STRUCTURE AND FUNCTION RELATIONSHIPS IN A COMPLEX SYNTHESIZING GLYCOGEN *DE NOVO* FROM *ASCARIS SUUM*

THESIS

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

A. Chris Heath, B.A.
Denton, Texas
December, 1989

R.G.D.

Heath, A. Chris, Structure and Function Relationships in a Complex Synthesizing Glycogen de novo from Ascaris suum. Master of Science (Biochemistry), December, 1989, 86 pp., 2 tables, 15 illustrations, bibliography, 74 titles.

A complex which synthesizes glycogen *de novo* has been purified from *Ascaris suum*. This complex (GS-2) consists of a 66 KDa protein, a 140 KDa protein, and a >330 KDa glycoprotein. The 66 KDa subunit can be dissociated from GS-2 and has a glucose 6-phosphate-dependent glycogen synthase activity, but no priming activity. The glycoprotein subunit is the primer for glycogen synthesis and is composed primarily of glycogen-like polysaccharide, but a portion of the polysaccharide is amylase-resistant and contains complex carbohydrate. The carbohydrate to protein linkage is likely a serine /threonine bond to N-acetylglucosamine. Collectively, the data establish that GS-2 is a multiprotein complex which is a unique glycogen synthase isozyme.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS--Continued

Figure Page

LIST OF ABBREVIATIONS

AMPS: ammonium persulfate

BSA: bovine serum albumin

Con A: Concanavalin A

cyclic AMP: adenosine 3',5'-monophosphate

DEAE: diethylaminoethyl

EDTA: ethylenediaminetetraacetic acid

FABMS: fast atom bombardment mass spectroscopy

G6-P: glucose 6-phosphate

GS: glycogen synthase

HPLC: high performance liquid chromatography

M_r: relative molecular weight

PAD: pulsed amperometric detection

PAS: periodic acid/Schiff base

PAGE: polyacrylamide gel electrophoresis

SDS: sodium dodecyl sulfate

TBS: Tris buffered saline, 20 mM Tris-Cl, pH 7.6, containing 0.5 M NaCl

TCA: trichloroacetic acid

TEMED: N,N,N',N'-tetramethylethylenediamine

TFMS: trifluoromethanesulfonic acid

Tris: Tris [hydroxymethyl] aminomethane

WGA: wheat germ agglutinin

CHAPTER I

INTRODUCTION

Members of the Nematode genus *Ascaris* are predominantly parasitic (1); moreover, many species pose severe problems to agriculture and public health worldwide. *Ascaris lumbricoides*, a species parasitic on humans, is an especially severe problem in Asia (2), and *Ascaris suum* infects the majority of swine in the United States, at significant expense to the agricultural industry (3). Investigations of the comparative biochemistry of *Ascaris sp.* and its hosts will provide insight into design of an effective and specific chemotherapeutic agent active against *Ascaris sp.* infection. Although similarities exist between the biochemistry of vertebrates and ascarids, the two groups are highly divergent. In addition to gross and cellular morphological distinctions (1,4), important differences exist in the cellular energy utilization in the two groups. Many parasitic helminths, including *Ascaris suum*, have adapted to an anaerobic environment by alteration of the glycolytic pathway. Phosphoenolpyruvate from glycolysis is converted to oxaloacetate, rather than pyruvate, followed by synthesis of malate from oxaloacetate, regenerating the NAD^+ consumed by the glyceraldehyde phosphate dehydrogenase enzyme during glycolysis (5). Malate is transported into the mitochondrion, where a portion of the pool is oxidized

by the malic enzyme to form pyruvate. Pyruvate is then converted into acetate which is excreted as short chain fatty acids. The fatty acid synthesis generates a reducing equivalent of NADH which is consumed in succinate synthesis from malate via fumarate (6). The reaction generating succinate from fumarate also yields ATP (5), and succinate is excreted (7). The TCA cycle of adult *Ascaris suum* appears to be nonfunctioning (5,7). Although some enzyme activities typically used in the Kreb's cycle are present, others are present in low concentration or perhaps missing altogether in adult ascarids (7). Adult *Ascaris suum* has also lost the ability to use fatty acids as fuel, even under aerobic starvation conditions (7). These adaptations force the adult worms to rely on glycogen as their sole source of energy during nonfeeding periods of the host (5).

Glycogen synthase (UDP-glucose:glycogen α-4-glucosyltransferase, EC 2.4.1.11) catalyzes the rate-limiting reaction in glycogen synthesis, the addition of glucose monomers onto a glucose polymer primer (8). This reaction can be written as follows:

$$\text{glucose}_{(n)} + \text{UDP-glucose} \longrightarrow \text{glucose}_{(n+1)} + \text{UDP} \quad (9).$$

Glycogen synthase occurs as two forms, GS I (independent) and GS D (dependent), depending on the phosphorylation state of the enzyme. GS I is phosphorylated by the cyclic AMP-dependent protein kinase to give GS D, a less active form of the enzyme (10). GS D has a strong dependence on the

presence of G6-P, an allosteric effector of the enzyme, for activity (10) and has no measurable activity under physiologic conditions (11,12). Activity is also regulated *in vitro* through multisite phosphorylation by several other kinases, including phosphorylase kinase, glycogen synthase kinase 3, glycogen synthase kinase 4, and glycogen synthase kinase 5 (Casein Kinase 2, $PC_{0.7}$) (9,10,13,14); in addition, phosphorylation has been shown to take place through cyclic AMP-independent pathways *in vivo* (15,16). Cooperativity between subunits also seems to play an important role in regulation of glycogen synthase *in vitro* (9).

The principal stored metabolic fuel in *Ascaris suum* is glycogen. In mammals, glycogen phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1), catalyzes the rate limiting step of glucose mobilization from glycogen (8). Glycogen phosphorylase is activated through phosphorylation by phosphorylase kinase, which is activated by the active cyclic AMP-dependent protein kinase in the presence of calcium (10). The synthetic and degradative pathways for glycogen are therefore coordinately regulated, preventing futile cycling of glucose.

Glycogen synthase has a requirement for the presence of a glucose acceptor for activity. Glycogen can be used by rat liver glycogen synthase as the acceptor for glucose from UDP-glucose (K_m=1 mM) (17), but small oligomers of glucose prove to be very poor substrates for glycogen synthase ($K_{m\,maltoheptalose}$=70 mM) (17). Glucose itself can be used as a substrate for rat skeletal muscle glycogen synthase, but the K_m for this substrate approaches 1 M (18). Since glucose and small oligomers of glucose are very

poor substrates at physiologic conditions, the origin of glycogen particles *in vivo* has, until recently, been unknown. In 1969, workers in Luis LeLoir's laboratory (19) found that the supernatant from a high speed centrifugation of rat liver homogenate catalyzed glycogen synthesis in the absence of added glycogen. The weight of the primer used by the synthase in this fraction had to be less than $5x10^6$ Da, since glycogen of this size or greater sedimented under the conditions used. Likewise, glycogen could only have been present in very small quantities, since a colorimetric test for glycogen did not indicate its presence (19). Subsequent studies showed that the endogenous primer was proteinaceous (20,21) and that it could be isolated as a complex with a glycogen synthase activity (designated GS-2) distinct from the classical enzyme which required an exogenous primer for activity (GS-1) (22).

Rabbit muscle glycogen appears to be bound to a protein, which can be isolated by amylase digestion of the glycogen (23). This protein, which has a M_r of 37 KDa, is very similar to a rabbit skeletal muscle protein which has been shown to be associated with glycogen synthase in rabbit skeletal muscle. The amino acid composition and immunoreactivity of these two proteins are identical (24,25). Interestingly, the smallest M_r protein serving as an acceptor for glucose in Krisman's GS-2 preparation also has an approximate molecular weight of 40 KDa (26). There is evidence indicating that the protein is bound to carbohydrate through a tyrosine residue (27,28) and that it has an autoglucosidation activity (25,29) which is presumed to create the carbohydrate primer used by glycogen synthase.

Nematodes are highly divergent from vertebrates morphologically and embryogenetically (1); however, *Ascaris suum* muscle has numerous biochemical similarities with vertebrate muscle. Tropomyosin, troponin, and myosin, myofibril proteins found in mammalian skeletal muscle, are present in *A. suum* muscle, and a myosin light chain kinase activity, found in mammalian muscle, has been detected in preparations of the worm (30). Cyclic AMP, an important second messenger in vertebrates (9) is present in *Ascaris suum* muscle (31). Production of cyclic AMP can be induced by introduction of serotonin into a muscle perfusion system (31), and a cyclic AMP-dependent protein kinase found in *A. suum* muscle is very similar to the mammalian enzyme by virtue of its chromatographic properties and substrate specificities (32). Inhibition of both the ascarid and mammalian cyclic AMP-dependent protein kinases by the mammalian heat-stable inhibitor indicates a particularly high amount of conservation in the active sites of the ascarid and mammalian kinases (32).

Enzymes of glycogen synthesis in *Ascaris suum* exhibit much similarity to their mammalian counterparts. Phosphorylase has been purified from *Ascaris suum* muscle and has been shown to be very similar to the mammalian enzyme (33). Glycogen synthase, as purified from *A. suum* by Hannigan *et al.* (34), is indistinguishable from the vertebrate GS-1 with respect to several kinetic parameters, including pH and temperature dependence. Glycogen synthesis in *A. suum* is regulated by cyclic AMP-dependent (32) and independent (35) pathways *in vivo*, much like the

vertebrate system, and the nematode enzyme can be inhibited by phosphorylation by both the mammalian cyclic AMP-dependent protein kinase and the glycogen synthase kinase 3 (34,36).

Glycogen is found in much larger quantities in *Ascaris suum* muscle than in mammalian tissues. *A. suum* muscle contains up to 15% glycogen by weight, while rat liver has only 5% and rabbit muscle only 1% (37). Glycogen synthase activity in crude homogenates of *A. suum* is 7.84 U/g (34), while rabbit skeletal muscle only has 0.42 U/g (10); however, the yields of GS-1 after purification from the two tissues are about equal. Most of the activity in the *A. suum* glycogen synthase preparation does not bind to the anion exchange resin used in the purification scheme and is lost in the column flow-through fractions. Investigation of the flow-through fraction of the anion exchange column showed that it contained glycogen synthesis activity in the absence of added glycogen. This activity appears to be analogous to the GS-2 activity discovered by Krisman and coworkers (22) and will be referred to as GS-2 throughout this work. Further purification showed the GS-2 activity to be associated with a complex of three subunits detected by SDS-PAGE (38). One of the subunits of this complex is a glycoprotein and serves as an acceptor of glucose from the substrate, UDP-glucose (39). The GS-2 complex differs from the known glycogen synthase in several ways. Assay of the purified GS-2 shows it to be completely dependent on the presence of G6-P for activity, and none of the proteins in the complex are phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase (39).

Studies presented in this thesis were performed in an attempt to characterize the glycogen primer complex of *Ascaris suum*, as well as to compare this complex to what is known about mammalian glycogen polymer initiation and synthesis.

CHAPTER II

METHODS

Tissue Collection

The presence of *Ascaris suum* infection in swine was investigated by palpation of the duodenum of dissected pigs at a local slaughterhouse. When *A. suum* organisms were found, a transverse incision was made through the intestine on both sides of the parasite, and the intestinal section was immersed into water and transported to the laboratory where the nematodes were transferred to a salt solution (40) continuously sparged with nitrogen and stored for less than 24 hrs. Adult female *A. suum* that were 10-30 cm in length were dissected longitudinally and the reproductive and gastro-intestinal tracts were discarded. The muscle layer was scraped from the underlying cuticle and used immediately or frozen immediately in liquid nitrogen. Frozen specimens were maintained at -80° C until needed.

Glycogen Synthase Assay

Glycogen synthase activity was assayed by a modification of the method of Thomas *et al.* (41) which measures the incorporation of [^{14}C]glucose from UDP[^{14}C]glucose into glycogen. The assay mixture consisted of 50 mM

Tris-Cl, pH 7.6, containing 6.7 mM UDP[U-^{14}C]glucose (Sp.Act.= 19.6 µCi/mmol), and 10 mM glucose 6-phosphate in a total volume of 0.1 ml. When glycogen was used in this assay, the final concentration of *A. suum* or oyster glycogen was 1%. The reaction mixture was incubated for 10 min at 30° C. The reaction was stopped by pipetting 50 µl aliquots onto 1 cm squares of Whatman ET-31 paper and washing the papers twice for 10 min each in 7 to 8 ml per paper of 66% ethanol. The papers were washed in acetone for 5 min, dried and [^{14}C]glucose incorporation into polysaccharide was quantitated by liquid scintillation counting. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the incorporation of 1 µmol [^{14}C]glucose per min at 30°C.

TCA Precipitation of Glycogen Synthase 2

GS-2 complex (2.5 µg protein) was incubated as described under Glycogen Synthase Assay at 30°C for variable time intervals from 10 to 40 min. The reaction was stopped by transferring an aliquot of the reaction mixture to ET31 paper which was immediately immersed in ice cold 10% trichloroacetic acid (m:v; 10 ml/paper). Papers were washed in 5% trichloroacetic acid as described by Reimann *et al.* (42) in order to remove unreacted UDP[U-^{14}C]glucose, and protein-bound radioactivity was determined by liquid scintillation counting of the dried papers.

Purification of Glycogen Synthase 1

Glycogen Synthase 1 (GS-1) was purified according to Hannigan *et al.* (34), with the following changes. The addition of K_2HPO_4 and 5'-AMP, and the corresponding incubation at 30° C were omitted. Addition of 2-mercap-

toethanol (40 mM) and phenylmethylsulfonyl fluoride (100 µM) was made to all buffers used. Chromatography using glucosamine 6-phosphate Sepharose was omitted.

Purification of Glycogen Synthase 2

GS-2 purification was accomplished by a modification of Ghosh *et al.* (39). *A. suum* muscle (50-100 g) was homogenized (3:1, m:v) in 20 mM Tris-Cl, pH 7.0, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride and 15 mM 2-mercaptoethanol (Buffer A). All procedures were carried out at 4^0 C unless otherwise stated. The homogenate was centrifuged at 10,000 x *g* for 30 min and the pellet was discarded. This fraction was designated Crude Extract. The Crude Extract was centrifuged at 105,000 x *g* for 60 min to remove the bulk of the glycogen. The supernatant fraction, designated Soluble Fraction, was dialyzed against 20 volumes of Buffer B (50 mM Tris-Cl, pH 7.5, 40 mM 2-mercaptoethanol, 1 mM EDTA, 10 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride and 5% sucrose).

The dialyzed Soluble Fraction was mixed with 150-200 ml of DEAE cellulose equilibrated with Buffer B. After 1 hr, the fraction which did not bind to DEAE cellulose, designated the DEAE Fraction, was collected either by filtration or by pouring the resin into a column if GS-1 was also to be purified. The DEAE Fraction, i.e. that protein which did not bind to the resin, was concentrated by precipitation with 0.56 g/ml ammonium sulfate. The precipitated protein was dissolved in 2-4 ml 50 mM β-glycerol phosphate, pH 7.0, 2 mM EDTA, 40 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl

fluoride, and 10% sucrose (Buffer C). The ammonium sulfate precipitate was dialyzed 2 hr against 500 volumes of Buffer C and then applied to a column (100 x 2.5 cm) of Sepharose 4B equilibrated with Buffer C. If the sample volume to be loaded onto the Sepharose column exceeded 10 ml, the sample was divided into two equal aliquots, and two Sepharose columns were used. Fractions were collected and the glycogen synthase activity of the fractions was determined. Fractions containing GS-2 activity were pooled and concentrated by precipitation with 0.56 g/ml ammonium sulfate. The precipitate was collected and dissolved in 10-15 ml Buffer D and dialyzed for 2 hrs against Buffer D (50 mM Tris-Cl, pH 7.4, 40 mM 2-mercaptoethanol, 1 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1 mM phenylmethylsulfonyl fluoride). The sample was applied to a column (20 x 0.9 cm) of Con A Sepharose equilibrated with Buffer D. The column was washed with 3 bed volumes of Buffer D and eluted with Buffer D containing 100 mM α-methylmannoside. Fractions were assayed in the presence and absence of glycogen and the active fractions were pooled and dialyzed against Buffer D containing 10% sucrose. The enzyme was stored in small aliquots at -80^0 C. The loss of enzyme activity in the stored enzyme preparation was less than 10% in six months.

Antibody Sensitivity

Antibodies were generated against the GS-2 complex according to Ghosh (38). Antibody reactivity of individual components of the GS-2 complex was determined by Western Blot analysis of samples from 10% polyacrylamide gels which were electrophoretically transferred to nitrocellulose in 25 mM

ethanolamine/glycine buffer, pH 9.5, with 20% (v:v) methanol. The Western blots were probed with the rabbit anti-GS-2 antibody (1:1,000) and horseradish peroxidase-linked goat anti-rabbit IgG.

Gel Electrophoresis

Protein analysis by gel electrophoresis was carried out according to (43) using 10% polyacrylamide gels (1.5 mm thick x 52.5 mm long) as resolving gels and 3.9% polyacrylamide stacking gels.

Samples were prepared for electrophoresis by adding sample buffer (1 part sample buffer:4 parts experimental sample) and incubating in a boiling water bath for 5 min. Samples were cooled to ambient temperature and applied to the stacking gel. Subsequent electrophoresis was done at 165 V until the dye front reached the bottom of the gel, about 40 min.

If the gel was to be stained, it was fixed in methanol:acetic acid:water (45:10:45) for at least 20 min. Gels were stained for proteins using Coomassie Brilliant Blue G-250 or Bio Rad silver stain or for carbohydrate using periodic acid/Schiff base reagent (44).

