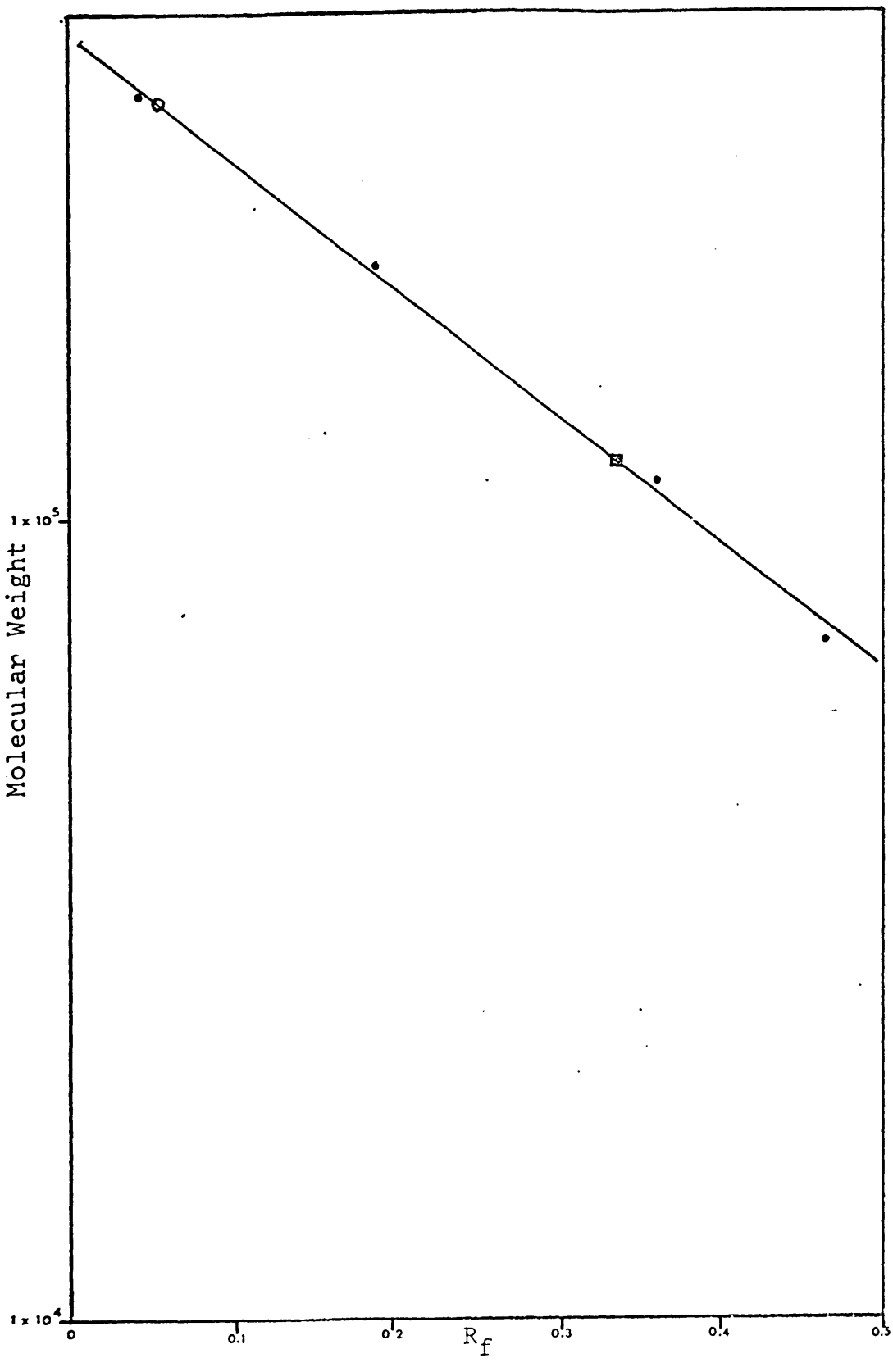

Molecular Weight
1 x 10^5
1 x 10^4
0
0.1
0.2
0.3
0.4
0.5
Rf

Figure 15. Electrophoretic analysis of dimethyl suberimidate crosslinked IgG. Forty µg each of uncrosslinked IgG (Lane 2) and IgG crosslinked with 1.0 and 2.0 mg/ml DMS (Lanes 3 and 1 respectively) were electrophoresed on a 7.5% SDS-polyacrylamide gel. After fixing overnight, the gel was stained with CBB R-250 in 50% TCA as described in Materials and Methods.

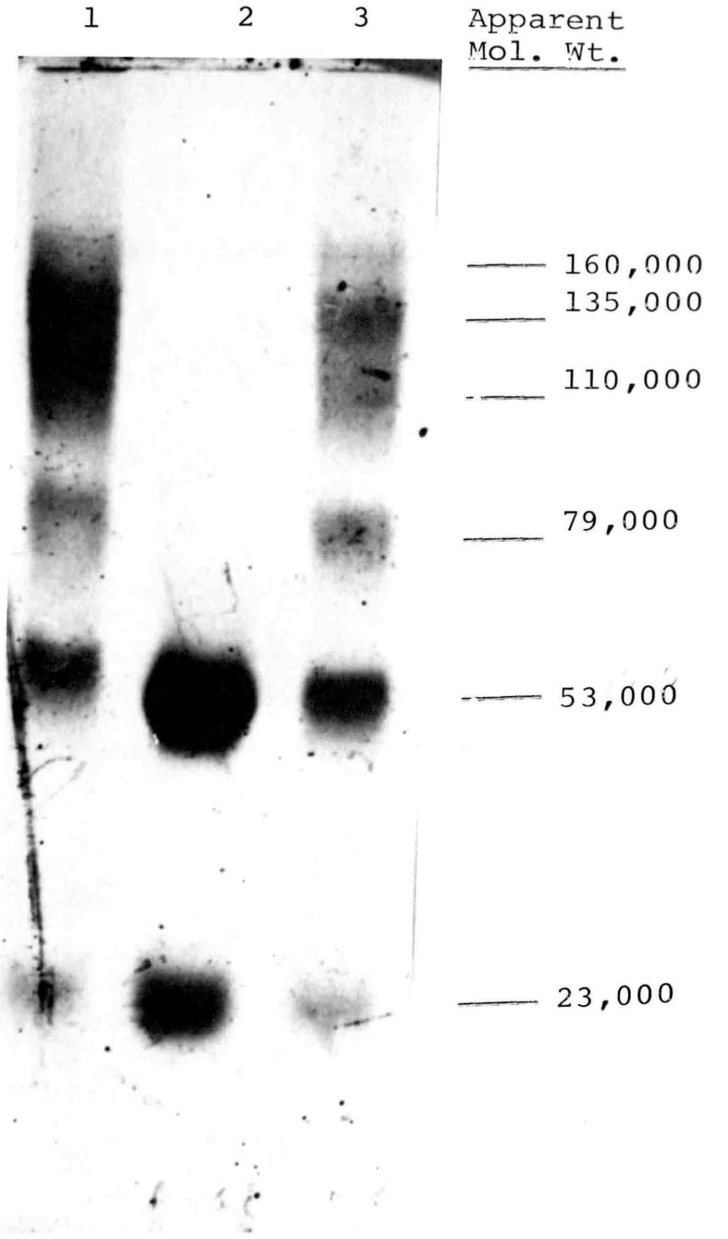
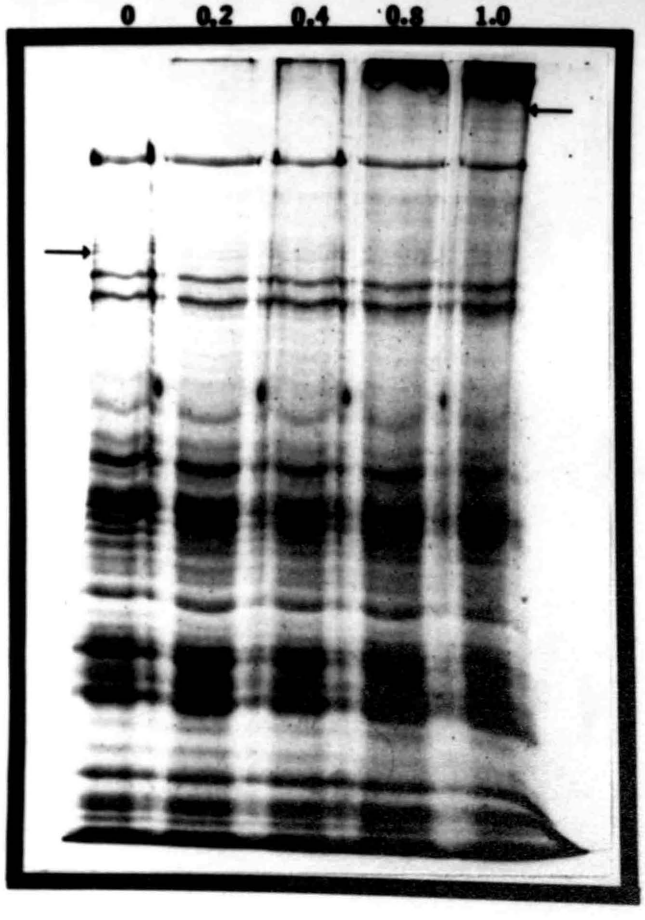

