PRESENCE OF GLYCOPROTEIN IN THE MEMBRANE OF *MICROCOCCUS LYSODEIKTICUS*

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CHAPTER I

INTRODUCTION

All proteins that contain covalently attached carbohydrate, regardless of the extent of glycosylation, are defined as glycoproteins. Even though Hammarsten (44) established the presence of a firm chemical linkage between the carbohydrate and protein moieties of a glycoprotein in 1888, it was not until almost seventy years later that these type-molecules received much attention. Numerous reports concerning glycoproteins have now been published, and the number of proteins discovered to be glycosylated has increased markedly (22, 46, 67, 95).

Because of the highly diverse nature of glycoproteins, it would be expected that these molecules carry out numerous biological functions. Specific examples are: Glycoproteins can function as enzymes [RNase A and B (78)] and hormones [chorionic gonadotrophin (35) and follicle stimulating hormone (34)]. They can also take part in antigen-antibody interactions [isolated glycoprotein from erythrocytes has A, B and MN blood group activities and also carries the receptor(s) for influenza viruses, phytohemagglutinin and wheat germ agglutinin (66)], in binding [corticosteroid-binding globulin (69)], transport [plasma glycoproteins such as transferrin (5)], and the clotting mechanism (20). In addition these molecules can act as lubricants [viscosity of the mucins equips them to serve as such (33)], and contribute to structural stability [possibly involved in maintaining the structural stability of collagen

brils above a certain diameter by cross-linking them (2)].

Recently the problem of cancer has been another area in which glyproteins appear to be involved (for recent review see ref. 26). Many ll properties are expressed at the surface of or mediated by the cytoasmic membrane, and among these are those which distinguish cancer lls from normal cells. It seems that particular carbohydrate expresons occur on tumor cells. It is not understood, however, whether an pression change is directly involved in, the result of or the cause r cancerous growth. Expression changes involving glycoproteins are assified essentially as two types. In one there is an increased glysylation restricted to certain surface glycoproteins; the other perins to exposure, rearrangement or concentration of the glycoprotein.

The phenomenon of increased glycosylation in cancer cells has en reported by several investigators. Buck, Glick and Warren (11,) found that an increased amount of a higher molecular weight fucosentaining glycoprotein is present on the surface of hamster cells tranormed by either polyoma, murine sarcoma or Rous sarcoma viruses. Lar Warren and co-workers (105) found that the higher molecular weight s due to the presence of increased levels of sialic acid in the transrmed mutant. Similar results have been obtained by Emmelot (26, 102). ey examined several established lines of malignant and non-malignant lls, and also found an increase in a surface fucose-containing glycootein.

Chiarugi and Urbano (21) also reported a finding which parallels e observations of Warren et al. and Emmelot. Transformation of BHK lls by polyoma virus was observed to cause increased glycosylation of particular glycoprotein released from the cell surface. In summary,

iarugi and Urbano stated that their data supports the idea that cell-
ar transformation involves alterations of cell membranes, and, partic-
arly, of the surface glycoproteins. Further examination of the data
these two investigators reveals that loss of some surface component
om a transformed cell could possibly expose (unmask from a cryptic
ate) other glycosylated components.

Studies involving surface components are usually carried out using
obes, such as wheat germ agglutinin (WGA) or concanavalin A (Con A).
ese probes agglutinate cells by binding to specific surface receptors
, 13, 15, 43, 51, 52). Overall, the results of these investigations
dicate that the surface membrane of cells contains binding sites that
n interact with certain agglutinins, and that a change in receptor
rypticity or number) occurs upon transformation.

Possible changes which receptor sites are thought to undergo on
e surface of tumor cells have been described by Inbar, Ben-Bassat and
chs (50).

a) Exposure of sites. Unmasking more of the same binding site by loss of protein cover from the tumor cell surface (loss of crypticity).

b) Concentration. Normal amount of binding sites on a tumor cell surface due to the smaller size of the cell.

c) Rearrangement. Although unchanged in amount, the sites are clustered; this promotes agglutination.