Non-reducing SDS-PAGE was carried out as described above, except the sample buffer contained no 2-mercaptoethanol. Before running non-reducing gels, sample was dialyzed for 2 hr against 30 ml Buffer D without 2-mercaptoethanol at 4^0 C.

Lectin Binding Experiments

The glycoprotein subunit of GS-2 complex was isolated by denaturation and subsequent chromatography on Con A Sepharose. Purified glycogen primer complex (30 µg protein, 6 mg carbohydrate) to which SDS was added

to a final concentration of 2% in a final volume of 125 µl, was heated in a boiling water bath for 5 min, then dialyzed against 30 ml Buffer D. A column of Con A Sepharose (0.2 ml) was poured and washed thoroughly with Buffer D. Small polypropylene chromatography columns (Bio Rad Econo-Column #731-1550) were used for all chromatography using ≤3 ml resin. Sample was applied to the column and the effluent reapplied 3 times, or the sample was applied at a very slow flow rate (<12 drops/min). The column was washed with 10 bed volumes Buffer D, and bound protein was eluted with 5 bed volumes of Buffer D containing 100 mM α-methylmannoside.

More glycogen primer complex was sometimes used, but the ratio of primer complex to Con A affinity resin (0.15:1 w/v), and the final SDS concentration were always kept constant. Addition of SDS was omitted where noted.

For WGA Sepharose chromatography, a column of this resin was poured (0.4 ml) and washed thoroughly with Buffer D. Glycoprotein subunit from the GS-2 complex (≤210 µg protein) was applied to the column and reapplied 3 times. The column was washed with 3 ml Buffer D (Wash Fractions), and bound material was eluted with 3 ml Buffer D containing 100 mM N-acetyl-glucosamine (Sugar Elution), followed by a 3 ml wash of Buffer D containing 1.0 M NaCl (Salt Elution). Three pools, consisting of the Wash Fractions, the Sugar Elution, and the Salt Elution, were collected. These were dialyzed against 8 mM Tris-Cl, pH 7.6, and concentrated by lyophilization.

Con A Sepharose and WGA Sepharose affinity resins were regenerated by

washing the column with 3-5 bed volumes regeneration buffer (50 mM Tris-Cl, pH 7.6; 1.5 M NaCl; 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$). If the resins were to be stored for more than a few days, they were washed with at least 3 volumes of storage buffer (20 mM Tris-Cl pH 7.6, 0.9% NaCl, 0.01% Thimerosal) and stored in this buffer.

To investigate the free WGA binding capability of the GS-2 complex, purified glycoprotein subunit (15 µg protein) of GS-2 treated with a 48 hr α-amylase digestion as described in Enzymatic Digestion of Carbohydrate or 30 µg of untreated purified GS-2 was subjected to SDS-PAGE, electro-blotted onto a nitrocellulose filter, and strips were cut out of the nitro-cellulose corresponding to the lanes containing proteins to be incubated with WGA. Each strip was incubated in 2 ml TBS (20 mM Tris-Cl, pH 7.6, 0.5 M NaCl) containing 0.1% periodate-treated BSA for 1 hr at ambient temperature. Strips were then incubated 2.5 hr in 2 ml each of TBS containing 10 µCi [^{125}I]WGA (3.7 µg). Strips were washed in 2 ml each of TBS for 3 changes of 10 min. Strips were dried and autoradiographed. Some strips were stained with amido black for protein. Periodate-treated BSA was made according to a procedure by Glass *et al.* (45). Periodic acid was added to a final concentration of 10 mM to 4% BSA in 0.1 M sodium acetate buffer, pH 5, and incubated 18 hr at ambient temperature. Glycerol was added to a concentration of 10 mM, and the reaction mixture was dialyzed for 6 hrs against two 20 volume changes of TBS. The BSA solution was diluted to a final concentration of 3% before use.

Enzymatic Digestion of Carbohydrate

To prepare a sample for glycosidic digestion, the glycoprotein subunit (15 µg protein) was isolated from purified GS-2 complex using Con A Sepharose chromatography in the presence of SDS, dialyzed overnight against 100 volumes 8 mM Tris-Cl, pH 7.6, and lyophilized. The sample was dissolved in 50 µl glycosidase buffer (20 mM sodium citrate, pH 6.0, 2 mM leupeptin, and 0.1% SDS). The solution was heated in a boiling water bath for 5 min, and Triton X-100 was added to a concentration of 1.25%. This sample will be referred to as the Glycosidase Sample.

Neuraminidase (5 mU) was added to the Glycosidase Sample or to 100 µl 2 mg/ml mucin in glycosidase buffer containing Triton X-100, 1.25%, and incubated at ambient temperature for 1 hr. Enzyme activity was terminated by immersion of the reaction tube into a boiling water bath for 5 min. Neuraminic acid released from mucin was assayed by the thiobarbituric acid assay (46). For determination of sialic acid content, a separate glycosidase sample prepared as above was incubated 1 hr at 37^{0} C with 20 mU neuraminidase. The reaction was heat-inactivated as above and dialyzed against 30 ml 1 mM sodium phosphate buffer, pH 6.0, for 3 hr. The dialysate was lyophilized and tested for free sialic acid using the thiobarbituric acid assay (46).

N-acetylglucosaminidase (7.8 mU) was added to the neuraminidase-treated Glycosidase Sample and incubated 24 hr at ambient temperature. This and all glycosidic digestions that were incubated longer than 5 hr were layered with toluene to prevent contamination by airborne bacteria, and all

liquid transfers into and out of the reaction once layered with toluene were done using a Hamilton syringe outfitted with a Teflon needle. As a positive control, the same amount of N-acetylglucosaminidase was added to 100 µl 1 mg/ml p-nitrophenyl-N-acetyl-β-D-glucosaminide in glucosidase buffer containing Triton X-100, 1.25%, and incubated 1 hr at ambient temperature. Release of p-nitrophenol from p-nitrophenyl-N-acetyl-β-D-glucosaminide was detected spectrophotometrically at 400 nm.

O-Glycanase (endo-α-N-acetylgalactosaminidase, 1mU) was added to the exoglycosidase-treated glycosidase sample or to 100 µl 2 mg/ml asialofetuin in glycosidase buffer containing Triton X-100, 1.25%, and incubated 24 hr at 37^0 C. Release of acylamino sugars from asialofetuin was detected by a modification of the Morgan-Elson assay (47).

An α-amylase digestion was performed on the glycoprotein subunit of GS-2 complex as follows. Purified glycoprotein subunit of the GS-2 complex (15 µg protein) in 300 µl 20 mM phosphate buffer, pH 5.8, containing 1 mM $CaCl_2$ and 2 mM leupeptin, was incubated with 9 U α-amylase from *Bacillus subtilis* and 140 mU amyloglucosidase at ambient temperature for 5 hr. The reaction mixture was dialyzed for 2 hr against 30 ml of the above phosphate buffer, after which 1.8 U *B. subtilis* α-amylase was added. The reaction was covered with toluene, and the reaction was allowed to incubate at ambient temperature for 15 hr. At the end of this incubation, the reaction mix was again dialyzed against 30 ml of the phosphate buffer for 2 hr, and another 1.8 U of *B. subtilis* α-amylase was

added. The reaction was allowed to proceed for 24 hr, at which time the reaction was stopped by immersion into a boiling water bath for 5 min. Reaction products were subjected to SDS-PAGE and electroblotted onto a nitrocellulose filter; proteins were probed either by antibodies against the GS-2 complex in a Western Blot procedure or [^{125}I]WGA binding and autoradiography.

A time course for the α-amylase reaction was performed on the glycoprotein subunit of the GS-2 complex for the purpose of following the loss of carbohydrate from the complex. Five 45 µl (13 µg protein each) aliquots of purified GS-2 complex were dialyzed 2 hr against 30 ml amylase buffer (Buffer D lacking 2-mercaptoethanol), and diluted to 1.35 ml. Human salivary α-amylase (3 U) was added at 0 min and 45 min to each aliquot and reactions were incubated at 30^0 C for 0, 30, 60, 90, or 120 min. At the end of each reaction, the enzymatic activities were stopped by immersion of the reaction tube into a boiling water bath for 10 min, and the solutions were dialyzed against three changes of 500 ml of 8 mM Tris-Cl, pH 7.6. Residual carbohydrate was quantitated by the anthrone assay for carbohydrate using glycogen for the standard curve, and protein was quantitated by the dye binding method of Bradford (48).

Partial α-amylase digestion for the purpose of separation of GS-2 subunits with retention of activity was performed as follows. All steps except for assay reactions were done at 4^0 C. Purified GS-2 complex (400 µl; 120 µg protein), was dialyzed against 30 ml amylase buffer

containing 2 μM leupeptin for 2 hr. Human salivary α-amylase (9 U) was added, and the mixture was incubated for 15 min. The reaction mixture was dialyzed against 30 ml amylase buffer for 1 hr. The reaction mixture was loaded onto a 0.6 ml column of Con-A Sepharose, the column was washed with 10 bed volumes of amylase buffer, and material was eluted with amylase buffer containing 100 mM α-methylmannoside. Fractions (8 drop) were collected and assayed for activity with and without the presence of glycogen. Wash and elution fractions were pooled, concentrated by lyophilization, and analyzed by SDS-PAGE.

Hydrolysis of GS-2 Glycoprotein

Hydrochloric acid-catalyzed hydrolysis was completed as follows. Glycoprotein subunit was isolated from purified glycogen primer complex by Con A chromatography in the presence of SDS. The sample was then dialyzed against approximately 100 volumes 8 mM Tris-Cl, pH 7.6, for 2 hr and lyophilyzed to dryness. This residue was then dissolved in an amount of water comparable to the volume before chromatography, and 6 N HCl was added to a final concentration of 1 N. The sample was incubated under vacuum in a Schlenk tube for 1 hr at 60^0, 70^0, 80^0, 90^0, or 100^0 C. At the end of the incubation, two volumes of 1 M ammonium bicarbonate (made fresh daily) was added to neutralize the solution, and the mixture was frozen and lyophilyzed. Products were analyzed by SDS-PAGE.

Hydrolysis using TFMS was completed by a modification of Bahl (49) as follows. Glycoprotein subunit was isolated from 180 μg of purified GS-2 complex by Con A Sepharose chromatography in the presence of SDS and was

then dialyzed against 30 ml water for at least 2 hr. The products were lyophilized to dryness. To the residue, 600 µl anhydrous trifluoromethane-sulfonic acid was added at 0^0 C, and the mixture was incubated at 0^0 C for 2 hr. This reagent was transferred only with glass instruments, and all reaction vessels, containers, and pipettes were purged with nitrogen gas immediately before use, as described in Shriver and Drezdzon (50). At the end of this incubation, the reaction mix was brought to around -75^0 C in a dry ice/acetone slush bath, and 800 µl pyridine:water (60:40 v/v) was added in small amounts (10 µl at a time initially). After addition of a portion of the pyridine solution, the reaction mixture began to freeze, so the solution was thawed slightly after each aliquot of base was added. After addition of the aqueous pyridine, the solution was dialyzed against 2 changes of 4 l of 0.1% ammonium bicarbonate for 4 hr.

Base-catalyzed hydrolysis was completed as follows. GS-2 glyco-protein subunit (15 µg protein) was isolated from purified glycogen primer complex by Con A chromatography in the presence of SDS. The protein, in 300 µl Buffer D, was dialyzed against 30 ml 10 mM NaCl for 3 hr. The sample was added to 0.2 M sodium hydroxide (1:1 vol:vol) and incubated under vacuum at 45^0 C for 15 hr. At the end of this time, the solution was neutralized with the addition of 1 N acetic acid, and dialyzed against 0.1 M ammonium acetate buffer, pH 7.0. The sample was then lyophilized to dryness, dissolved in sample buffer, and analyzed using SDS-PAGE.

Smith Degradation

Periodate oxidation followed by $NaBH_4$ reduction was done by a modification of the method of Smith (51,52). Glycoprotein subunit was isolated from purified GS-2 complex (210 µg protein) by Con A Sepharose chromatography in the presence of SDS. This sample was dialyzed against 30 ml 8 mM Tris-Cl, pH 7.6, for 2 hr and lyophilized. The residue was dissolved in 400 µl 60 mM sodium acetate buffer, pH 5.0, containing 30 mM $NaIO_4$, and incubated at 4^0 C for 28 hr. After this incubation, ethylene glycol was added in ten-fold molar excess to the $NaIO_4$ in the form of a 1:1 aqueous solution.

The reaction mixture was then titrated to pH 8.0 with 1 N NaOH, and $NaBH_4$ was added in twenty-five-fold molar excess of the periodate as a 0.6 M solution in 0.6 M sodium borate buffer, pH 8.0. This mixture was incubated at 4^0 C for 16 hr. The entire mixture was then titrated to pH 5.0 with glacial acetic acid, dialyzed against 30 ml 200 mM sodium acetate buffer, pH 5.0, for 2 hr, and then dialyzed overnight against 30 ml 10 mM NaCl. This product was hydrolyzed for 2 hr at 80^0 C under vacuum by the addition of 1 N HCl to a final concentration of 0.1 N. The mixture was cooled to ambient temperature at the end of the incubation and was neutralized by the addition of 1 N NaOH.

High Performance Liquid Chromatography

HPLC was performed using a variation of the technique described by Hardy *et al.* (53). The column used for sugar separation was a Dionex 10µ HPIC AS-6 pellicular anion exchange column, and eluted sugars were

detected by a Dionex PAD system with time interval settings of T1=300 msec, T2=120 msec, and T3=300 msec, and intensity settings of E1=-0.05V, E2=0.60V, and E3=-0.80V. Eluants were degassed, after which they were sparged with ultrapure carrier grade helium for ≥1 hr. The column was equilibrated with 20 mM NaOH for ≥1 hr after sample chromatography or startup. Samples were eluted by an isocratic elution of 20 mM sodium hydroxide at 0.5 ml/min with a postcolumn addition of 0.6 M NaOH added at 0.1 ml/min. The column was washed with 150 mM NaOH at 0.5 ml/min for ≥30 min after chromatography of sugar standards, or with 150 mM NaOH containing 0.6 M sodium acetate at 0.4 ml/min for ≥30 min followed by 20 mM NaOH at 0.5 ml/min for at least 1 hr after chromatography of samples containing protein or oligosaccharides. All NaOH solutions were dilutions of commercially prepared 50% NaOH, which was used only until 30 days after opening because of the possible buildup of carbonates in the concentrated solution.

To prepare sample for HPLC analysis, 200 µl (60 µg protein) purified GS-2 complex was dialyzed 3 hr against 30 ml amylase buffer (Buffer D lacking 2-mercaptoethanol, containing 2 µM leupeptin). Human salivary α-amylase (0.1 U, 0.1 µg) was added at 0, 45, and 90 min, and the reaction mixture was incubated for a total time of 135 min. The reaction was stopped by immersion of the reaction tube into a boiling water bath for 5 min, and the solution was dialyzed for 18 hr against 3 changes of 8 mM Tris-Cl, pH 7.6. The sample was lyophilized, dissolved in 100 µl warm 2 N HCl, and incubated 2 hr at 100^{0} C. After cooling, the acid was neutralized by the addition of 200 µl 2 N ammonium bicarbonate, and the sample was

degassed for 10 min to remove excess CO_2. This sample will be referred to as HPLC Unknown.

Protease Digestion

Trypsin digestion was completed as follows. Purified glycogen primer complex (12 µg protein in 40 µl Buffer D, 10% sucrose) was added to 20 µl trypsin solution (2.1 U) and incubated for 0, 20, 40, or 60 min. The trypsin solution was made by combining 12 µl 0.24 mg/ml bovine pancreatic trypsin, 108 µl 1 mM HCl, and 120 µl trypsin buffer (40 mM Tris-Cl pH 8.0, 45 mM 2-mercaptoethanol). Reactions were stopped by the addition of 10 µl SDS-PAGE sample buffer and heating at 90^0 C for 5 min in a heat block. Products were analyzed using SDS-PAGE.

Pronase digestion was completed as follows. Purified GS-2 complex (12 µg protein in 40 µl Buffer D, 10% sucrose) was dialyzed against 30 ml pronase buffer (0.1 M potassium phosphate buffer, pH 7.5, 0.45 mM $CaCl_2$, 0.25 mM $MgCl_2$) for 2 hr. Pronase (*Streptomyces griseus* protease, 0.6 U), as 10 µl of a 0.7 mg/l solution, was added, and the reaction mixture was incubated for 60 min at 37^0 C. Protease activity was stopped by immersion of the reaction vessel into boiling water for 5 min.

Alkaline Phosphatase

Purified GS-2 complex (30 µg protein) was dialyzed against 30 ml 0.1 M Tris-Cl, pH 8.0, for 2 hr. Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, 28.5 U), as 100 µl of a 5 mg/ml solution, was added, and the mixture was incubated at 30^0 C for 30 min. Aliquots (50 µl) were taken out at 15 and 30 min. The reaction was stopped by adding 10 µl of SDS-

PAGE sample buffer and heating at 90^0 C for 5 min in a heat block. Products were analyzed using SDS-PAGE.