1
2
3
Apparent
Mol. Wt.
160,000
135,000
110,000
79,000
53,000
23,000

Figure 16. Electrophoretic analysis of dimethyl suberimidate crosslinked membranes of A. niger. Membranes were crosslinked with from 0 to 1.0 mg/ml DMS for 15 min at 20 C followed by electrophoresis on 7.5% SDS-polyacrylamide slab gels, transfer to nitrocellulose sheets, and incubation with Fluram-labeled anti-α-glu_E IgG. The arrow in Lane 5 of the polyacrylamide gel indicates the position of the α-glucosidase containing component. The arrow in Lane 1 shows the position of α-glu_M.

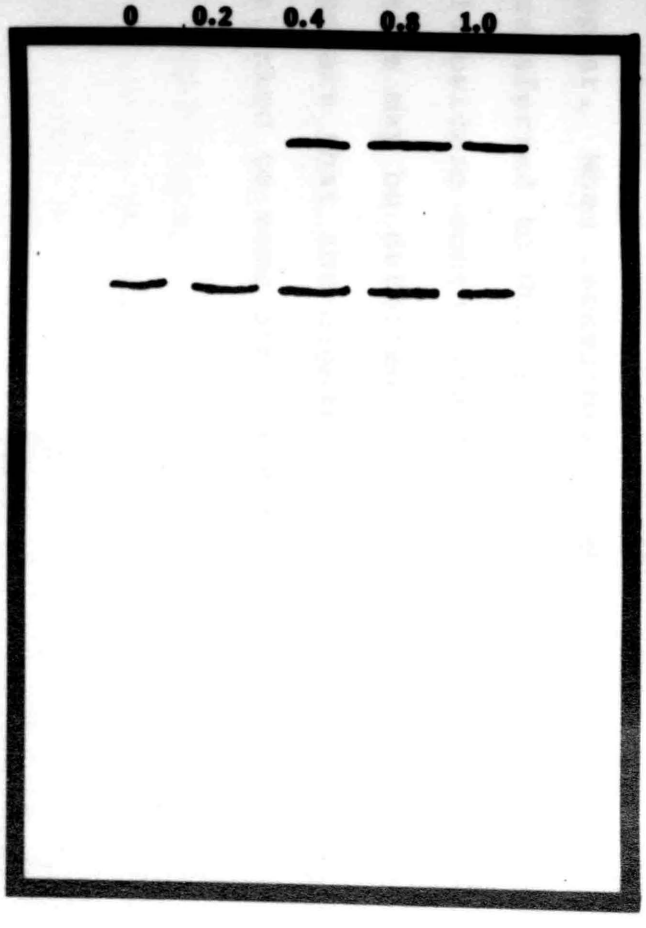
7.5% SDS-Polyacrylamide Gel

CONCENTRATION OF DMS (MG/ML)