Support for the view that a change in agglutinin receptors is re-
ted to the phenotypically altered pattern of transformed cell growth
ransplantability and thus malignancy) is summarized in Table I. It
ould be pointed out that alterations in carbohydrate content may also
due to glycolipids (reviewed in ref. 26). Glycolipids of transformed
lls usually have a more simple carbohydrate composition than normal

TABLE I

CHANGES IN CARBOHYDRATE EXPRESSION
OF THE TUMOR CELL SURFACE

a) Transformation causes loss of cell contact inhibition, which correlates with an increase in cell transplantability (1, 80, 81) and agglutination by WGA (79).

b) Revertants (23, 47) and mutant-virus infected as well as transformed cells grown at non-permissive temperatures (4, 24) possess normal surface carbohydrate expressions.

c) After interaction with Con A, transformed cells exhibit contact inhibition (16).

d) Specific structural and metabolic changes on the surface membrane of transformed cells allow agglutination by Con A, and are associated with malignancy in vivo or transplantability (50).

e) A synthetic antigen, containing the presumed receptor site (N-acetyl-glucosamine) for WGA, elicits an immune response in mice able to crossreact with WGA receptor sites on myeloma tumor cells; mice thusly immunized are able to reject five times as many transplanted tumor cells as do control mice (91).

f) Agglutinin receptors on normal cells can be unmasked by trypsin treatment, and this change (or alteration) induces DNA synthesis and cell proliferation in confluent monolayers of cells (13, 14, 15).

lls and such changes are usually described as minimal alterations
04).

Overall, the above investigations concerning cancer cells have
tablished that tranformation involves alteration of the cell surface,
d can be recognized by several key events, one which is a change in
rface-related expressions of certain glycoproteins.

When my study was initiated, only one report indicated possible

esence of glycoproteins in bacteria. Okuda and Weinbaum (74) report- the presence of several glycoproteins in the envelope complex of cherichia coli B. They arrived at their conclusion after studying in- rporation of label from ^{14}C-D-glucosamine. It should be emphasized at it was not ascertained whether the glycoprotein components were of ll wall or membrane origin.

The work of Bosman (8) substantiates the conclusion of Okuda and inbaum although glycoproteins were not directly investigated. That , five glycoprotein glycosyltransferases were partially purified from iton X-100 solubilized preparations from E. coli B and characterized ing exogeneous glycoprotein acceptors. The enzymes were shown to be stinct from lipopolysaccharide transferases in that addition of ten mes the amount of transferase normally used in experimentation did t increase the synthesis of lipopolysaccharide above that obtained ing particulate preparations. Evidence was also presented which indi- ted that the product of each transferase was glycoprotein in nature, e., no phosphorous was detected in any of the products of the enzymes might be expected if the products were lipopolysaccharide, glycolipid nucleic acid containing materials. Consequently, it was concluded at the enzymes are glycoprotein glycosyltransferases.

Glycoproteins may also be present in the cell envelope of Halo- cterium halobium (56). Although evidence was not published, Konce- cz stated that three Periodic Acid-Schiff (PAS)-positive components re detected after phenol extraction of envelopes followed by deter- nt gel electrophoresis of material migrating into the aqueous phase. nce it is generally assumed that a positive PAS reaction indicates esence of carbohydrate, it was concluded that glycoproteins are pres-

t in the envelope of this organism. However, since procedures were t given, caution should be exercised regarding the conclusion with- t further supporting evidence. This is important because anomalies y occur and erroneous interpretations may be made unless proper PAS aining and subsequent washing procedures are followed (31). In sup- rt of the conclusion, it should be pointed out that pronase treatment the envelope caused a change in the electrophoretic mobility of the S-positive components. Such behavior could indicate that glycopro- in was present.

Although not extensive, some evidence has been published for pres- ce of two glycoproteins in the envelope of Pseudomonas aeruginosa 7). The data obtained using detergent gel electrophoretic analysis conjunction with the PAS staining procedure described by Holden et . (48). According to the criteria of Glossman and Neville (31), this thod does not appear to produce anomalies; therefore, the evidence ems to be valid.