Materials

Sepharose 4B, 2-mercaptoethanol, phenylmethylsulfonyl fluoride, β-glycerophosphate, Tris base, WGA Sepharose 6MB, type XI bovine pancreatic trypsin, *Eschericia coli* alkaline phosphatase, human salivary α-amylase, *Aspergillus niger* amyloglucosidase, *Aspergillus niger* β-D-N-acetyl-glucosaminidase, bovine pancreatic ribonuclease b, and BSA were purchased from Sigma Chemical Company. DEAE cellulose (DE52) and ET31 paper was from Whatman Biosystems, Ltd. Con A Sepharose was purchased from Pharmacia. Trifluoromethanesulfonic acid and Teflon syringe needles were from Aldrich Chemical Company, Inc. Ultrapure helium was obtained from a local supplier of Air Products and Chemicals, Inc. of Tamaqua, PA. Sodium hydroxide solution, 50 % w:w, was from Fischer Scientific. Thiobarbituric acid was purchased from Fluka. Schlenk tubes were from Pierce Chemical Company. Schlenk flasks were made by a glassblower on the campus of the University of North Texas; Schlenk fittings for those were obtained from Chemglass, Inc., Vineland, NJ. *Streptomyces griseus* protease (Pronase) was purchased from Calbiochem. *Diplococcus pneumoniae* endo-α-N-acetylgalactosaminidase (O-glycanase) and *Bacillus subtilis* α-amylase were from Boehringer Mannheim Biochemicals. Neuraminidase from nonpathogenic *Streptococcus sp.* was from Genzyme Corporation. Dialysis of samples of less than 1 ml were performed using a microdialyzer, the Pierce Chemical MEGA System (#HPI-6300). Uridine diphosphate glucose,

[D-glucose-U-^{14}C] (Sp.Act.=200 mCi/mmol), D-glucose-6-phosphate [D-glucose-U-^{14}C] (Sp.Act.=200 mCi/mmol), and [^{125}I]wheat germ agglutinin (Sp.Act.=1.09 mCi/mmol) were purchased from ICN Radiochemicals. Where SDS-PAGE gels were silver stained, a Bio Rad Silver Stain Kit #161-0443 was used.

CHAPTER III

RESULTS

Correlation of Structure with Function of Glycogen Synthase Activity in the GS-2 Complex of *Ascaris suum*

GS-2 complex was subjected to a 15 or 30 min amylase digest and chromatographed using Con A Sepharose in an attempt to isolate any subunits peripherally bound to the carbohydrate, possibly including the synthase catalytic subunit. If the catalytic subunit acts on and is associated with peripheral portions of the glycogen-like carbohydrate, it is predicted that the enzyme will be released from the complex as this portion of the carbohydrate is digested. SDS-PAGE of the fractions obtained from chromatography of the digest (Figure 1) showed that the 66 KDa subunit appears in the fraction not bound by Con A, indicating its release upon carbohydrate digestion. A second digestion followed by an assay for activity was performed (Table 1). The wash fraction, which contains only 66 KDa subunit, contains GS activity which is dependent on the presence of glycogen.

To investigate the susceptibility of the complex to proteolysis, and therefore indicate the peripheral or central location of the subunits with

relation to the carbohydrate, a tryptic digestion was performed and analyzed using SDS-PAGE (Figure 2). Fragments were cleaved from the subunits in the resolving gel after only 20 min, suggesting their peripheral position on the complex. Protein retained at the stacking gel/resolving gel interface did not undergo detectable digestion; however, after digestion with pronase, this band disappeared, confirming its proteinaceous nature.

To investigate possible homologies between the GS-2 complex and GS-1, GS-1 purified from *Ascaris suum* muscle was subjected to SDS-PAGE, followed by a Western Blot using antibodies generated against the GS-2 complex as described in Methods. Glycogen synthase, the major band in the GS-1 preparation, did not react with the antibodies (Figure 3). These data suggest that the proteins seen in the GS-2 preparation can not be proteolytic fragments of GS-1, since they share no epitopes recognized by the polyclonal antibodies generated against the GS-2 complex. The only immunoreactive band in the GS-1 preparation migrated closely with the 66 KDa subunit and was possibly a contamination of GS-2 in the GS-1 preparation.

The activity of the *A. suum* GS-2 complex is completely dependent on the presence of G6-P for activity (38). This differs from the activities of both GS-I and GS-D, which are reduced but measurable in the absence of G6-P. Trehalose synthase, an enzyme present in *Ascaris suum* (54), uses both G6-P and UDP-glucose as substrates for the reaction it catalyzes. To investigate the use of G6-P as a substrate, GS-2 was incubated with [^{14}C]glucose 6-phosphate and nonradiolabelled UDP-glucose, as described in

Methods. [^{14}C]Glucose was not incorporated into ethanol-precipitatable material (Figure 4), indicating that G6-P is used as an effector of the enzyme and that trehalose synthase activity is not responsible for the glucosyl transferase activity detected.

The 66 KDa subunit appears in some preparations as a doublet, and in some preparations even as a triplet of bands in SDS-PAGE. Phosphorylated GS-1 displays similar electrophoretic patterns due to differing phosphorylation states of the enzyme (9). To determine the phosphorylation state of the *A. suum* complex, a sample of the complex was incubated with alkaline phosphatase, and the reaction product was compared to untreated GS-2 by SDS-PAGE. The 66 KDa subunit, which occurs in many preparations as well as this one as a triplet of bands, collapses almost completely into a single band after the alkaline phosphatase incubation (Figure 5), suggesting that the GS-2 66 KDa subunit is purified as a phosphorylated enzyme.

Correlation of Structure with Function of the Glycoprotein Subunit of the GS-2 Complex of *Ascaris suum*

To confirm that the glucose was transferred to a glycoprotein primer molecule, the glycogen synthase reaction was stopped by immersion of the papers in a TCA solution rather than the ethanol solution normally used. Recovery of radiolabel as a TCA precipitate (Figure 6) indicates a proteinaceous primer is used for synthesis of the polysaccharide. This confirms the result of Ghosh (38) who demonstrated that radiolabel was incorporated into the protein retained at the stacking gel/resolving gel interface during SDS-PAGE.

To determine the amount of carbohydrate present in the complex that was glycogen-like in nature, purified GS-2 complex was digested with human salivary α-amylase and analyzed using SDS-PAGE. A time course for the amylase digestion was performed as described in Methods, and the weight ratio of bound carbohydrate to protein was calculated (Table 2). The ratio ranged from 120:1 to 206:1 (w:w) carbohydrate:protein in GS-2 preparations before digestion (n=3). In the presence of amylase, the GS-2 carbohydrate was initially degraded at a rate of 26 pmol/min (Table 2). After 30 min, the rate began to decrease until a plateau was observed at 60 min. This plateau does not represent the generation of a deglycosylated moiety, since carbohydrate can be detected in the sample after thorough dialysis.

To further characterize the amylase digestion product, a reaction performed as above and incubated for 90 min was subsequently chromatographed on Con A Sepharose in order to demonstrate that glycoprotein was still present. The fraction which bound to Con A Sepharose and was eluted by α-methylmannoside was analyzed by SDS-PAGE. Material staining for both carbohydrate and protein in the stacking gel collapsed into a tight band at the stacking gel/resolving gel interface after amylase treatment (Figure 7). The amylase digestion apparently reduced the mass of the glycoprotein in the stacking gel although the retention of the glycoprotein subunit at the stacking gel/resolving gel interface and the intense staining of the product with PAS confirmed that substantial quantities of carbohydrate remained bound to the protein.

More extensive digestion of the carbohydrate in the glycoprotein subunit was performed using *Bacillus subtilis* α-amylase, three-fold more units than was used before, and amyloglucosidase from *Aspergillus niger*. The reaction was incubated for 48 hr and was dialyzed to remove reaction products at 5 and 24 hr. The non-dialyzable reaction product was analyzed by SDS-PAGE followed by a Western Blot using polyclonal antibodies generated against the GS-2 complex. Material contained solely in the stacking gel before enzymatic treatment enters the resolving gel upon extensive amylase digestion (Figure 8). Multiple immunoreactive bands were detected in the resolving gel; the lowest M_r observed was 39 KDa, possibly representing the minimum M_r for this reaction product.

To investigate the glycoprotein linkage type as well as to confirm the molecular weight derived from the amylase experiment, the glycoprotein subunit of the GS-2 complex was treated with neuraminidase, followed by an N-acetylglucosaminidase digestion, and finally incubation with O-glycanase. Products were analyzed by SDS-PAGE followed by a Western Blot using antibodies generated against the GS-2 complex as described in Methods. After enzymatic digestion, a strong band appeared at 40 KDa, similar to the result obtained after amylase digestion (Figure 8). These results indicate the presence of an O-type linkage in the glycoprotein; however, the identification of this protein as the apoglycoprotein is not conclusive. A series of bands also occur at a higher M_r, the strongest of which migrates at 75 KDa and comigrates with a distinct band seen in the amylase digestion, but not in the starting material. Positive controls using

mucin for neuraminidase substrate and asialofetuin for O-glycanase substrate showed both enzymes to be active under the reaction conditions used.

The glycoprotein subunit of the GS-2 complex was hydrolyzed with 1 N HCl for 1 hr at various temperatures to digest the carbohydrate bound to this protein. Products were analyzed by SDS-PAGE to obtain the apoprotein M_r. The results of these reactions showed two bands of M_r 58 and 53 KDa that appeared only during hydrolysis above 80^0 C (Figure 9). The appearance of these bands coincided with the progressive loss of Coomassie Blue-staining material from the stacking gel (Figure 9).

A Western Blot was done of a HCl acid hydrolysis product to investigate the reactivity of the "apoprotein" with the polyclonal antibodies generated against the complex. The purified complex was hydrolyzed using 1 N HCl for 1 hr at 85^0 C, a temperature just above the lowest shown to hydrolyze the glycoprotein. Antibodies were shown to react with bands at 55 and 48 KDa in the reaction product (Figure 10); these bands were not present in the starting material. Antibodies also reacted strongly with substance trapped in the stacking gel in the lane containing starting material; this material did not appear in the reaction product. A hydrolysis using TFMS yielded similar protein bands after SDS-PAGE; however, low yields from this reaction caused problems in electrophoretic mobility similar to those encountered during base hydrolysis (data not shown).

Purified GS-2 glycoprotein subunit was subjected to base catalyzed β-elimination to investigate the possibility of the carbohydrate being linked through an acetal linkage to threonine or serine in the polypeptide, since

these are the only common glycosidic linkages that are base labile (55,56). Analysis of the base hydrolysis product using SDS-PAGE showed two bands of M_r 53 and 58 KDa which migrated similarly to bands produced by acid hydrolysis, confirming that the carbohydrate is bound through a serine or threonine hydroxyl moiety in the polypeptide, since these are the only known amino acid linkages that can undergo base catalyzed β-elimination; however, extremely low yields during this reaction precluded efficient SDS-PAGE electrophoresis. Electrophoresis of quantities of base-hydrolyzed product sufficient for detection by Coomassie blue stain overloaded the gels, causing reverse osmotic effects and anomalous migration. Loading quantities small enough for correct electrophoresis produced protein bands in the resolving gel that were barely detectable, even after silver staining.

Susceptability of the protein:carbohydrate bond to O-glycanase cleavage indicates the presence of an O-type linkage in this glycoprotein; however, this does not conclusively prove the identity of the penultimate sugar residue as N-acetylgalactosamine. Although the protein:carbohydrate linkage typically presented in the literature (57,58,59) involves a serine/threonine: N-acetylgalactosamine moiety, these are extracellular proteins such as those involved in secretion and intercellular matrices. A class of glycoproteins containing a serine/threonine:N-acetylglucosamine moiety has been discovered just recently (60). This type of linkage is found specifically on intracellular proteins, often on cytoplasmic domains of membrane proteins. The ability for O-glycanase to cleave O-linked serine/threonine:N-acetylglucosamine is not known (personal communication, technical staff at Boehringer Mannheim Biochemicals); however,

since N-acetylglucosaminidase, an exoglucosidase, can cleave both O-linked N-acetylglucosamine and N-acetylgalactosamine, it is possible that a carbohydrate bound by N-acetylglucosamine could be cleaved by O-glycanase.

To investigate the presence of sugars other than glucose in the glycoprotein, purified GS-2 was electrophoresed by SDS-PAGE, stained for protein with Coomassie Blue, electroblotted onto a nitrocellulose filter as described under Antibody Generation in Methods, and incubated with [^{125}I]WGA. WGA binds with high affinity and specificity to N-acetyl-β-D-glucosamine residues (61,62,63). WGA also binds to N-acetylneuraminic acid (64,65), probably by a different mechanism (62,66). The glycoprotein subunit bound strongly to the WGA, as shown by an autoradiograph of the blots after incubation with [^{125}I]WGA (Figures 11,12), indicating the presence of either N-acetylglucosamine or N-acetylneuraminic acid in the glycoprotein, while other subunits of the GS-2 complex did not bind to WGA. Since treatment with sialidase as in Methods yielded no detectable free sialic acid detectable by the thiobarbituric acid assay, the presence of N-acetylglucosamine is indicated. Treatment of mucin by sialidase yielded detectable amounts of neuraminic acid.

To show that the protein that binds to WGA in the stacking gel is the glycoprotein containing glycogen-like carbohydrate, an amylase digestion was done on purified glycoprotein subunit, with subsequent SDS-PAGE, electroblotting, and incubation with [^{125}I]WGA (Figure 12). The disappearance of the WGA-binding band from the stacking gel/running gel interface

after amylase digestion indicates that the migration of the WGA-binding protein(s) was altered by amylase treatment (Figures 11,12).

Purified GS-2 glycoprotein was subjected to the degradation of Smith (52) to investigate the possible presence of carbohydrate other than glycogen in the glycoprotein. Glycogen will be completely degraded by the Smith degradation, but saccharides that contain no *vic*-diols, such as carbohydrates at the reducing end of many glycoproteins, will escape degradation.

The glycoprotein subunit purified from GS-2 complex was subjected to the Smith degradation and subsequent chromatography using WGA Sepharose. Fractions from the WGA chromatography were analyzed using SDS-PAGE (Figure 13). Two bands appeared that migrated very similarly to proteins seen after acid hydrolysis in both the wash and elution fractions (see Figure 10). The appearance of "apoprotein" in the elution fractions indicates the presence of N-acetylglucosamine on this protein. The resin bound specifically to the two bands at 63 KDa and 58 KDa, since smaller M_r material (<30 KDa) appears solely in the wash fraction and did not bind at all. The appearance of "apoprotein" in the wash fraction may be due to hydrolysis of the periodate-resistant "core" containing the N-acetylglucosamine by the acid used in the last step of the Smith chemical sequence. Polysaccharides have been shown to be hydrolyzed by as low as 0.05 N H_2SO_4, albeit incompletely (67).

To further investigate the presence of sugars in addition to glucose in the glycoprotein moiety, anion exchange HPLC analysis of an acid

hydrolysate of purified GS-2 was performed. At very high pH, saccharides ionize to yield anions ($pK_{a_{glucose}}$=12.28); these anions can easily be separated using anion exchange HPLC. Chromatography of 10 µl of an acid hydrolysate of the GS-2 complex, labelled "HPLC Unknown" as described in Methods, yielded a huge peak (peak E, Figure 14) which coeluted with glucose; at high sensitivities a small shoulder was consistently seen on this peak. Other peaks occurred on this chromatogram; however, only one peak (peak G) occurred near the glucose elution. This peak is due to an instrument artifact; it occurred even when no sample was injected.

To investigate the nature of the shoulder on the glucose peak, 15 µl of the HPLC Unknown was injected into the HPLC system without the PAD system connected, and 250 µl fractions were collected. These fractions were rechromatographed, and Figure 15 shows the rechromatography of a fraction corresponding to the end of the glucose peak. The glucose peak is reduced, and the chromatogram clearly shows the appearance of a second peak (peak D). This second peak coelutes with mannose, as shown by its comigration with the mannose standard spike in the chromatogram which is overlaid on this figure; however, this peak could also represent the presence of N-acetylglucosamine or N-acetylgalactosamine, since these sugars also coelute with mannose (personal communication, technical personnel at Dionex).

CHAPTER IV

DISCUSSION

The 66 KDa subunit of the *Ascaris suum* GS-2 complex appears to contain the glucosyl transferase activity. This subunit is an integral part of the complex, since it copurifies with the complex in all GS-2 purifications performed (n=10). It is not, however, bound to the complex by disulfide bridges, since proteins electrophoresed on SDS-PAGE appeared identical in the absence or presence of reducing agents; moreover, after α-amylase treatment, the 66 KDa protein is released from the complex (Figure 1, Figure 7), indicating a peripheral positioning of the 66 KDa protein in the complex and association with the glycogen-like carbohydrate. This subunit contains glucosyltransferase activity, since a glycogen-dependent activity was recovered in a fraction containing only 66 KDa protein after α-amylase digestion and Con A Sepharose chromatography (Figure 1, Table 1).

Similarities exist between the 66 KDa subunit and the known GS-1. In addition to their similar catalytic activities, the 66 KDa subunit appears to exist in different phosphorylation states, as demonstrated by alkaline phosphatase data (Figure 5). Significant differences exist, however; polyclonal antibodies generated against the GS-2 complex do not cross-

react with GS-1 (Figure 3), which indicates a large amount of divergence in the primarystructure of the two proteins. Further evidence of this divergence is shown by the inability to phosphorylate GS-2 by the catalytic subunit of cAMP-dependent protein kinase (38).

The protein migrating at the stacking gel/resolving gel interface is a glycoprotein, as shown by its ability to stain with both Coomassie blue and PAS stains on SDS-PAGE (Figure 7). This glycoprotein appears to serve as the primer for glycogen synthase, since this is the only protein in the complex staining positively for carbohydrate (Figure 8) and since incubation with UDP[^{14}C]glucose followed by SDS-PAGE showed radiolabelled material to appear only in the stacking gel (38). The glycogen-like nature of the carbohydrate bound to the protein is demonstrated by α-amylase treatments of the glycoprotein which digest most of the carbohydrate in the glyco-conjugate (Table 2, Figure 8). These data are supplemented by incubations with phosphorylase a, which also digest this carbohydrate (38).

The carbohydrate is very large, also in agreement with the similarities to glycogen. The fact that the protein cannot migrate into resolving gels during SDS-PAGE shows the glycoprotein to have a M_r in excess of 300 KDa, and quantitation of the amounts of carbohydrate and protein using the Bradford and anthrone reagents show the glycoprotein to consist mostly of carbohydrate. In addition, complete digestion of the carbohydrate using α-amylase and amyloglucosidase requires long incubation times of 48 hr.