Nitrocellulose Sheet

CONCENTRATION OF DMS (MG/ML)



crosslinker is increased, higher molecular weight bands, which presumably result from the crosslinking of membrane proteins, appear. When crosslinked membrane is electrophoresed, transferred to NC, and incubated with anti-α-glu_E IgG, an α-glucosidase containing component of approximately 330,000 daltons may be detected (Figures 13 and 16). It therefore appears that the higher molecular weight α-glu_M may be crosslinked to some other membrane protein or proteins.

DISCUSSION

Aspergillus niger produces and secretes an α-glucosidase as well as several other secretory glycosidases (Rudick and Elbein, 1974). Prior to secretion, the α-glucosidase is synthesized and glycosylated on membrane-bound polysomes (Long and Rudick, 1979) and then appears to remain associated with membranes for some time before being released (Fitzgerald, 1979). The primary purpose of this study was to examine the nature of the α-glucosidase-membrane interaction, since this should lead to further understanding of the synthesis and transport of the secretory glycoprotein.

Immunochemical Assay of Membranes

Not all of the α-glucosidase can be displaced from the membrane by treatment with high salt (Fitzgerald, 1979). This indicates that α-glucosidase is not exclusively a peripheral membrane protein, since one of the characteristics of a peripheral protein is its ease of removal from the membrane by relatively mild treatments which would disrupt the ionic interactions between the two (Singer and Nicolson, 1972).

Furthermore, an analogue of the nonreducing end of the oligosaccharide chain, α-methylmannoside, also did not cause the removal of α-glucosidase from the membrane (Fitzgerald, 1979). This indicates that the oligosaccharide is not involved in the protein-membrane interaction.

Lysosomal enzymes are directed to their proper intracellular location by the presence of a phosphorylated mannose residue on their oligosaccharide chains (Hasilik and Neufeld, 1980a). Therefore, it was considered possible that a similarly modified mannose residue might be important in holding the α-glucosidase to the membrane. To test this possibility, membranes were chromatographed on an Agarose A-5m column following treatment with mannose-6-phosphate (Figure 5) or bacterial alkaline phosphatase and incubation with rabbit anti-α-glucosidase IgG coupled to horseradish peroxidase. Since mannose-6-phosphate did not displace $\alpha\text{-glu}_M$, a phosphorylated mannose residue can be ruled out as the attachment point of α-glucosidase to the membrane.

The same chromatographic separation was carried out following treatment of the membranes with dithiothreitol. Again, the elution profile of the treated membrane was the same as that of the control membrane (Figure 6) indicating

that no sulphydryl linkages are involved in the interaction.

Thus, since the association of α-glu_M with the membrane is not disturbed by treatment with α-methylmannoside, bacterial alkaline phosphatase, or mannose-6-phosphate, the oligosaccharide chain, or a phosphorylated mannose residue of the oligosaccharide chain, can be eliminated as the important component in the α-glu_M-membrane interaction. Similarly, dithiothreitol treatment rules out sulphydryl bridges as requirements for the association.