At the near termination of my work, Estrugo et al. (27) published ntative evidence to indicate that at least three glycoproteins may be esent in the cell membrane of Micrococcus lysodeikticus.

At present, the preceding reports are the only ones available which dicate the possible presence of glycoproteins in the surface struc- res of bacteria. Lack of definitive data prompted Leaback (57) to blish a theory concerning bacterial glycoproteins. It was stated that ycoproteins occur rarely, if at all, as secreted products of bacteria. e major rationale presented is that procaryotes lack cytoplasmic mem- ane systems of the endoplasmic reticulum type; thus, the necessary bi- ynthetic machinery is probably not present. This theory cannot have

.idity since phytotoxic glycoprotein has been reported to be present
cluture filtrates of Corynebacterium sepedonicum (99).

From the preceding literature survey, it can be seen that only a
v investigations of bacterial glycoproteins have been undertaken, and
ɜse relate to presence or absence of glycoproteins in surface struc-
ɜes. Unfortunately, none of the investigators attempted to distingu-
ı between cell wall and the membrane. Thus, adequate data are lack-
ɜ to definitely prove that glycoproteins are present in the membrane
true bacteria. Certainly no information exists which relates to pos-
ɔle function(s). A recent report by Terry and Zupnik (101) indicates
ıt glycoproteins are not present in the plasma membrane of Achole-
ısma (formerly Mycoplasma) laidlawii.

My study was originally started as a result of several observations
ie during an earlier investigation of the M. lysodeikticus membrane
ɔ). Whole membranes of this organism were found to contain a compo-
ıt, which could be resolved by polyacrylamide gel electrophoresis
i yielded a positive reaction upon treatment with PAS reagent. This
formation indicated that the component could be glycoprotein. In ad-
ɪion, the PAS-positive component was found to enter into reaggregated
ıbrane sheets under proper conditions of dialysis. Since glycopro-
ins possess numerous important functions, the observations were in-
ɪguing. Consequently, we decided to investigate this PAS-positive
mponent. Our first interest was to confirm that it is a glycoprotein.
definitive evidence for such a protein species could be obtained, it
ɜ hoped that isolation and purification could be done, and that fur-
ɜr studies regarding chemical characterization as well as determina-
ɔn of possible function could be accomplished.

CHAPTER II

MATERIALS AND METHODS

Growth Medium and Membrane Isolation

M. lysodeikticus was grown in defined medium (37), and membranes
:e isolated by the technique described by Butler, Smith and Grula
'). All membrane preparations were washed three times in cold Tris
:fer (0.0025 M, pH 7.4) by centrifuging at 54,500 x g for 30 min after
ich they were used immediately or lyophilized to dryness and stored
-30 C. Unless otherwise specified, the concentration and pH of Tris
:fer utilized for various analyses were always as given as above.

Electrophoresis

Detergent (sodium lauryl sulfate, SLS) gel electrophoresis was per-
rmed utilizing essentially the system of Weber and Osborn (106) as de-
ribed and further refined (41, 85), with the exception of sample sol-
ilization. The solvent consisted of 14.4 mM $Na_2HPO_4 \cdot 7H_2O$, 5.65 mM
$H_2PO_4 \cdot H_2O$, 1.6% glycerine, 1% SLS and 1% B-mercaptoethanol. Membrane
mples were incubated in solvent (3 mg/ml) either at 37 C for 2 hr or
ated to 100 C for 15 min (occasionally at both, with 37 C first) prior
electrophoresis. Carbohydrate was always detected using Periodic
id-Schiff (PAS) reagent (31) regardless of the system utilized for
ectrophoresis.

Non-detergent gel electrophoresis was performed using the pH 2.7

tem of Neville (72) as previously described (85).