Treatment of the glycoprotein with O-glycanase causes the appearance of a protein at 40 KDa (Figure 8). A minor band appears at 75 KDa. Presence

of a large amount of immunoreactive material in the stack, although possibly indicative of unreacted material, may also be due to the carbohydrate product of the reaction, since glycogen has been shown to have a 2^0 and possibly a 3^0 structure (23) and is immunoreactive (68). Since this enzyme is believed to cleave the glycosidic bond between serine/threonine and N-acetylgalactosamine, these data indicate that the glycogen-like carbohydrate is linked through an acetal glycosidic linkage to serine or threonine in the peptide. Enzymatic cleavage at this linkage is an indication of the presence of another carbohydrate in a core region of the glycogen-like carbohydrate, namely N-acetylgalactosamine. The presence of an O-linkage in the glycoprotein is also suggested by the susceptability of the glycoprotein to base-catalyzed hydrolysis.

The presence of N-acetylglucosamine was investigated using [^{125}I]wheat germ agglutinin. Binding of this protein specifically to the glycoprotein subunit of the *A. suum* GS-2 complex (Figure 11, Figure 12) indicates the presence of either N-acetylglucosamine or sialic acid. Since sialic acids were not detected after neuraminidase treatment, the presence of N-acetylglucosamine is indicated. The presence of this sugar in the glycogen primer protein, and not in another protein with similar electrophoretic migration, was confirmed by the disappearance of the WGA-binding protein after amylase treatment, indicating the glycogen-like nature of the WGA-binding protein.

Appearance of Smith degradation product in the elution fractions from WGA Sepharose chromatography indicates that the N-acetylglucosamine is located in a Smith-degradation resistant core polysaccharide. Further evidence for N-acetylglucosamine in the primer glycoprotein is given by the HPLC data, which show a minor peak which could correspond to N-acetyl-glucosamine, N-acetylgalactosamine, or mannose.

The finding of an O-linked glycoprotein in a cytosolic fraction is unusual. Although many O-linked glycoproteins exist, these are typically secreted proteins and have an N-acetylgalactosamine moiety as the ultimate residue (57,58,59). Conversely, glycoproteins with N-acetylglucosamine as the ultimate sugar residue are usually N-linked to protein and are found on extracellular domains of membrane proteins, as well as blood plasma proteins. It is unusual that the glycoprotein subunit of the GS-2 complex may have an O-linked N-acetylglucosamine, but this linkage is not novel. Torres and Hart (69) have identified such a linkage on the cytosolic domains of certain lymphocyte membrane proteins, and it has been hypothesized that this may be a general linkage for cytosolic glycoproteins. Work on a Trematode, *Schistosoma mansoni*, shows that most of the O-linked sugars in this organism are O-linked N-acetylglucosamine (70); however, all of the examples of O-linked N-acetylglucosamine found to date have been mono-saccharyl-protein linkages. If this glycoprotein proves to have an N-acetyl-glucosamine penultimate to a polysaccharide as these data suggest, it will represent a heretofore undocumented glycoprotein type. The large size and occurence in the cytosol of this glycoprotein indicate it to be a novel type

of glycoprotein, and the existence of other relatively unique features would not be surprising.

The apoprotein M_r appears to be 58 KDa, since this is the molecular weight of the glycoprotein subunit after being subjected to acid hydrolysis (Figure 10). This reaction goes to completion, since no material corresponding to the starting material is left in the stacking gel. A minor second band occurs at 48 KDa. Hydrolysis with anhydrous TFMS yields the same bands from the glycoprotein subunit as was found after HCl hydrolysis (data not shown).

There seems to be some ambiguity with the molecular weight determinations made on the primer subunit of *A. suum* GS-2 complex. If the carbohydrate portion of the glycoprotein subunit is mostly or entirely α 1,4 and α 1,6 glucose polymer, the limiting M_r obtained after α-amylase digestion should approximate that of the apoprotein. After thorough α-amylase digestion, the digested glycoprotein displayed a minimum molecular weight of 40 KDa as shown by SDS-PAGE (Figure 8). An enzymatic digestion using neuraminidase followed by O-glycanase gave a similar M_r; however, chemical digestions of the glycoprotein using hydrochloric acid, trifluoromethanesulfonic acid, sodium hydroxide, or the sequential periodic acid /sodium borohydride/mild hydrochloric acid hydrolysis of the Smith degradation yield two proteinaceous products that migrate at about 58 and 53 KDa.

One possible explanation for this apparent ambiguity is that two large glycoproteins exist in the complex or copurify with it. This idea is not

likely; the presence of a second glycoprotein would have been indicated by SDS-PAGE of the hydrochloric acid product (Figure 10) which shows only the doublet of bands in the 55 KDa range, and no bands at 40 KDa.

Another, more feasible possibility is that the 40 KDa protein is a product of proteolysis of the 58 and 53 KDa proteins by a protease. The presence of small bands at 58 and 53 KDa as well as the major band at 40 KDa in O-glycanase digests is evidence for this. A similar protease may be common to both the α-amylase preparation and either the neuraminidase or the O-glycanase preparation used. Since extensive digest times were used during both the α-amylase (48 hr) and the O-glycanase (24 hr) digestions, effects would appear from the presence of even very small amounts of protease. The presence of a protease contaminant is indicated in the neuraminidase preparation (technical bulletin supplied with the enzyme). More evidence for the 40 KDa protein being a proteolytic fragment is shown in Figure 9. In lane D, enzymatic activities were not terminated at the end of incubation times, as they were in lane E. If proteases were present in the neuraminidase preparation, they would have been allowed to act much longer in lane D than in lane E. The appearance of bands in the 70 KDa range may represent unreacted substrate of the protease and would indicate the true apoprotein M_r. The observation of a 34 KDa protein appearing after only two hr of α-amylase treatment by an earlier investigator (38) may also be caused by contamination by this protease of the α-amylase preparation used.

These data indicate a different linkage than what has been found for the mammalian glycogen primer. Early work by Krisman *et al.* showed the TCA-precipitable glycogen primer to be acid-labile, but stable to base treatment (20), indicating the carbohydrate was bound by some linkage other than the serine/threonine O-glycosidic bond. Rodriguez and Whelan (27) employed chemical modification of tyrosine residues in the primer protein (designated "glycogenin") isolated from rabbit muscle as evidence of the existence of a novel tyrosine-carbohydrate linkage. Isolation of a small glycosylated proteolytic peptide after incubation with UDP[^{14}C]glucose, and identification of the radiolabelled residue as tyrosine in the laboratory of Cohen confirmed these data (28). The difference in the two carbohydrate primers is not unexpected, however. The two tissues studied are widely divergent, as discussed in "Introduction". In other studies a protein found by Juana Tandecarz and coworkers to serve as a primer for starch synthesis in potato tuber (71,72) has been shown to contain carbohydrate bound to a more typical serine or threonine residue, similar to what the data suggest for the *A. suum* GS-2 complex. An interesting observation was made by Rodriguez and Whelan (27) that the genetic codons for tyrosine and serine differ by only a single base. This is not altogether true; of the six codons coding for serine (UCU, UCC, UCA, UCG, AGU, and AGC), only two differ from the tyrosine codons (UAU, UAC) in a single base. However, two of the serine codons not similar to those of tyrosine (UCA and UCG) are rarely used in yeast (73). The chance that one of the two similar codons (UCU or UCC) were used for serine in this protein and underwent a point mutation to one

of the tyrosine codons early in the divergence of chordates is finite and conceptually could have represented a viable mutation.

These data indicating the presence of other sugars besides glucose in the primer glycoprotein are also in disagreement with data collected on proteinaceous glycogen primers from other sources. Fast Atom Bombardment Mass Spectroscopy (FABMS) of a small peptide from rabbit muscle glycogenin corresponding to the glycosylation site observed in Cohen's laboratory (28) showed it to be a mixture of two peptides, containing either one or two unsubstituted hexose units. Methylation data showed these to be glucose. A starch protein primer found in potato tuber has also been found to contain only glucose (71,72). These are based on paper chromatography of Smith degradation products. If the ultimate saccharide were derivatized, it would be Smith degradation-resistant, and would not have appeared on the paper chromatogram. The presence of N-acetylglucosamine was suggested by the work of Whelan *et al.* (23) but was attributed to contaminating proteins.

Glycogen is an important energy source for *Ascaris suum*. Glucose from glycogen represents the only energy available to the worm during nonfeeding periods of the host. The GS-2 complex described in this work may represent the evolution of a second pathway of synthesis of this all-important polymer, in which case this complex may only occur in parasitic helminths. The complex displays similarities to GS-2 isolated from rat muscle. GS-2 occurs in rat muscle as a complex chromatographing in the flow-through fraction of DE52 cellulose and displays a dependence on the

presence of G6-P for activity (22). These two complexes display distinct differences, however. The GS-2 complex found in rat contains no 140 KDa protein, and activity is increased (12X) in the presence of exogenous glycogen. The *Ascaris suum* complex is likewise dissimilar to the starch synthase complex found in potato tuber, since the starch synthetic complex dissociates during chromatography (72). The observation of a complex in *Echinococcus multilocularis* (74) similar to the one described here for *Ascaris suum* provides further evidence for the existence of two pathways of glycogen biosynthesis in parasitic helminths.

Another possible metabolic role for the GS-2 complex is in the maturation of glycogen. The GS-2 complex could build a glycogen particle *de novo* until it got to a defined size, at which time the nascent particle could be transferred to glycogenin to mature fully. Although the glycogenin protein found in rabbit muscle has an autoglucosidation activity, it can only synthesize a small oligomer of glucose (29). Such an oligomer may only be used with difficulty by glycogen synthase (rat muscle glycogen synthase $K_{m_{maltoheptalose}}$=70 mM (17)), but it might be used readily by a "glycogen transferase", which would transfer the nascent glycogen particle from a primer protein to the glucose oligomer on glycogenin. Moreover, a 40 KDa contaminant in preparations of GS-1 from *Ascaris suum* muscle (34) could possibly be glycogenin, since glycogenin is found as a contaminant in rabbit skeletal muscle GS-1 (24).

This scenario also creates two possibilities for the role of the 140 KDa subunit. One possibility is that the 140 KDa subunit acts as the "glycogen transferase", transferring the maturing glycogen particle to a primed glycogenin molecule. This activity is not much different conceptually from that of a branching enzyme, and such an enzyme could contain both branching and transferase activities. The second possibility is that the 140 KDa subunit acts as the "glycogen initiator synthase" which would transfer the first glucose molecule to the primer protein during glycogen initiation, as theorized by Krisman (21) and discussed by Cohen (29).

Energy generation in *Ascaris suum* is different in many ways from that of its mammalian host. These differences may be used as the basis of new chemotherapies which can be targeted to the parasite while doing no harm to the host. A more thorough understanding of the sugar metabolism of *Ascaris suum* will shed new light on these differences and will be paramount to the design of efficient and economical drugs against ascarid infections. Studies presented in this work have been performed in an attempt to understand a previously unknown and possibly unique complex involved in energy storage in this organism.

Table 1

Glycosyltransferase activity of fractions separated from the A. suum GS-2 complex by α-amylase digestion followed by Con A Sepharose chromatography.

Purified *A. suum* GS-2 complex (30 µg protein) was incubated for 15 min with 1 U α-amylase at 4^0 C. Material was loaded onto a 0.2 ml Con A Sepharose column, washed with 2 ml Buffer D, and material was eluted with 1 ml Buffer D + 0.1 M α-methylmannoside. Fractions were assayed for activity as described in Methods.

TABLE 1

Glucosyltransferase Activity of Fractions Separated by α-Amylase Digestion Followed by Con A Sepharose Chromatography

Eluant	Fraction	Glycogen synthase activity (pmoles glucose transferred/min) +glycogen	-glycogen
Buffer D	1	121	0
	2	121	0
	3	0	0
	4	105	2
	5	168	0
	6	2	0
Buffer D + 0.1 M	1	0	0
α-methylmannoside	2	864	0
	3	253	0
	4	0	0

Table 2

Digestion of GS-2 carbohydrate by α-amylase.

Purified GS-2 (4.7 mg carbohydrate, 23 µg protein) was incubated with 3 units α-amylase at 30^0 C. Amylase was added in two portions at 0 and 60 min. Aliquots were withdrawn from the incubation mixture at the designated time points and the reaction was stopped by heating in boiling water for 3 min. Samples were dialyzed and protein and carbohydrate were determined as described in Methods.

TABLE 2

Digestion of GS-2 Carbohydrate by α-Amylase

Time (min)	[Protein] (mg/ml)	[Carbohydrate] (mg/ml)	Ratio (carbohyd/prot)
0	0.017	3.50	206
30	0.019	0.44	23
60	0.037	0.26	7
90	0.025	0.23	9
120	0.037	0.11	3

Figure 1. Separation of the 66 KDa subunit from the GS-2 complex by mild amylase digestion. Purified GS-2 complex (22 µg protein) was incubated with 0.5 U of α-amylase for 15 min (lanes B-D) or 30 min (lanes E-G). Amylase-digested samples were applied to a Con A Sepharose column (0.5 ml), washed with Buffer D, and eluted with Buffer D + 0.1 M α-methylmannoside. Fractions obtained were analyzed using SDS-PAGE and stained using Coomassie blue. Lane A: undigested material. Lanes B and E: Flow through during loading procedure. Lanes C and F: elution using Buffer D as eluant. Lanes D and G: elutions using Buffer D + 0.1 M α-methylmannoside as eluant. The stacking gel/running gel interface is labelled "S".

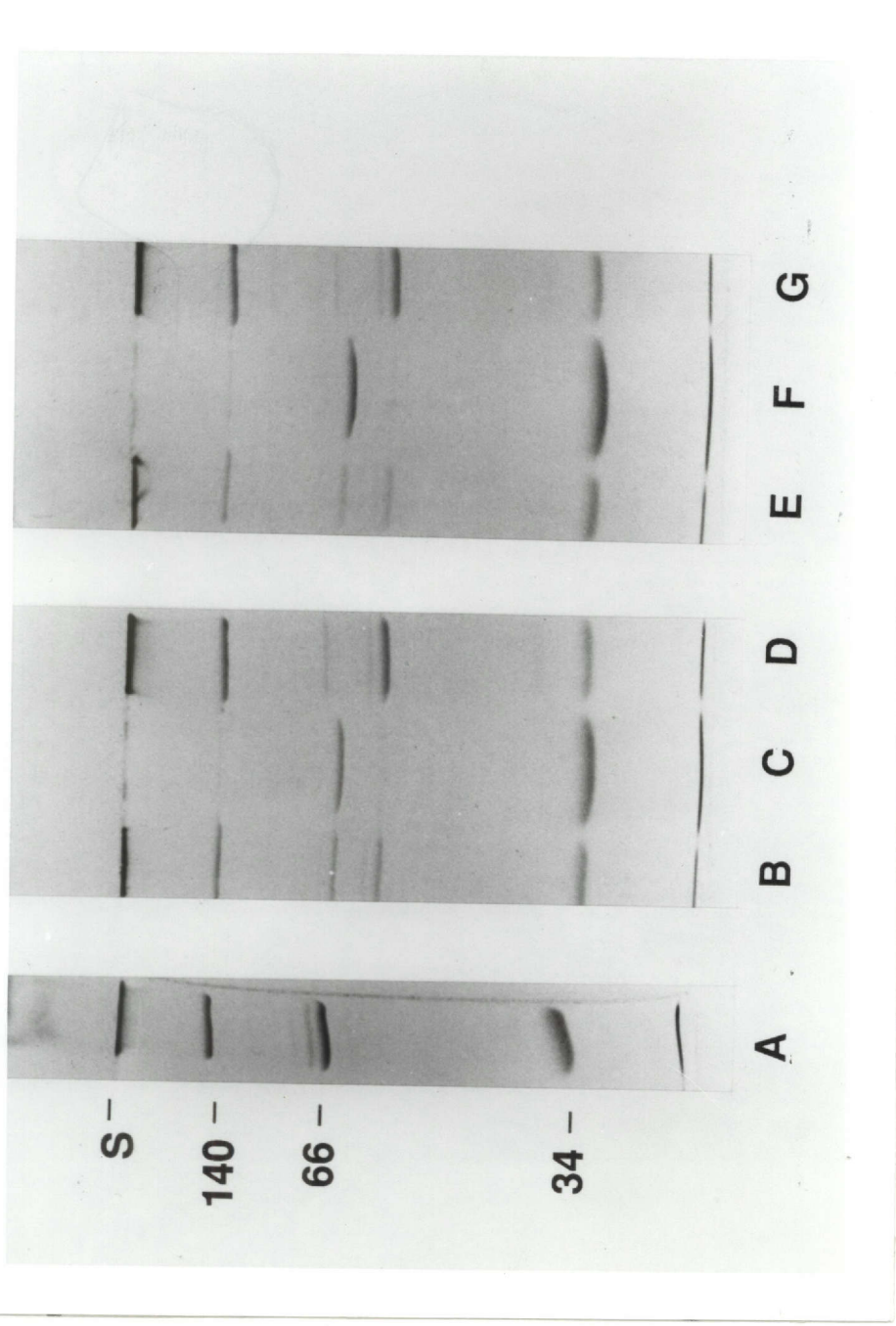

S –
140 –
66 –
34 –
A
B
C
D
E
F
G

Figure 2. Proteolytic digestion of GS-2. Purified GS-2 (12 µg protein) was digested with 2.4 µg trypsin for 0 min (lane A), 20 min (lane B), 40 min (lane C), and 60 min (lane D) and the reaction products were analyzed by SDS-PAGE and Coomassie Blue staining. In lane E purified GS-2 (12 µg protein) was digested with Pronase (0.6 U) for 20 min prior to SDS-PAGE analysis. S denotes the stacking gel/running interface.