Western Blotting

To examine possible molecular weight differences between α-glu_M and α-glu_E, it was necessary to develop a technique whereby a specific protein could be identified on a polyacrylamide gel, even in the presence of a large number of other proteins. Initially, radiolabeled proteins were electrophoresed and transferred to DBM-paper according to the method described by Renart et al (1979). They reported an efficiency of transfer varying from 11 to 16% and attributed this low efficiency to the fact that a substantial portion of the protein remains in the gel. Even after overnight incubation at 37 C, no radiolabeled protein was detected on the DBM-paper. This is probably not only due to the fact that a large amount of protein is unable to diffuse from the gel, but also that the unstable diazonium

groups of the paper do not remain active overnight. Diazonium salts slowly decompose, even at ice-bath temperature, and therefore should be used immediately after preparation (Morrison and Boyd, 1975).

To increase the rate of transfer, proteins were transferred electrophoretically to DBM-paper from polyacrylamide gels. With time, Fluram-labeled ovalbumin could be seen to move out of the gel but, due to the orange color of the paper, could not be detected on the DBM-paper. Therefore, nitrocellulose sheets were substituted for DBM-paper. NC's major advantages over DBM-paper are its ease of storage and lack of pre-transfer preparation. Aminobenzyloxymethyl (ABM)-paper must be maintained dessicated at -4 C. This paper can be stored for only a few weeks and must be converted to DBM-paper immediately prior to use. The conversion procedure is time consuming and must be completed no more than 15 min before transfer (Alwine et al., 1977). NC also allows Fluram-labeled antibody to be used for the detection of a specific antigen.

It was important to determine the length of time necessary to transfer proteins from a polyacrylamide gel to NC. To do this, radiolabeled protein was transferred for varying lengths of time to NC sheets followed by fluorography of both the gel and the sheet. Figure 11 shows that

all the radioactivity appears to be transferred from the gel to the NC after 90 min. However, some of the lower molecular weight protein may be lost from the sheets with increasing times of transfer (after 60 min). Towbin and coworkers (1979) developed an electrophoretic transfer technique using NC and found that RNA and small molecules were not adsorbed.

After total membrane proteins are transferred to NC, $\alpha\text{-glu}_M$ can be detected by incubating the sheet with Fluram-labeled anti-$\alpha\text{-glu}_E$ IgG. Before incubation with antibody, it is necessary to block the NC with BSA to prevent nonspecific binding of the labeled probe to the sheet. Blockage can be accomplished by incubating the sheet, to which proteins have been transferred, with 200 mg/ml BSA at 4 C for 30 min (Figure 10). It has been reported that incubation with 3 mg/ml for 1 h at 40 C will prevent nonspecific binding (Towbin et al., 1979).

Western blotting allows total membrane proteins to be electrophoresed, transferred to NC and one specific protein identified by incubating the sheet with labeled antibody to that protein. Since the antigen is fixed to the sheet, the antibody is not required to form a precipitate with the antigen, eliminating the necessity of determining the proper antigen to antibody ratio necessary for immunoprecipitation.