Proteolysis

The procedure was carried out at 37 C for 3 hr in Tris buffer and tained per ml: 3 mg membrane and 500 ug pronase. Insoluble materi- were sedimented by centrifugation at 80,000 x g for 2 hr; both pel- and supernatant were lyophilized and stored at -30 C.

Membrane Disaggregation

When necessary, membranes (in 0.0025 M Tris buffer) were disaggre- ed by incubation in the presence of 0.02 M SLS (prepared in distil- l water) for 2 hr at 37 C. Insoluble materials, if any, were removed centrifugation at 80,000 x g for 30 min.

Alkali-Catalyzed β-elimination

Carbohydrate side-chains of disaggregated membrane samples were re- ased by alkali-catalyzed β-elimination (107). Equal volumes (preheat- to 50 C) of sample (2 mg/ml) and 1 N NaOH were mixed and the reaction rried out at 50 C) was followed by an increase in absorption at 240 . The resulting acetodehydro amino acid product was converted to the spective keto-acid form (pyruvate from serine and α-ketobutyrate from reonine) by mild acid hydrolysis of the insoluble residue (3 N HCl at) C for 1.5 hr). Detection of pyruvate and/or α-ketobutyrate was ac- plished by enzymatic end-point analysis (107, 70), utilizing lactic d dehydrogenase (LDH) and nicotinamide adenine dinucleotide (NADH), l by chromatographic analysis after preparation of the respective 4-dinitrophenylhydrazine derivative (98).

Concanavalin A (Con A) Interaction

Precipitation of membrane material by Con A was accomplished at C in Tris buffer. Proportions used in this study were 2 mg membrane ple to 600 ug Con A (corrected value since large amounts of NaCl are sent in the commercial product). Dissociation of Con A-membrane re- tor complexes, in the presence or absence of SLS, was accomplished lizing either α-methyl-D-mannoside or α-methyl-D-glucoside (0.01 M al concentration).

Isolation of the Con A Receptor Component

A flow diagram for this procedure is presented in Figure 1. Mem- nes were disaggregated by adding SLS; all insoluble materials were oved by centrifugation as described above. Con A (600 ug final con- tration) was then added to the supernatant and the solution allowed react at 25 C for 12 hr. Precipitated membrane material (Con A-re- tor complexes) was collected by centrifuging at 54,500 x g for 30 min 4 C, resuspended in Tris buffer and dissociated by adding α-methyl- lucoside (0.01 M final concentration). To separate the dissociated A and receptor molecules, hot phenol partitioning was utilized. aqueous phase, which contained the receptor components, was collect- by centrifugation at 10,500 x g for 30 min at 4 C and acetone (90% al concentration) added to cause component precipitation. This pre- itate was collected, washed with acetone (2 times), lyophilized and red at -30 C until analyzed.

Figure 1. Flow Chart for Isolation of Con A Receptor Component From *M. lysodeikticus* Membranes.

branes suspended in Tris buffer (2 mg/ml) were disaggregated by adding SLS (0.02 M final concentration)

oluble material removed by centrifugation (80,000 x g for 30 min at 4 C)

A added to supernatant (600 ug/ml final concentration) and mixture incubated 12 hr at 25 C

cipitated membrane material collected by centrifugation (54,500 x g for 30 min at 4 C), suspended in Tris buffer and dissociated by adding α-methyl-D-glucoside

al volume of freshly prepared hot (67 - 70 C) phenol (80%) added

ct at 67 - 70 C for 1 hr with occasional stirring

trifuge at 10,500 x g for 30 min at 4 C

lect aqueous phase and add acetone to 90% final concentration

cipitate collected by centrifugation (10,500 x g for 30 min at 4 C) and washed with 90% acetone (2 times)

cipitate lyophilized to dryness and stored at -30 C

Protoplast Formation

Cells from 100 ml medium were sedimented by centrifugation at 00 x g for 10 min, resuspended in Tris buffer (0.05 M, pH 7.4) conning 0.156 N NaCl and 0.01 M Mg^{++}, washed twice and then resuspended 19 ml of the same buffer. One ml lysozyme (200 ug/ml final concention) in this buffer was then added, and the suspension incubated at C for 2 hr. Formation of protoplasts was monitored utilizing phase trast as well as electron microscopy. After formation, protoplasts e collected by centrifugation at 8,000 x g for 15 min at 4 C, gently uspended (without using a pipette) and washed twice in the Tris buf- described above without lysozyme present.