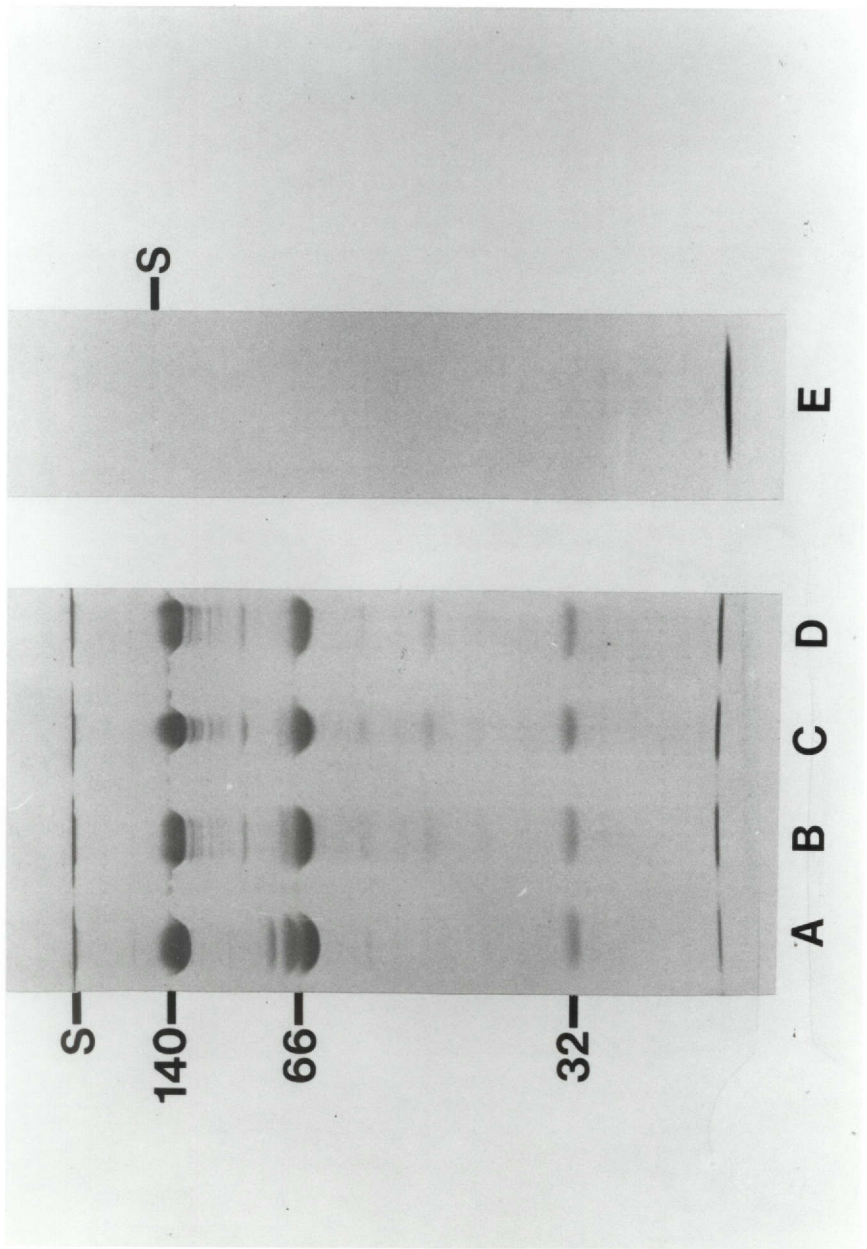

S
140
66
32
A
B
C
D
S
E

Figure 3. Western Blot of GS-1 and GS-2. Prestained molecular weight standards (lane A), 40 µl (12 µg protein) *A. suum* GS-2 complex (lane B) and *A. suum* GS-1 (40 µl in both lanes C and D) were subjected to SDS-PAGE and transblotted onto nitrocellulose. Lanes A, B, and C were cut away from lane D and probed with antibody generated against the GS-2 complex. Lane D was stained with amido black stain for visualization of proteins.

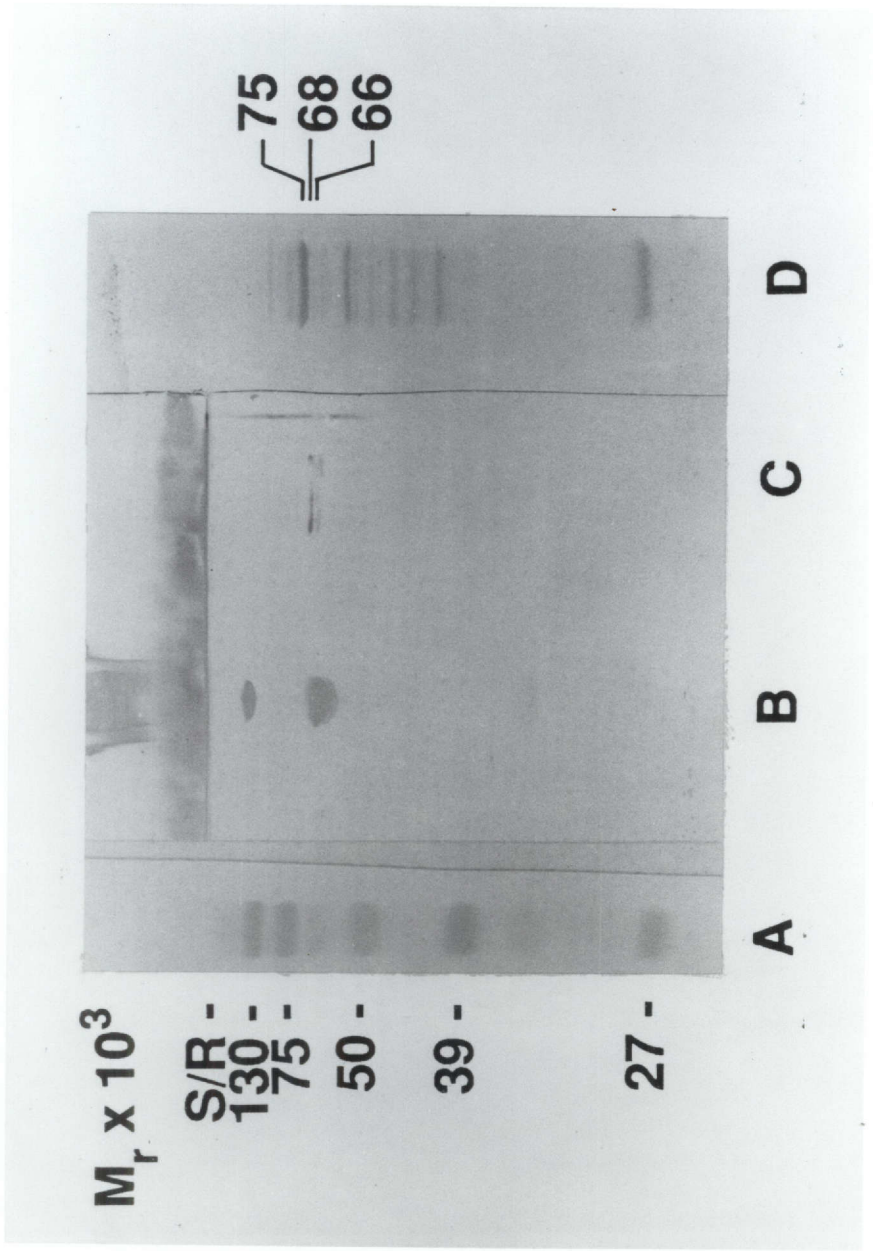

75
68
66
M_r x 10^3
S/R -
130 -
75 -
50 -
39 -
27 -
A
B
C
D

Figure 4. Incorporation of radiolabel from UDP[^{14}C]glucose or [^{14}C]glucose 6-P into ethanol precipitable product. Designated amounts of purified glycogen primer complex were incubated with 6.7 mM UDP[^{14}C]glucose, 10 mM G6-P, and 1% glycogen (open squares); 6.7mM UDP[^{14}C]glucose, and 10 mM G6-P (closed diamonds); 6.7 mM UDP-glucose, 10 mM [^{14}C]G6-P and 1% glycogen (closed squares); or 6.7 mM UDP-glucose and 10 mM [^{14}C]G6-P (open diamonds) for 10 min at 30^{0} C, and reactions were stopped by pipetting 50 µl of the reaction mixture onto paper squares and immersing into water:ethanol (34:66 v/v), as stated in Methods. The specific activity of radiolabelled compounds was 19.6 µCi/mmol.

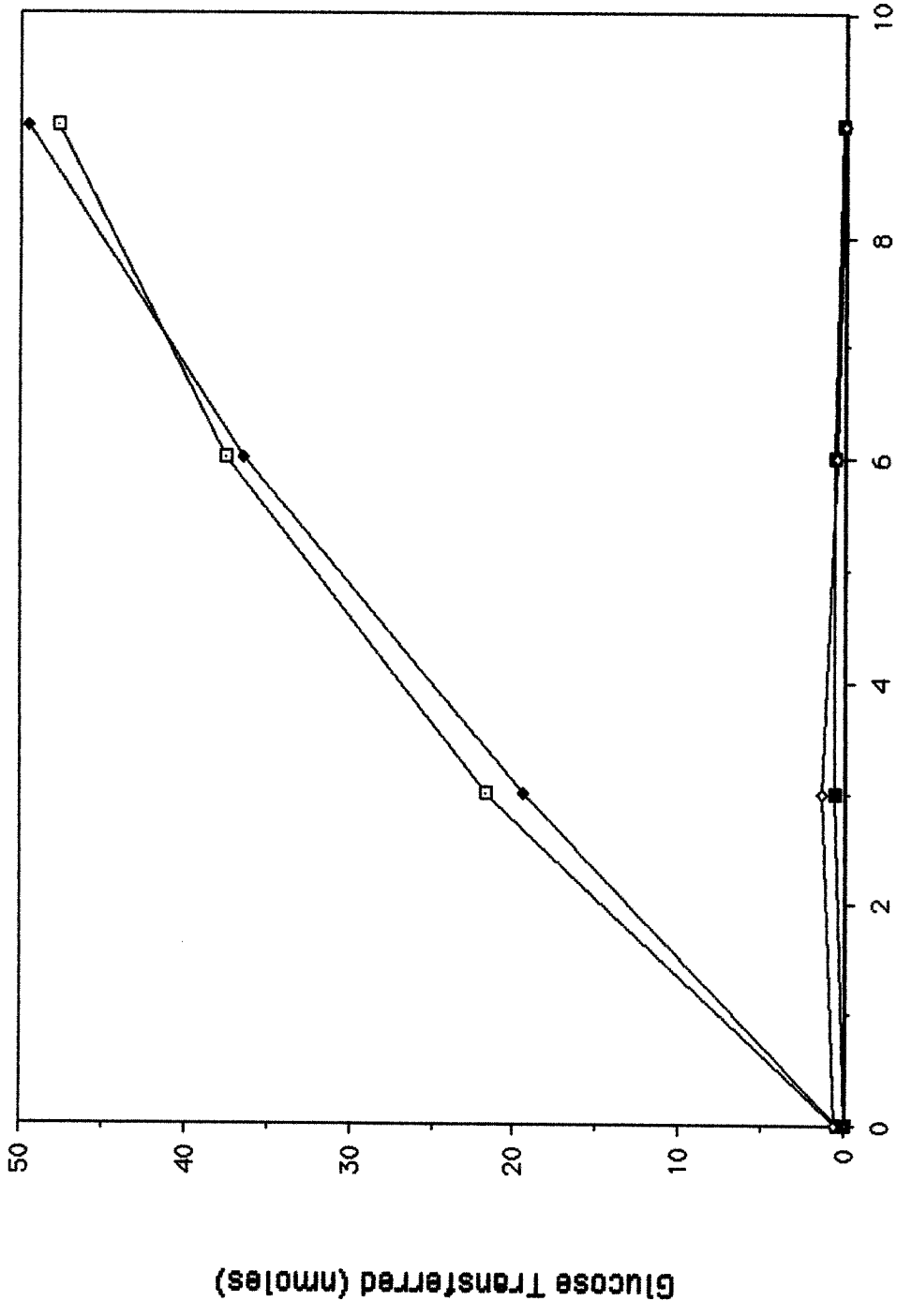

Glucose Transferred (nmoles)
0
10
20
30
40
50
0
2
4
6
8
10
[Enzyme] (µg)

Figure 5. Alkaline phosphatase treatment of the GS-2 complex. Purified GS-2 complex (30 µg protein) was incubated with 28.5 U alkaline phosphatase in 200 µl 0.1 M Tris-Cl pH 8.0, at 30^0 C for 30 min. Aliquots (50 µl) were removed after 15 min (lane C) and 30 min (lane D), electrophoresed on SDS-PAGE, and stained using Coomassie blue. Lane B contains untreated GS-2 complex. Lane A contains low molecular weight standards.

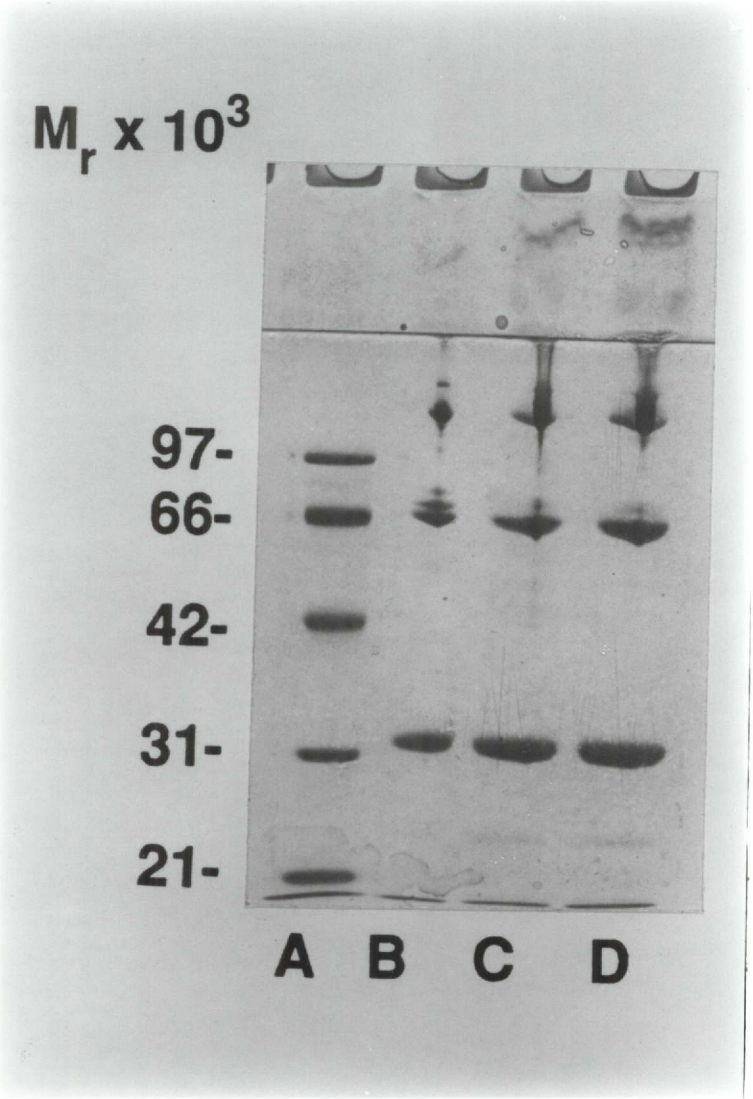

M_r x 10^3
97-
66-
42-
31-
21-
A
B
C
D

Figure 6. Incorporation of radiolabel from UDP[^{14}C]glucose into ethanol or TCA precipitable product. Purified glycogen primer complex (3 µg protein) was incubated with 10 mM G6-P and 6.7 mM UDP[^{14}C]Glucose (Sp.Act.=19.6 µCi/mmole) at 30^{0} C for the indicated times, and reactions were stopped by pipetting 50 µl of the reaction mixture onto paper squares and immersing into either water:ethanol (34:66 v/v) (open squares) or cold 10% TCA in water (closed diamonds) as stated in Methods.