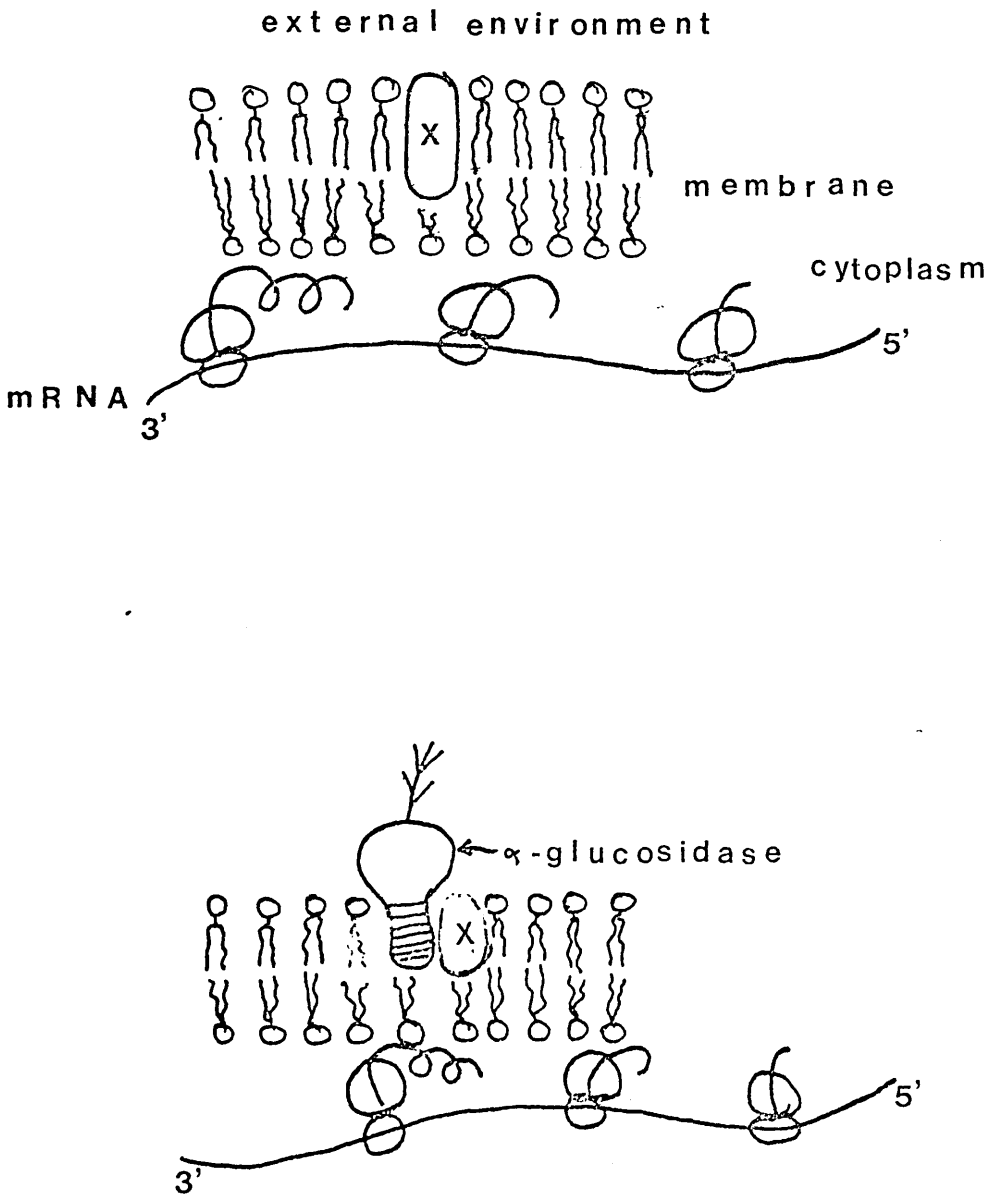
Association of α-glucosidase with Membranes

Gel filtration chromatography of Triton-disrupted membranes isolated from A. niger suggests that α-glu_M has a higher molecular weight than α-glu_E. α-glu_M eluted at the void volume whereas α-glu_E was in the included volume of the Sephacryl S-200 (Figure 8). However, it is also possible that the apparent higher molecular weight is due to the incomplete break down of protein-protein interactions in the membrane by Triton X-100 (Hjelmeland, 1980) or to the formation of aggregates by the detergent with the enzyme (Tanford and Reynolds, 1976). Either of these two conditions would result in α-glu_M eluting from the column at the void volume and lead to an erroneous conclusion concerning the molecular weight of α-glu_M.