Electron Microscopy

Electron Microscopy was performed as previously described (85).

Glutaraldehyde Treatment

Glutaraldehyde treatment was accomplished essentially by the proure reported by Capaldi (18). Freshly isolated membranes were susded (4 mg/ml) in cold Tris buffer, and mixed (1:1, v/v) with a cold ution of 8% glutaraldehyde (prepared by diluting with the Tris buf-). This mixture was then placed at 0 C (ice bath) and stirred for r. After treatment, ten volumes of cold distilled water were added, the mixture was centrifuged at 54,500 x g for 30 min at 4 C. The ernatant was discarded, and the pellet washed (2 times) with Tris fer before being analyzed.

Amino Acid and Carbohydrate Analyses

Amino acid and carbohydrate analyses were performed in the Depart-
t of Biochemistry under the supervision of Dr. B. G. Hudson.

no Acids

Amino acid analyses were performed on a Beckman Model 120 C amino
d analyzer after hydrolysis of membrane sample with glass-distilled
stant-boiling HCL (6N) under reduced pressure for 24 hr at 110 C (94).
al half-cystine content was determined as cysteic acid after oxida-
n of membranes with performic acid and hydrolysis as described above
, 86).

bohydrates

Hexosamine analyses were made using the short column of the amino
d analyzer after hydrolysis with 4N HCl at 100 C for 6 hr. Sialic
d analyses were made by thethiobarbituric acid assay after hydroly-
in 0.1N H_2SO_4 at 80 C for 1 hr (71, 103). Neutral monosaccharides
e determined on a Technicon Model SC-1 Auto Analyzer (58) after hy-
lysis with 2N H_2SO_4 for 4 hr at 100 C and passage of hydrolysate
ough coupled columns of Dowex 50 and Dowex 1 (95).

Chemicals

Con A, LDH and NADH were purchased from Sigma Chemical Co., pro-
e from Calbiochem and glutaraldehyde from Eastman Kodak Co..

CHAPTER III

RESULTS

In this study of the M. lysodeikticus membrane glycoprotein compo-
:(s), the techniques of Butler et al. (17) were employed for cell
vth and membrane isolations. Experiments were generally monitored
lizing electron microscopy, polyacrylamide gel electrophoresis (de-
gent and non-detergent systems) and, when necessary, measurement of
ıt absorption at 440 (for carotenoid pigments), 540 and 660 nm.

The membrane of M. lysodeikticus was disaggregated using SLS and
ggregated into membranous sheet structures in the presence or absence
different types of phospholipids (17, 38). Until systems of poly-
ylamide gel electrophoresis (classified either as detergent or non-
ergent) were tested and the effects of each were observed on the mem-
ıe proteins of M. lysodeikticus (41, 85), the number and kinds of
ponents in or associated with this membrane were not known. Results
ained using the non-detergent type system were found to be unsatis-
tory in that they were not reproducible, 'stacking' on top of the gel
urs and protein migrations fluctuate due presumably to ammonium per-
fate interactions. On the other hand, it was found that reproducible
ponent patterns could be obtained using the detergent system.

Electrophoretic resolution of M. lysodeikticus membrane proteins
the detergent (SLS) gel system revealed that at least 43 protein
cies are present (41, 85); the majority have a molecular weight (MW)

n the range of 20,000-80,000 daltons (Fig. 2A). Although one or two roteins may have a MW approaching 100,000 daltons, it may be signifiant that proteins of high MW, such as the actomysin-like components 250,000 daltons) of the red blood cell membrane (42), do not appear to e present.