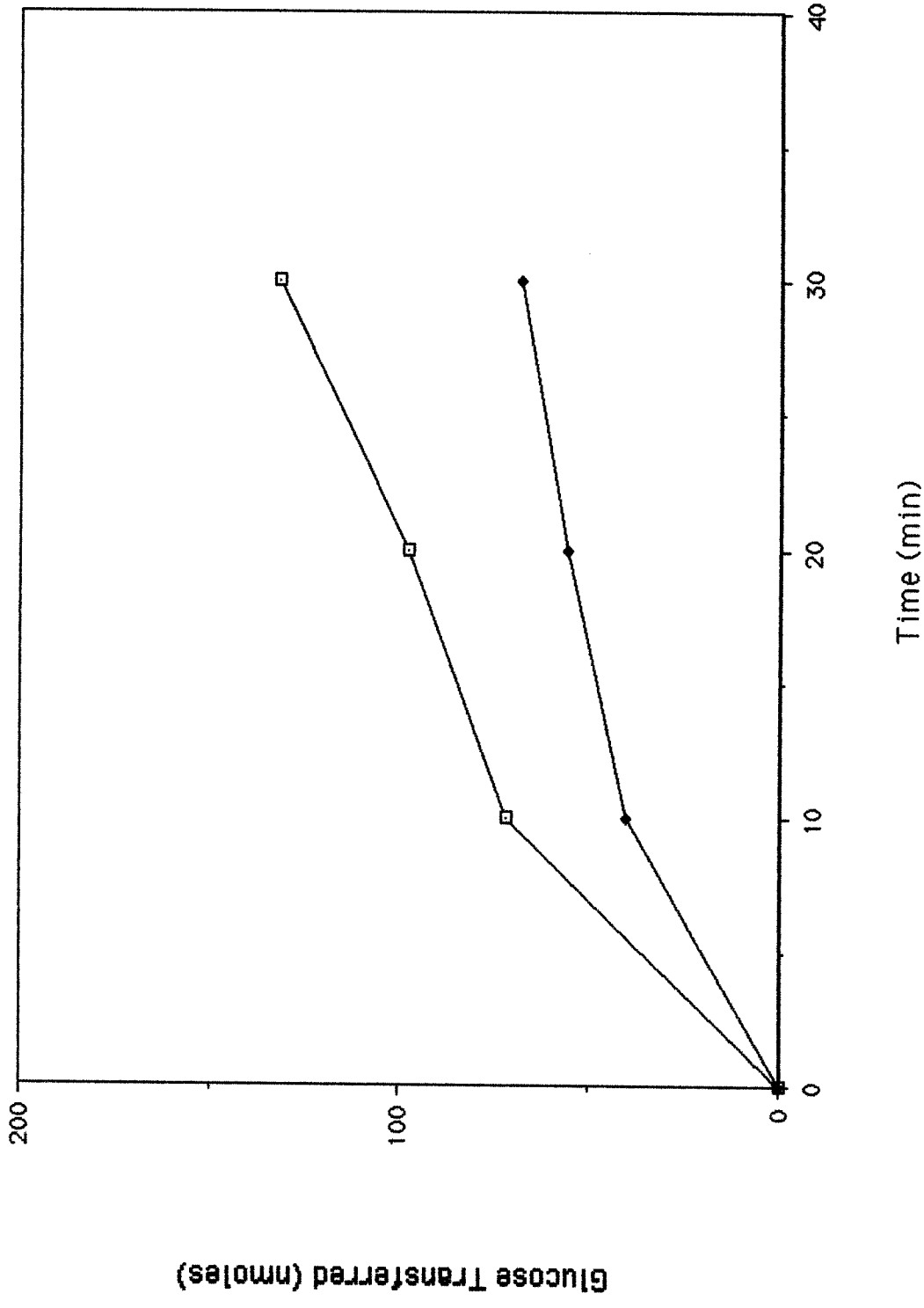

Glucose Transferred (nmoles)
200
100
0
0
10
20
30
40
Time (min)

Figure 7. Electrophoresis of GS-2 after amylase digestion using Coomassie and PAS stains. Purified GS-2 complex (9 µg protein) was incubated with 3 U human salivary α-amylase for 90 min and was subsequently chromatographed using Con A Sepharose. Lanes A and B contain untreated purified GS-2 complex. Lanes C and D contain amylase digested material which bound to Con A Sepharose. Lanes A and C were stained using Coomassie Blue, lanes B and D were stained with PAS stain.

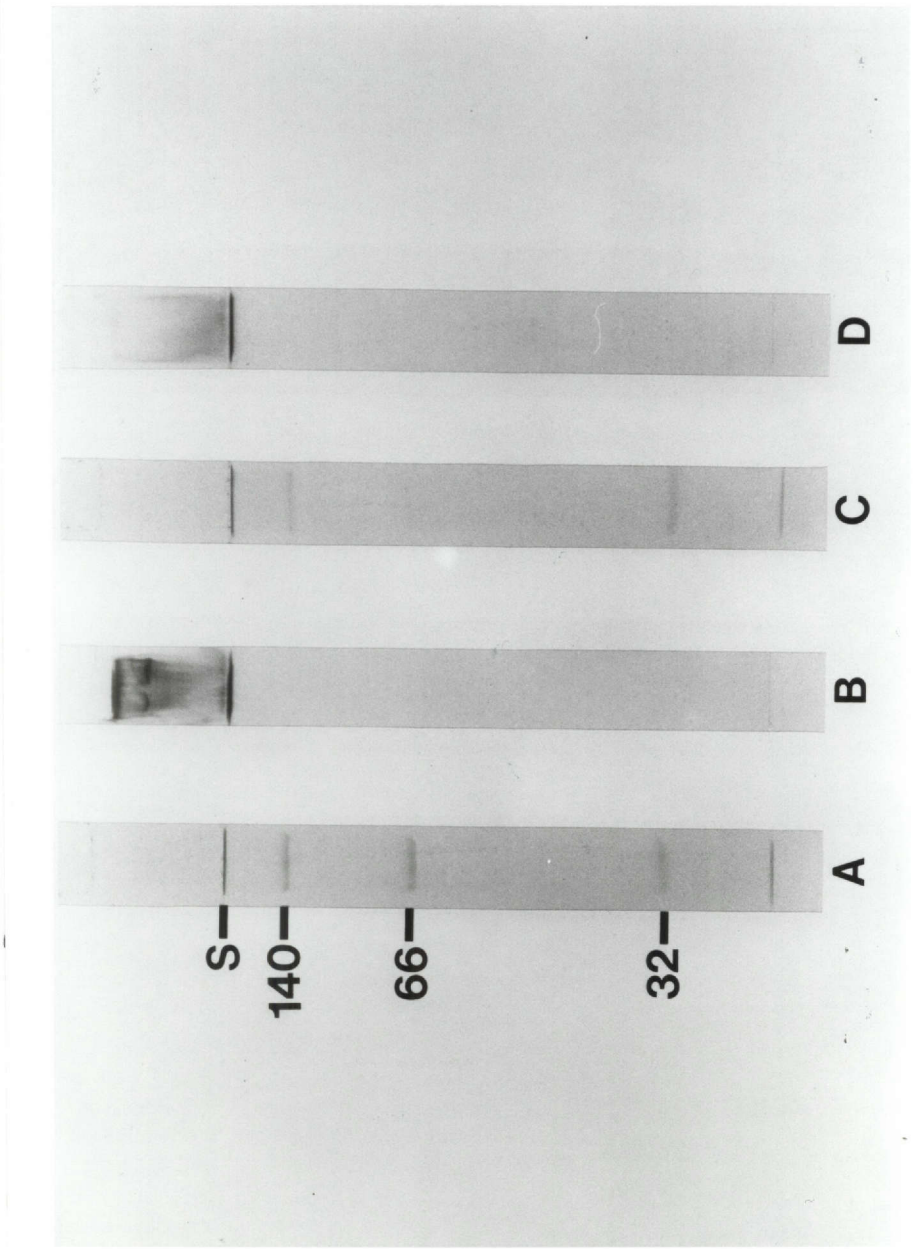

S
140
66
32
A
B
C
D

Figure 8. Glycosidic digestion of the GS-2 glycoprotein subunit. Purified glycoprotein subunit of the GS-2 complex (15 µg protein) was incubated with 9 U *B. subtilis* α-amylase and 140 mU *A. niger* amyloglucosidase for 48 hr as described in Methods (lane C). Purified glycoprotein subunit (15 µg protein) was digested with neuraminidase for 1 hr, followed by incubation with N-acetylglucosaminidase for 24 hr, and finally digested with O-glycanase for 24 hr. Half of this sample was used for SDS-PAGE analysis (lane D). Purified glycoprotein subunit (15 µg protein) was digested with neuraminidase for 1 hr, followed by a 24 hr digest with O-glycanase. Half of this sample was used for SDS-PAGE analysis (lane E). Prestained molecular weight standards appear in lane A, and undigested complex appears in lane B. After SDS-PAGE, proteins were Western Blotted using antibodies generated against the GS-2 complex.

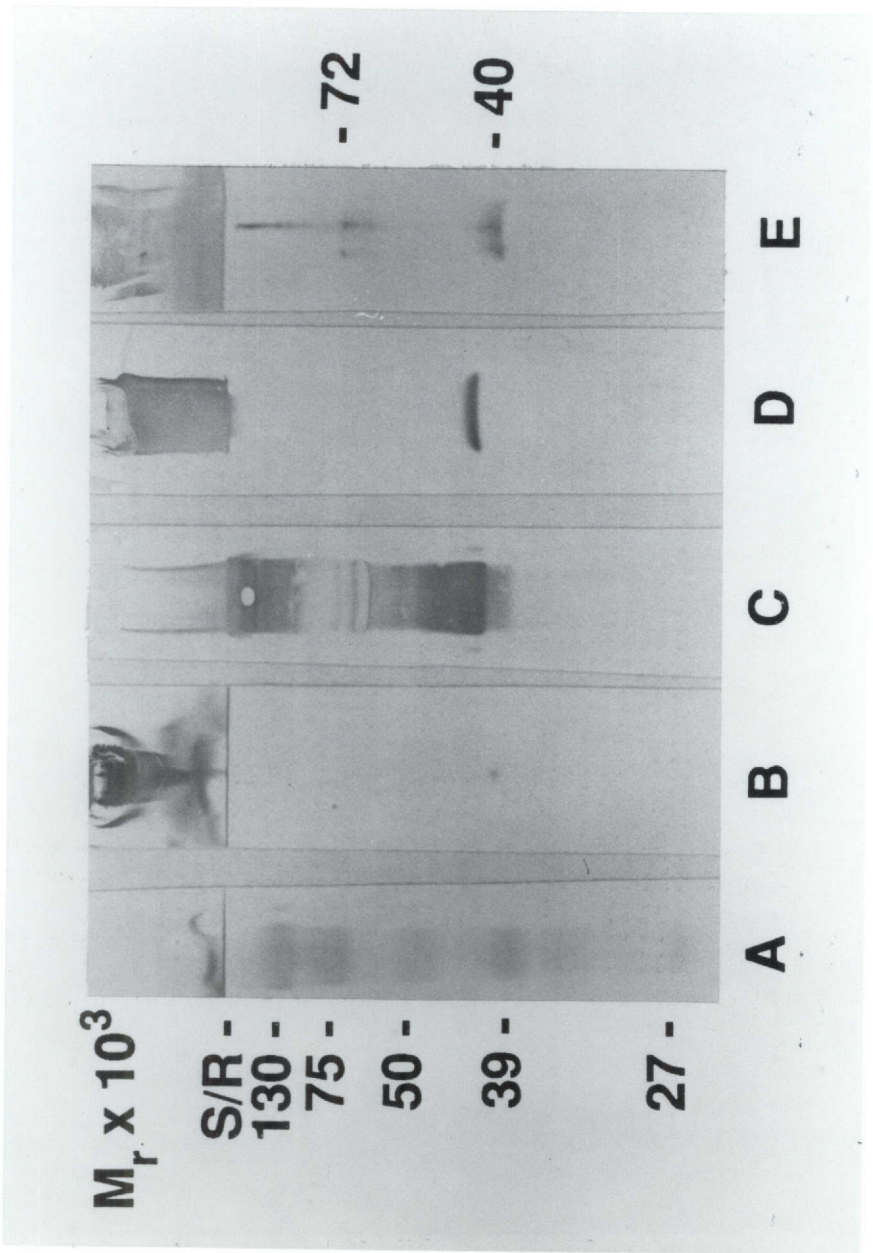

M_r x 10^3
S/R -
130 -
75 -
50 -
39 -
27 -
- 72
- 40
A
B
C
D
E

Figure 9. Temperature course of a HCl acid hydrolysis of the glycoprotein subunit of GS-2. Glycoprotein subunit (~18 µg protein) was isolated by Con A chromatography in the presence of SDS from 600 µl (180 µg protein) purified GS-2 complex. The subunit was dialyzed against 30 ml water for 4 hr, divided into 6 equal aliquots, and lyophilized. One aliquot was electrophoresed without further treatment (lane A). The other 5 aliquots were then dissolved in warm (45^0-55^0 C) 1 N HCl, transferred to Schlenk tubes, and heated under vacuum for 1 hr at 60^0 (lane C), 70^0 (lane D), 80^0 (lane E), 90^0 (lane F), or 100^0 C (lane G). Products were cooled and neutralized with 2 volumes 1 N ammonium bicarbonate and dialyzed against 30 ml water for 2 hr, followed by SDS-PAGE and silver staining. Lane H contains low molecular weight standards.

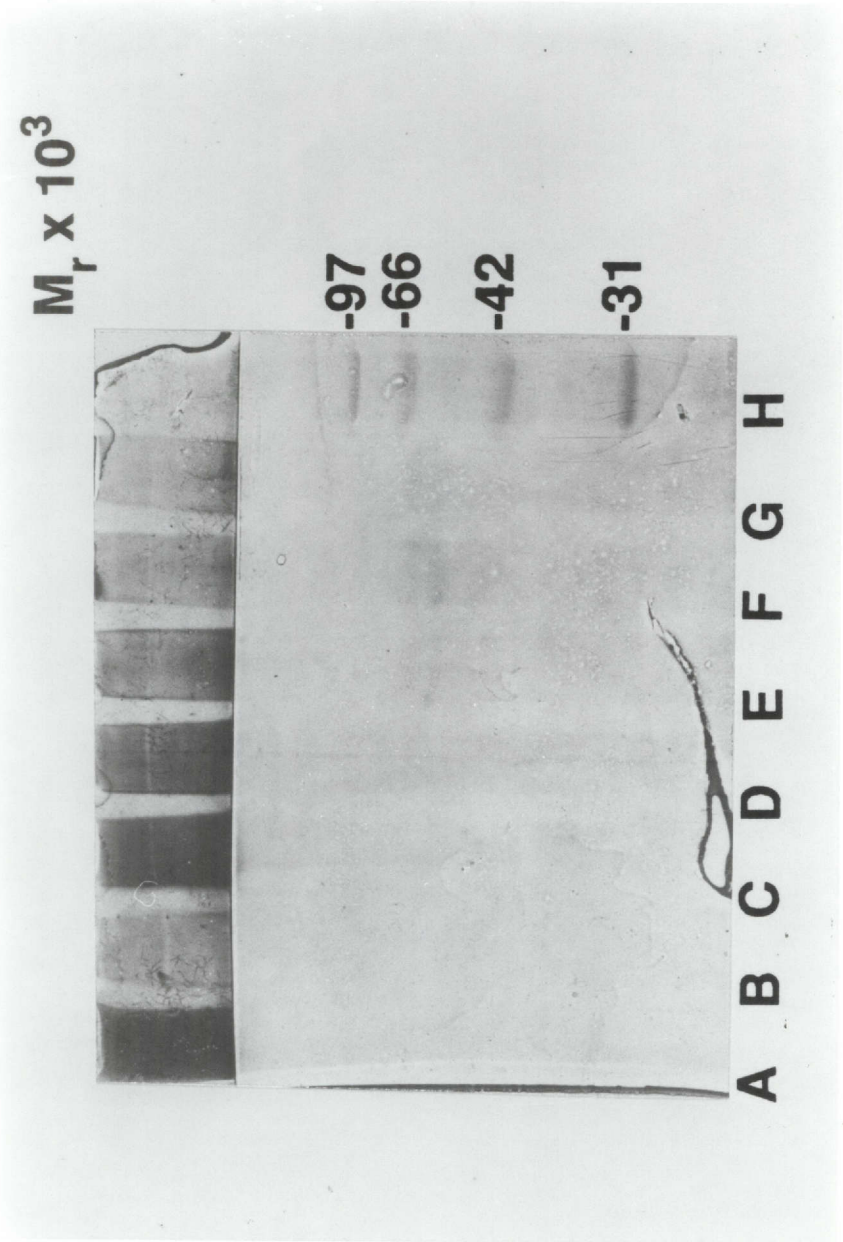

M_r x 10^3
-97
-66
-42
-31
A B C D E F G H

Figure 10. Western Blot of a HCl acid hydrolysis product of the GS-2 glycoprotein subunit. Glycoprotein subunit (~3 µg) was isolated from 100 µl (30 µg) purified GS-2 complex by Con A Sepharose chromatography in the presence of SDS. The sample was dialyzed for 4 hr against 30 ml water and lyophilized. The dry sample was dissolved in water (0.1 ml) and 6 N HCl was added to a final concentration of 1 N. The mixture was transferred to a Schlenk tube and heated under vacuum for 1 hr at 85^{0} C. After heating, the mixture was cooled and neutralized by the addition of 2 volumes of 1 M ammonium bicarbonate. Low molecular weight standards (lane A), 25 µl of purified glycoprotein subunit (~1 µg) (lane B), and the reaction product (lane C) were subjected to SDS-PAGE and Western blotted using antibody generated against the GS-2 complex. Material at the dyefront is labelled "D"; the stacking gel/resolving gel interface is labelled "S/R".