SDS-PAGE followed by Western blotting was used to determine whether or not α-glu_M did in fact have a higher molecular weight than α-glu_E. Figure 13 shows that α-glu_M appears to have a molecular weight which is slightly more than twice that of α-glu_E

Thus, the additional amino acid residues are probably responsible for the α-glu_M-membrane association (Figure 17). This would be similar to the situation with the membrane-bound IgM of lymphocytes (Vassalli et al., 1980). The heavy chain of the membrane-bound IgM differs

Figure 17. Model of the association of α-glucosidase with the membranes of A. niger.


external environment
X
membrane
cytoplasm
5'
mRNA
3'
α-glucosidase
X
5'
3'

from the secretory form in the following: the membrane form has a higher molecular weight; the membrane form has a different isoelectric point; and the membrane form has a larger carboxy-terminal cyanogen bromide fragment. Electron microscopy of A. niger membranes labeled with IgG-HRPO show that label is distributed on the plasma membrane symmetrically (Fitzgerald, 1979). Whenever label was seen on the cytoplasmic side, it was also seen on the extracellular side, demonstrating antigenic sites on both sides of the membrane.

The available data are consistent with a model (Figure 17) in which the insertion of $\alpha\text{-glu}_M$ into the membrane and its association with that membrane are interpreted. It is proposed that α-glucosidase is not inserted into the membrane as soon as synthesis of the polypeptide begins. This would explain the presence of $\alpha\text{-glu}_E$ antigenic sites on the cytoplasmic side of the membrane. As synthesis continues a sequence of amino acids is formed which allows $\alpha\text{-glu}_M$ to enter and cross the membrane. This series of amino acids also allows the protein to remain membrane bound until a protease processes the enzyme into the mature secretory form. This internal sequence could be similar to the case of ovalbumin which

has been found to have an internal signal sequence (Lingappa et al., 1979).

Available evidence precludes making a definitive statement about whether or not $\alpha\text{-glu}_M$ is a precursor of $\alpha\text{-glu}_E$. It may be that there are actually two independent forms of the enzyme, one which remains membrane-bound and the other which is secreted, as has been shown to be the case with IgM (Vassalli et al., 1980). This would yield the results obtained after treatment of the membranes with high salt. The $\alpha\text{-glu}_E$ is associated loosely with the membrane and is easily removed whereas the $\alpha\text{-glu}_M$ requires harsh treatment for removal.

If $\alpha\text{-glu}_M$ is a precursor to the secretory form, a protease would be required to remove a large portion (more than half) of the membrane-bound molecule. This rather large loss is not unprecedented. For example, the 29,000 dalton β-chain of human fibroblast hexosaminidase A is derived from a 63,000 dalton precursor (Hasilik and Neufeld, 1980a). An example of another transmembrane protein which is a precursor to the secretory form is secretory component, a glycoprotein synthesized by glandular epithelial cells in the rabbit. This protein serves a receptor function in its transmembrane form and

then, after proteolysis, is found as a secretory protein (Mostov et al., 1980).

When IgG was crosslinked and electrophoresed, a variety of crosslinked species appeared. Protein staining of the gel (Figure 15) revealed bands ranging from the light subunit alone (23,000 daltons) to a complex consisting of 2 heavy and 2 light chains (160,000 daltons). The only two crosslinked species not appearing were those corresponding to the 46,000 dalton light-light complex and the 99,000 dalton heavy-light-light complex. This result is to be expected, since the 2 light chains are not in proximity in the immunoglobulin molecule.

The α-glu$_M$ can be crosslinked in the membrane to form a higher molecular weight α-glucosidase containing component. One possibility is that α-glu$_M$ is being crosslinked to other α-glu$_M$ molecules. This is unlikely, since a protein with a molecular weight corresponding to that of the dimer is not seen. In addition, the large, α-glucosidase-containing component has an apparent molecular weight of 330,000 daltons while an α-glu$_M$ trimer has a molecular weight of 390,000 daltons. Whether the protein to which α-glu$_M$ is crosslinked is necessary for anchoring the α-glu$_M$ to the membrane is unknown. However, this appears unlikely in light of the fact that α-glu$_M$ could not

be dissociated from the membrane by any of the treatments described above, one of which would be apt to disrupt any noncovalent interaction between the two proteins. The protein which is crosslinked to α-glu_M could possibly be a part of the translocation apparatus or perhaps a protease involved in the processing of the membrane-bound to the secretory α-glucosidase.

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