In the early phases of this study, it was observed that one proein (CBB-positive) in the M. lysodeikticus membrane reacts well with he Periodic Acid-Schiff (PAS) reagent (85). Several others, all mirating in the MW range of 42,000-55,000 daltons, also gave a slight eaction with this reagent. Since it is generally accepted that a postive PAS reaction indicates presence of carbohydrate, it was tentativey concluded that these components are glycoproteins. A definitive conlusion could not be reached since Glossman and Neville (31) have shown hat anomalies may occur and erroneous interpretations made unless propr PAS staining and washing procedures are followed, particularly when tilizing detergent gel systems.

After these PAS-positive components were re-examined utilizing the AS staining procedure as recommended by Glossman and Neville, only one omponent was observed to react positively (Fig. 2B). This component ossesses a mobility rate similar to protein component number 17 (estiated MW of 55,000 daltons). Such data, although not conclusive, indiate that this component may be a glycoprotein. It should be pointed ut that relatively large amounts of mannose are present in the membrane f M. lysodeikticus (30); consequently, it is possible that this polymer ould, under some conditions of treatment, behave as a CBB-positive rotein.

The above information is interesting, particularly when related to

omposition and sequences of known glycoproteins (reviewed in refs. 95, 7). Carbohydrate heterogeneity of glycoproteins appears to be a commor ccurrence, with units possessing rather conservative structural patter- s and preferred sequences. One frequently occurring sequence contains ialic acid, N-acetylglucosamine, galactose and mannose. Sialic acid is he terminal residue and is linked to N-acetylglucosamine through ga- actose. This unit is then linked to a core of mannose and a few N-ace- ylglucosamine residues. The simplest type unit mentioned contains on- y mannose and N-acetylglucosamine. In light of this information, the arbohydrate sequences of the possible M. lysodeikticus membrane glyco- rotein (PAS-positive component) would appear to be rather unique in hat it probably consists totally, if not completely, of mannose. Such homogeneous sequence, however, may be more widespread than previousl hought since the cell wall of bakers yeast contains a mannan glycopro- ein (90). This homopolymer of mannose is O-glycosidically linked to he protein through serine and threonine residues.

If the PAS-positive component in the membrane of M. lysodeikticus s not a glycoprotein, the other alternatives are that it is either a annan or glycolipid. Both of these components are present in the mem- rane of this organism (65, 59).

It is unlikely that the component is glycolipid for several reason: he membrane lipids of M. lysodeikticus have been shown (85) to migrate ear the electrophoretic gel front in the region of component number 43 Fig. 2A), and carbohydrate can be detected in this area when resolved hole membrane components are treated with the PAS reagent. These car- ohydrate-containing lipids are most likely mannose-containing glycolip- ds since stripping (removal of phospholipids and carotenoids using 90%

›ld acetone containing 50 ul ammonium hydroxide per 100 ml) removes ıem. Also the suspected glycoprotein component has a rather slow mo- ılity rate (near to or similar to component number 17). This informa- ion lends credence to the belief that the PAS-positive component re- ıining after stripping is not glycolipid.

A recent report (101) supports the idea that this PAS-positive com- ›nent could be a homopolymer of mannose. A polymer of hexosamine, ıich is not covalently attached to protein, has been shown to migrate ›ut very poorly) during electrophoretic gel resolution of A. laidlawii ›mbrane components. It is only weakly associated, however, with the ›mbrane, and can easily be removed by mild sonicating procedures. To ›termine if the PAS-positive component of the M. lysodeikticus membrane a homopolymer of mannose or a glycoprotein, other experiments were ›rformed.

Commercially prepared mannan (Sigma), isolated from yeast cell ll, was electrophoretically analyzed, and the results were compared to ›se of the PAS-positive membrane component (Table II). The only sim- arity is that both molecules yield a positive reaction when treated th the PAS reagent.