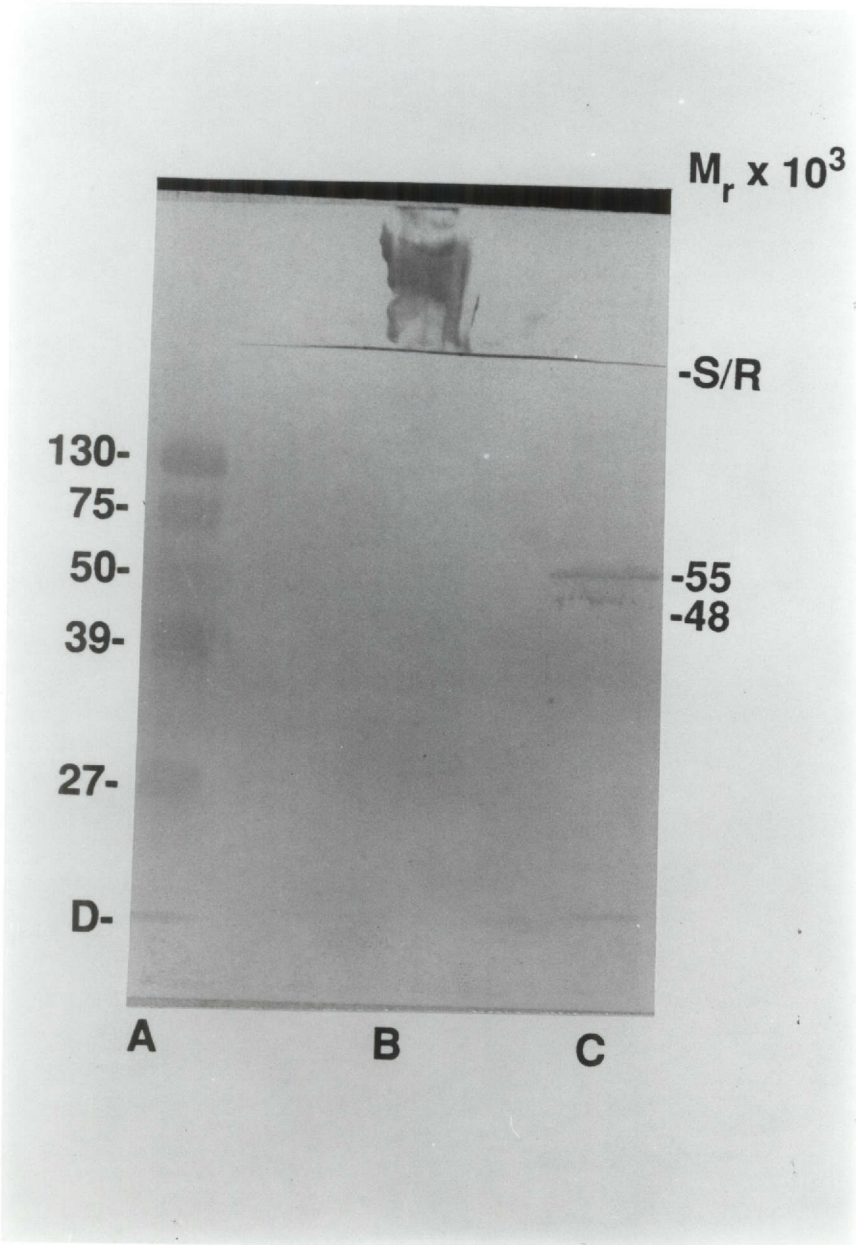

M_r x 10^3
-S/R
130-
75-
50-
39-
27-
D-
-55
-48
A
B
C

Figure 11. Amylase digestion of the GS-2 complex for WGA binding study. GS-2 complex (12 µg protein; lane A), purified glycoprotein subunit (15 µg protein) digested for 48 hr with *Bacillus subtilis* α-amylase (lane B), and 12.6 U *B. subtilis* α-amylase (lane C) were subjected to SDS-PAGE, stained with Coomassie Blue, and transferred to a nitrocellulose sheet. The stacking gel/resolving gel interface is labelled "S/R"

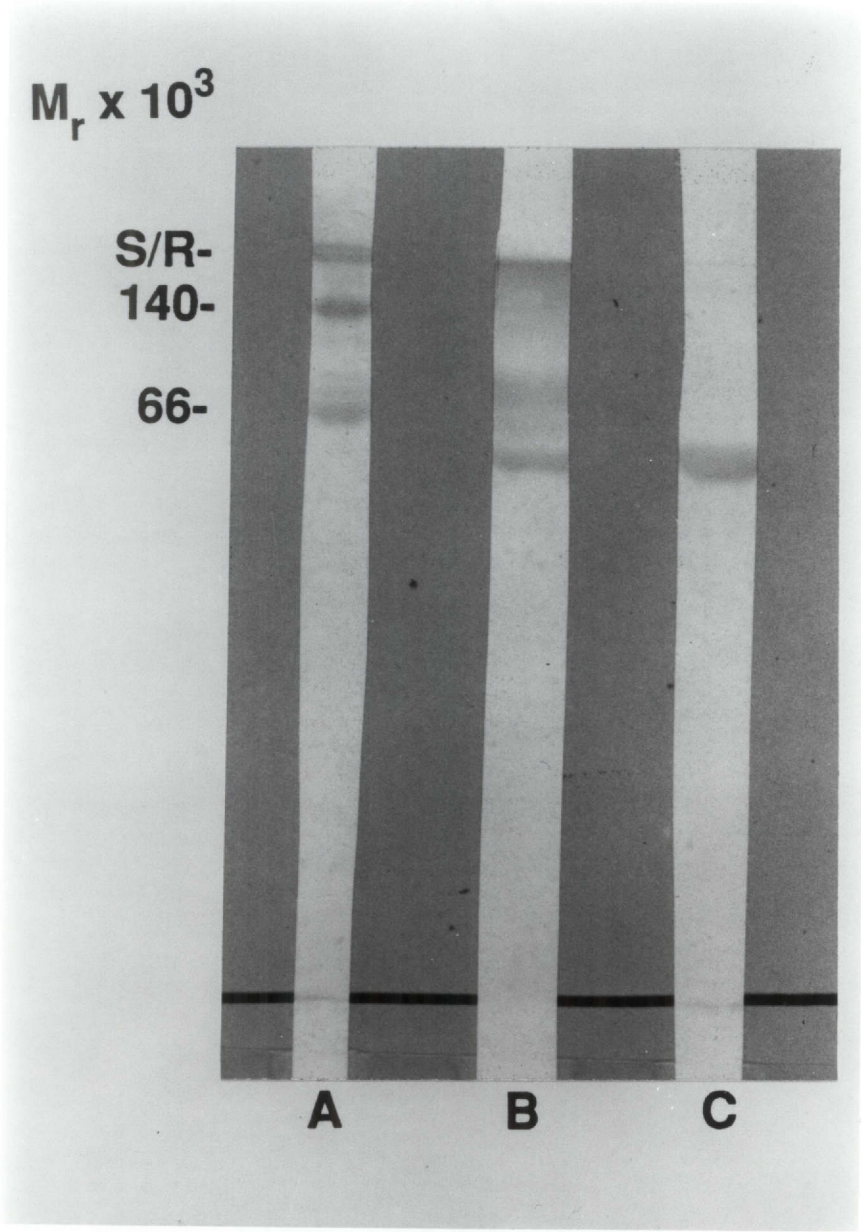

M_r x 10^3
S/R-
140-
66-
A
B
C

Figure 12. Binding of [^{125}I]Wheat Germ Agglutinin to the GS-2 complex. GS-2 complex (12 µg protein; lane A), purified glycoprotein subunit (15 µg protein) digested for 48 hr with *B. Subtilis* α-amylase (lane B), and 12.6 U *B. subtilis* α-amylase (lane C) were subjected to SDS-PAGE, stained with Coomassie Blue, and transferred to a nitrocellulose sheet. Strips were cut out corresponding to individual lanes and each was incubated in 2 ml TBS containing 0.1 % periodate-treated BSA for 1 hr, followed by an incubation in TBS containing 3.7 µg [^{125}I]WGA (10 µCi). Strips were washed with three 10 min changes of TBS, and dried. An autoradiograph (shown) was prepared using Kodak X-omat film. The exposure conditions were 45 min at -20^{0} C. The stacking gel/resolving gel interface is labelled "S/R".

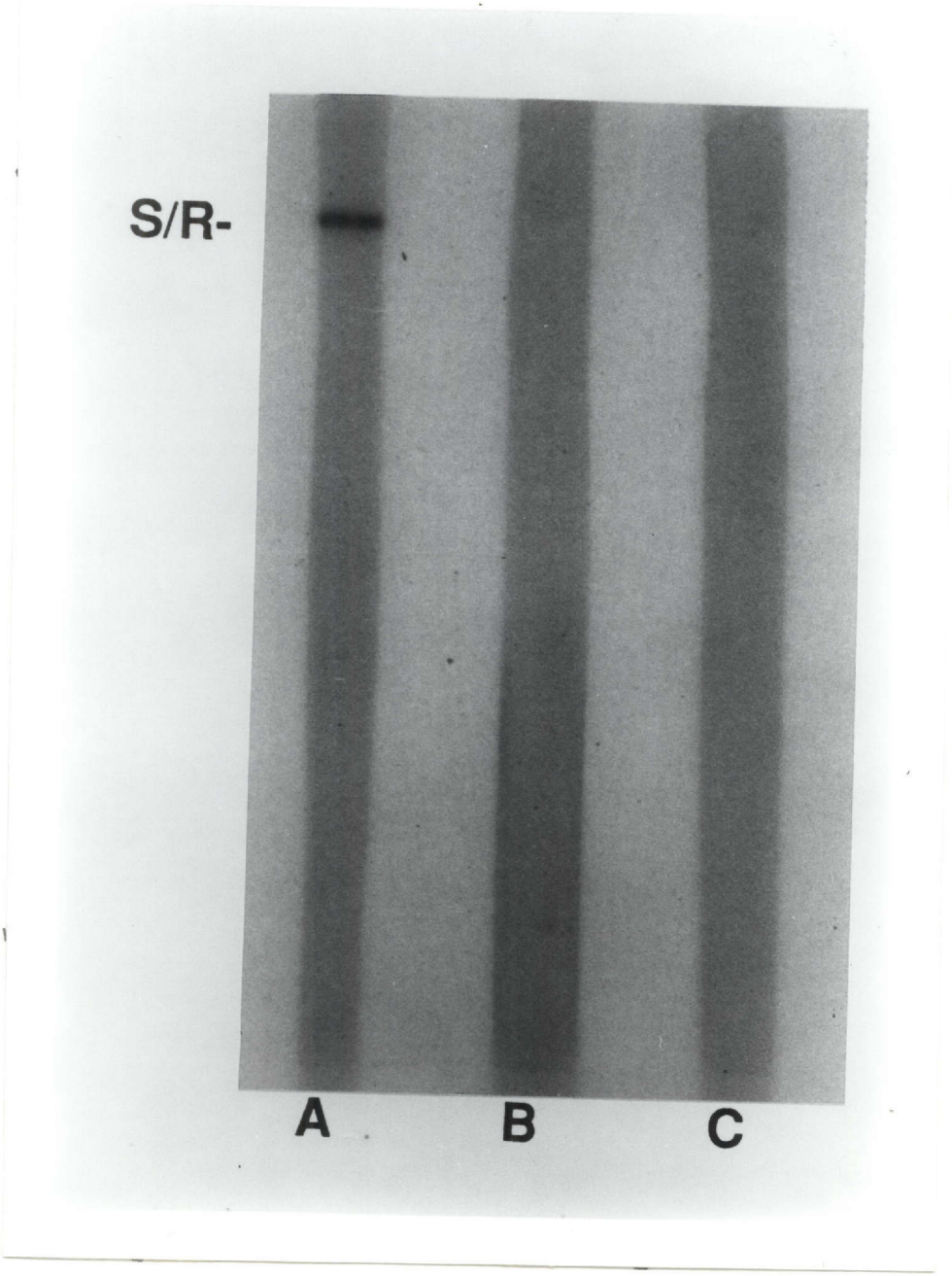

S/R-
A
B
C

Figure 13. WGA Sepharose chromatography of the glycoprotein subunit after Smith degradation. The glycoprotein subunit (~20 µg protein) was isolated from 210 µg GS-2 complex and subjected to Smith degradation as described in Methods. WGA Sepharose affinity chromatography was performed on the Smith product as indicated in Methods, and low molecular weight standards (lane A), the Buffer D wash fraction (lane B), the elution using Buffer D + 0.1 M N-acetyl-glucosamine (lane C), and the elution using Buffer D + 1.0 M NaCl (lane D) were analyzed by SDS-PAGE and silver stained.

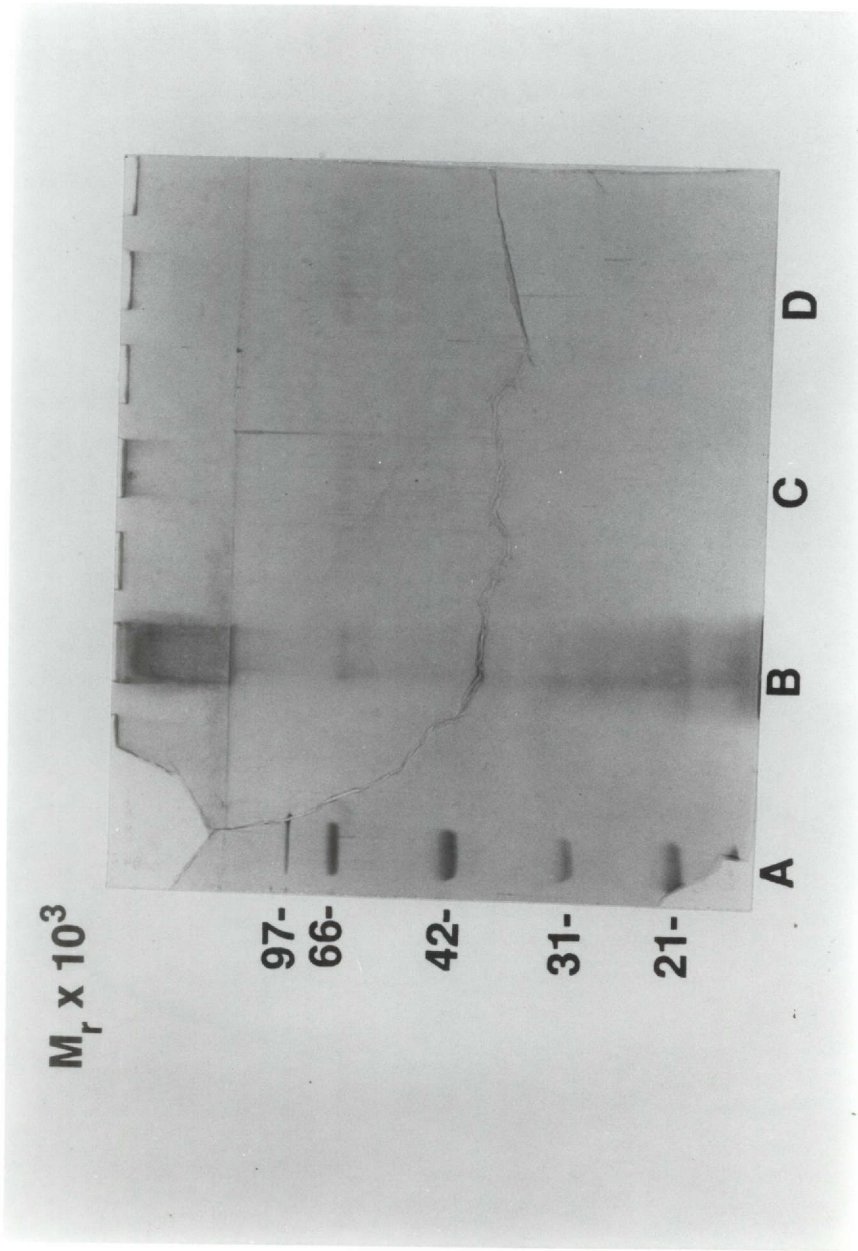

M_r x 10^3
97-
66-
42-
31-
21-
A
B
C
D

Figure 14. Analysis of sugars in an acid hydrolysate of the GS-2 complex by HPLC. Purified GS-2 complex (60 µg protein) was subjected to an α-amylase digest with 0.3 µg amylase as described under High Performance Liquid Chromatography in Methods. Product was dialyzed thoroughly and lyophilized. Dry sample was dissolved in 100 µl warm (45^0-55^0 C) 2 N HCl and heated 2 hr at 100^0 C. The hydrolysate was cooled and neutralized by the addition of 200 µl 2 N ammonium bicarbonate. Sample was analyzed for sugar content by injecting 10 µl of this sample into an HPLC instrument outfitted with an ion exchange column and a PAD system. The instrument had been equilibrated with 20 mM NaOH. Settings on the PAD are listed in Methods, and sensitivity was set at 1 KnA. Elution time is labelled on the abscissal axis, and peaks are labelled A through H.

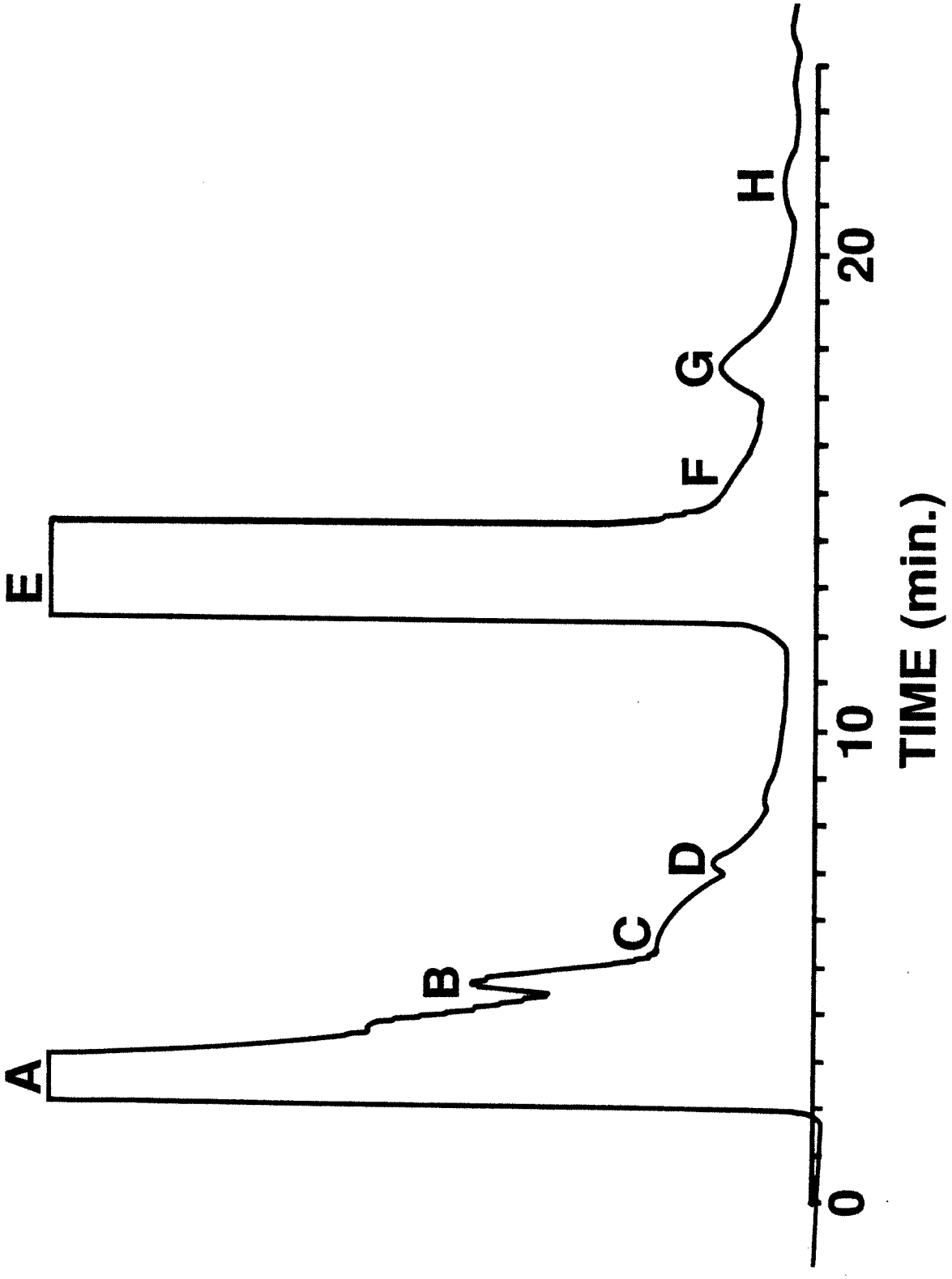
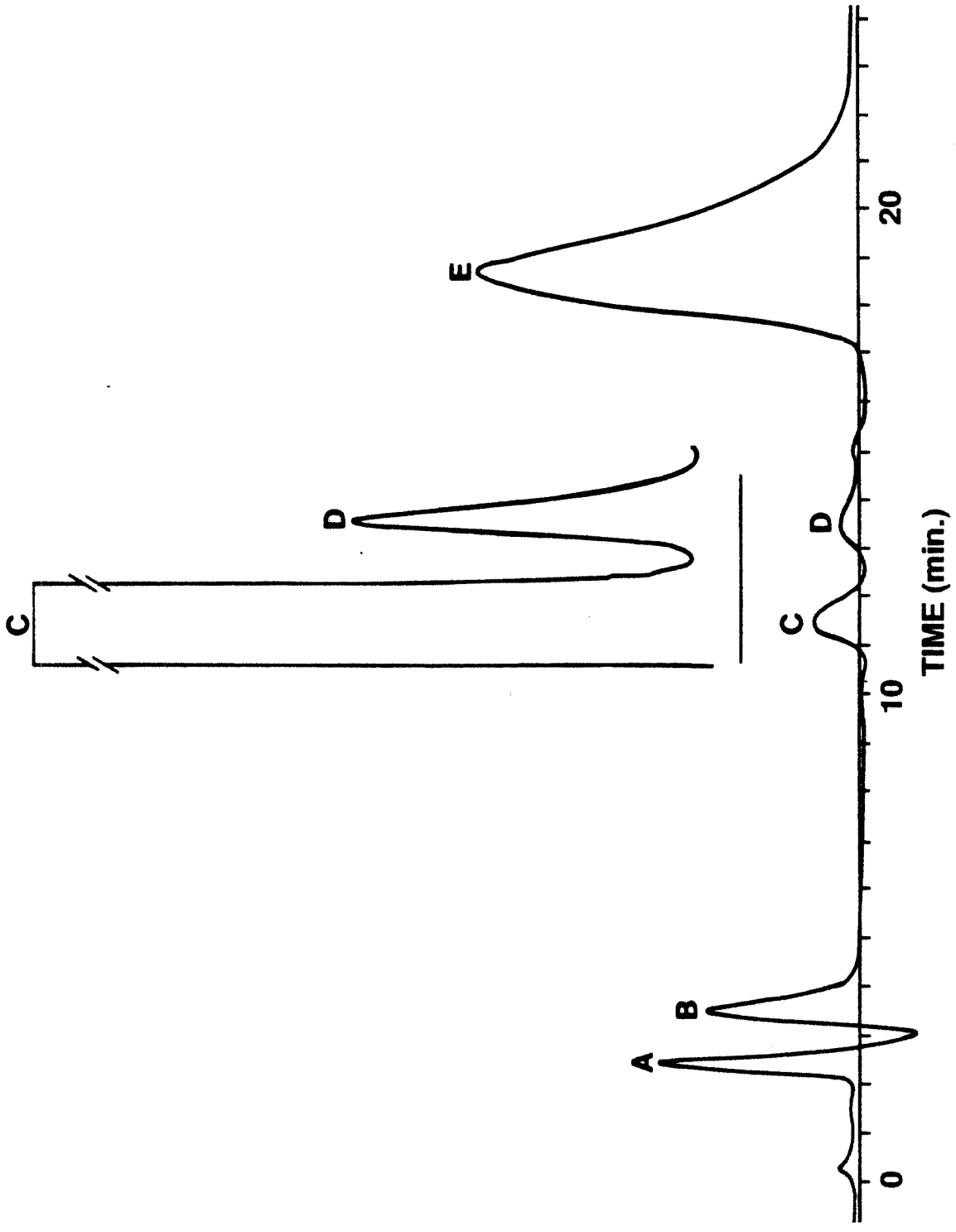

A
B
C
D
E
F
G
H
0
10
20
TIME (min.)

Figure 15. Analysis of sugars in an acid hydrolysate of the GS-2 complex by rechromatography of fractions obtained during HPLC. Purified GS-2 complex (60 µg protein) was subjected to an α-amylase digest with 0.3 µg amylase as described under High Performance Liquid Chromatography in Methods. Product was dialyzed thoroughly and lyophilized. Dry sample was dissolved in 100 µl warm (45^0-55^0 C) 2 N HCl and heated 2 hr at 100^0 C. The hydrolysate was cooled and neutralized by the addition of 200 µl 2 N ammonium bicarbonate. An aliquot (15 µl) of this solution was injected into an HPLC instrument outfitted with an anion exchange column equilibrated with 20 mM NaOH, and 250 µl fractions were obtained. A portion (100 µl) of a fraction corresponding to the end of the glucose peak (fraction 31) was rechromatographed using a PAD system. Settings on the PAD are listed in Methods, and sensitivity was set at 1 KnA. Elution time is labelled on the abscissal axis, and peaks are labelled A through E. A chromatogram was performed on 10 µl of the hydrolysate before treatment with 2.8 nmoles mannose added. A portion of this chromatogram is superimposed above the corresponding peaks on this figure.


A
B
C
D
E
C
D
0
10
20
TIME (min.)

BIBLIOGRAPHY

1. Barnes, R. D. (1980) *Invertebrate Zoology, Fourth Edition,* Saunders College, Philadelphia.
2. Levine, N. D. (1980) *Nematode Parasites of Domestic Animals and of Man, Second Edition,* Burgess Publishing Company, Minneapolis.
3. Chaudhuri, J., Martin, R. E. and Donahue, M. J. (1987) *Successful Farming* **February,** 18-T.
4. Rosenbluth, J. (1965) *Journal of Cell Biology* **26,** 579-591.
5. Saz, H. J. (1970) *Journal of Parasitology* **56,** 634-642.
6. Saz, H. J. (1972) in *Comparative Biochemistry of Parasites* (Van den Bossche, H. ed) pp. 33-47, Academic Press, New York.
7. Von Brand, T. (1973) *Biochemistry of Parasites, Second Edition,* Academic Press, New York and London.
8. Hers, H. G., DeWulf, H., and Stalmans, W. (1970) *FEBS Letters* **12,** 73-82.
9. Roach, P. J. (1981), in *Current Topics in Cellular Regulation, Vol. 20* (Horecker, B. L. and Stadtman, E. R., eds) pp. 45-105, Academic Press, New York.

10. Soderling, T. R., Hickenbottom, J. P., Reimann, E. M., Hunkeler, F. L., Walsh, D. A., and Krebs, E. G. (1970) *The Journal of Biological Chemistry* **245,** 6317-6328.

11. Hers, H. G. (1976) *Annual Review of Biochemistry, 45th Edition* (Snell, E. E., Boyer, P. D., Meister, A. and Richardson, C. C., eds) pp. 167-189. Annual Reviews, Incorporated, Palo Alto.

12. DeWulf, H., Stalmans, W. and Hers, H. G. (1968) *European Journal of Biochemistry* **6**, 545-551.

13. Camici, M., Ahmad, Z., DePaoli-Roach, A. A., and Roach, P. J. (1984) *The Journal of Biological Chemistry* **259,** 2466-2473.

14. DePaoli-Roach, A. A., Ahmad, Z., Camici, M., Lawrence, J. C. Jr., and Roach, P. J. (1983) *The Journal of Biological Chemistry* **258,** 10702-10709.

15. Craig, J. W., Rall, T. W., and Larner, J. (1969) *Biochemica et Biophysica Acta* **177,** 213-219.

16. Lawrence, J. C. Jr., Hiken, J. F., DePaoli-Roach, A. A. and Roach, P. J. (1983) *The Journal of Biological Chemistry* **258,** 10710-10719.

17. Goldemberg, S. H. (1962) *Biochimica et Biophysica Acta* **56,** 357-359.

18. Salsas, E. and Larner, J. (1975) *The Journal of Biological Chemistry* **250,** 1833-1837.

19. Parodi, A. J., Mordoh, J., Krisman, C. R., and Leloir, L. F. (1969) *Archives of Biochemistry and Biophysics* **132,** 111-117.

20. Krisman, C. R. (1972), *Biochemical and Biophysical Research Communications* **46,** 1206-1212.

21. Krisman, C. R. and Barengo, R. (1975) *European Journal of Biochemistry* **52,** 117-123.

22. Blumenfield, M. L. and Krisman, C. R. (1985) *The Journal of Biological Chemistry* **260,** 11560-11566.

23. Kennedy, L. D., Kirkman, B. R., Lomako, J., Rodriguez, I. R., and Whelan, W. J. (1985) in *Membranes and Muscle* (Berman, M. C., Gevers, W., and Opie, L. A., eds) pp. 65-84, ICSU Press/IRL Press, Oxford.

24. Pitcher, J., Smythe, C., Campbell, D. G., and Cohen, P. (1987) *European Journal of Biochemistry* **169,** 497-502.

25. Rodriguez, I. R. and Fliesler, S. J. (1988) *Archives of Biochemistry and Biophysics* **260,** 628-637.

26. Blumenfield, M. L. and Krisman, C. R. (1986) *European Journal of Biochemistry* **156,** 163-169.

27. Rodriguez, I. R., and Whelan, W. J. (1985) *Biochemical and Biophysical Research Communications* **132,** 829-836.

28. Smythe, C., Caudwell, F. B., Ferguson, M., and Cohen, P. (1988) *The EMBO Journal* **7,** 2681-2686.

29. Pitcher, J., Smythe, C., and Cohen, P. (1988) *European Journal of Biochemistry* **176,** 391-395.

30. Donahue, M. J., Michnoff, C. A., and Masaracchia, R. A. (1985) *Comparative Biochemistry and Physiology* **82B,** 395-403.

31. Donahue, M. J., Yacoub, N. J., Michnoff, C. A., Masaracchia, R. A., and Harris, B. G. (1981) *Biochemical and Biophysical Research Communications* **101,** 112-117.

32. Donahue, M. J., de la Houssaye, B. A., Harris, B. G., and Masaracchia, R. A. (1981) *Comparative Biochemistry and Physiology* **69B,** 693-699.

33. Yacoub, N. J., Allen, B. L., Payne, D. M., Masaracchia, R. A., and Harris, B. G. (1983) *Molecular and Biochemical Parasitology* **9,** 297-307.

34. Hannigan, L. L., Donahue, M. J., and Masaracchia, R. A., (1985) *The Journal of Biological Chemistry* **260,** 16099-16105.

35. Donahue, M. J., Yacoub, N. Y., and Harris, B. G. (1982), *The American Journal of Physiology* **242,** R514-521.

36. Donahue, M. J., Saunders, L., Magnino, P. E., and Masaracchia, R. A. (1984) *Federation of American Societies for Experimental Biology Proceedings* **43,** 1468.

37. Jett, M. F. and Soderling, T. R. (1979) *The Journal of Biological Chemistry* **254,** 6739-6745.

38. Ghosh, P. (1987) Dissertation for the Degree of Doctor of Philosophy in Biology, North Texas State University, Denton, Texas.

39. Ghosh, P., Heath, A. C., Donahue, M. J., and Masaracchia, R. A. (1989) *European Journal of Biochemistry* ***183,*** 679-685.

40. Donahue, M. J., Yacoub, N. J., Kaeini, M. R., Masaracchia, R. A., and Harris, B. G. (1981) *Journal of Parasitology* **67,** 505-510.

41. Thomas, J. A., Schlender, K. K., and Larner, J. (1968) *Analytical Biochemistry* **25,** 486-499.

42. Reimann, E., Walsh, D. A., and Krebs, E. G. (1971) *The Journal of Biological Chemistry* **246,** 1986-1995.

43. Laemmli, U. K. (1970) *Nature (London)* **227,** 680-685.

44. Zacharius, R. M., Zell, T. E., Morrison, J. H., and Woodlock, J. J. (1969) *Analytical Biochemistry* **30,** 148-152.

45. Glass, W. F. II, Briggs, R. C., and Hnilica, L. S. (1981), *Analytical Biochemistry* **115,** 219-224.

46. Warren, L. (1959) *The Journal of Biological Chemistry* **234,** 1971-1975.

47. Reissig, J. L., Strominger, J. L., and Leloir, L. F. (1955) *The Journal of Biological Chemistry* **217,** 959-966.

48. Bradford, M. M. (1976) *Analytical Biochemistry* **72,** 248-254.

49. Sojar, H. T. and Bahl, O. P. (1987) *Methods in Enzymology* **138,** 341-350.

50. Shriver, D. F. and Drezdzon, M. A. (1986) *The Manipulation of Air-Sensitive Compounds, Second Edition,* John Wiley and Sons, New York.

51. Prentice, N., Cuendet, L. S., Geddes, W. F., and Smith, F. (1958) *American Chemical Society Journal* **81,** 684-688.

52. Spiro, M. J., and Spiro, R. G. (1972) *Methods in Enzymology* **28,** 625.

53. Hardy, M. R., Townsend, R. R., and Lee, Y. C. (1988) *Analytical Biochemistry* **170,** 54-62.

54. Feist, C. F., Read, C. P., and Fisher, F. M. Jr. (1965) *The Journal of Parasitology* **51,** 76-78.

55. Marshall, R. D., and Neuberger, A. (1968) *Advances in Carbohydrate Chemistry and Biochemistry* **25,** 407-478.

56. Carlson, D. M., Iyer, R. N., and Mayo, J. (1970) in *Blood and Tissue Antigens* (David Aminoff, ed) pp.229-247, Academic Press, New York.

57. Lennarz, R. J. (1980) *The Biochemistry of Glycoproteins and Proteoglycans,* Plenum Press, New York.

58. Ivatt, R. J. (1984) *The Biology of Glycoproteins* , Plenum Press, New York.

59. Hughes, R. C. (1983) *Glycoproteins,* Chapman and Hall, New York.

60. Hart, G. W., Holt, G. D., and Haltiwanger, R. S. (1988) *Trends in Biochemical Sciences* October 1988, 380-384.

61. Goldstein, I. J., Hammarstrom, S., and Sundblad, G. (1975) *Biochemica et Biophysica Acta* **405,** 53-61.

62. Goldstein, I. J. and Hayes, C. E. (1978) in *Advances in Carbohydrate Chemistry and Biochemistry vol. 35* (R. S. Tipson and D. Horton, eds) Academic Press, New York.

63. Ebisu, S., Lonngren, J., and Goldstein, I. J. (1977) *Carbohydrate Research* **58,** 187-191.

64. Bhavanandan, V. P., Umemoto, J., Banks, J. R., and Davidson, E. A. (1977) *Biochemistry* **16,** 4426-4437.

65. Bhavanandan, V. P. and Katlic, A. W. (1979) *Journal of Biological Chemistry* **254,** 4000-4008.

66. Burger, M. M. and Goldberg, A. R. (1967) *National Academy of Science Proceedings* **57,** 359-366.

67. Spiro. R. G., (1966) *Methods in Enzymology* 8, 26-52.

68. Heidelberger, M., Aisenberg, A. C., and Hassid, W. Z. (1954) *Journal of Experimental Medicine* **99,** 343-353.

69. Torres, C.-R. and Hart, G. W. (1984) *The Journal of Biological Chemistry* **259,** 3308-3317.

70. Nyame, K., Cummings, R. D., and Damian, R. T. (1987) *The Journal of Biological Chemistry* **262,** 7990-7995.

71. Tandecarz, J. S. and Cardini, C. E. (1978) *Biochemica et Biophysica Acta* **543,** 423-429.

72. Moreno, S., Cardini, C. E., and Tandecarz, J. S. (1987) *European Journal of Biochemistry* **162,** 609-614.

73. Guthrie, C. and Abelson, J. (1982) in *The Molecular Biology of the Yeast Saccharomyces, Metabolism and Gene Expression* (Strathern, J. N., Jones, E. W., and Broach, J. R., eds) pp 487-528, Cold Spring Harbor Laboratory, New York.

74. Sarciron, M. E., Azzar, G., Got, R., and Petavy, A.-F. (1988) *Experimental Parasitology* **66,** 1-6.