Retardation coefficient differences could possibly be explained by e properties of globular molecules in SLS. This detergent is a po- nt protein denaturant and solubilizing agent which binds to and thus ncels intrinsic charge differences. It converts most proteins to rod- ke particles whose lengths vary uniquely with the MW. The logarithm mobility of this type molecule during SLS gel electrophoresis is also near with respect to MW. On the other hand, the logarithm of mobility a globular molecule is linear with the square of the molecule radius

TABLE II

ELECTROPHORETIC CHARACTERIZATION OF COMMERCIALLY PREPARED MANNAN AND THE M. LYSODEIKTICUS PAS-POSITIVE MEMBRANE COMPONENT*

aracteristics	Mannan	PAS-positive Component
S Reaction	+	+
tardation Coefficient**	0.05	0.25
solution	broad zone	sharp band
B Reaction	-	+

*Samples analyzed (SLS gel system) simultaneously.

**Migration distance of membrane component number 43 set at 1.0 (Fig. 2A).

7, 106). The mannose homopolymer would probably be of the globular pe. Therefore, any electrophoretic mobility of this molecule would be rectly affected by the extent of branching and size. If the M. lyso- ikticus PAS-positive membrane component is a mannose polymer and dif- rs from the commercial mannan in size and extent of branching, then fferences in retardation coefficients would be expected. It should pointed out, however, that carbohydrate attached to protein could al- account for differences in retardation coefficients.

Comparison of the electrophoretic gel resolutions of the two mole- les lends support to the idea that the M. lysodeikticus PAS-positive mbrane component is a glycoprotein. Regardless of the amount of sam- e analyzed, mannan always migrates as a broad zone; whereas, the mem-

rane PAS-positive component always migrates and appears as a sharp and. Also contributing significantly to this idea is the observation hat mannan is CBB-negative and the PAS-positive membrane component is BB-positive.

Overall, the data shown in Table II strongly indicate that the M. ysodeikticus membrane PAS-positive component is a glycoprotein rather han a homopolymer of mannose.

To ascertain if the PAS-positive component contains protein, proeolysis of intact membranes by pronase was accomplished. It was reaoned that if a protein is attached to carbohydrate, proteolysis would hange the electrophoretic migration rate of the component. It was ound that after pronase digestion, the PAS-positive component as well s all protein peaks disappear; only a broad CBB-positive zone near the eading edge of the gel (thought to be small peptides) can be demonstraed (shown in Fig. 2C). The supernatant was also analyzed after proase digestion to determine if the PAS-positive component is merely reeased rather than degraded. Although the data are not shown, the PASositive component could not be found in the supernatant and, therefore, ppears to have been degraded by pronase. Data from these types of exeriments support the conclusion that this PAS-positive membrane compoent consists of protein and carbohydrate moieties covalently linked toether, and therefore cannot be considered a homopolysaccharide.

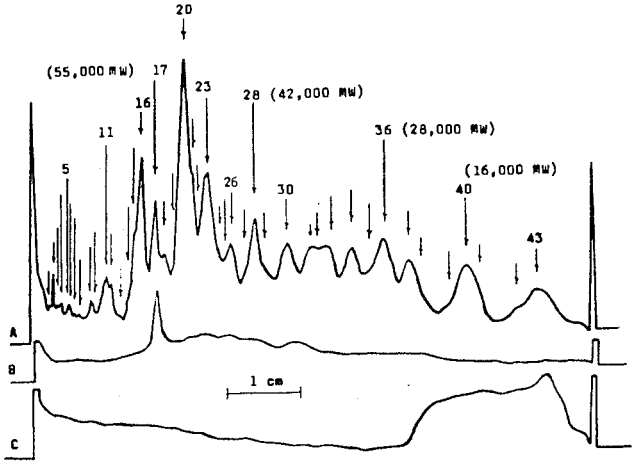
A non-detergent electrophoretic gel system [pH 2.7 system of Nevlle (72)] was also utilized to determine if a PAS-positive reaction ould occur on gels electrophoresed in the absence of detergent. Even hough this system will not resolve all membrane proteins (41), it has ne significant advantage over detergent systems since detergent induced

gure 2. Electropherogram Component Patterns (Detergent Gel System) of M. lysodeikticus Membranes Treated with:

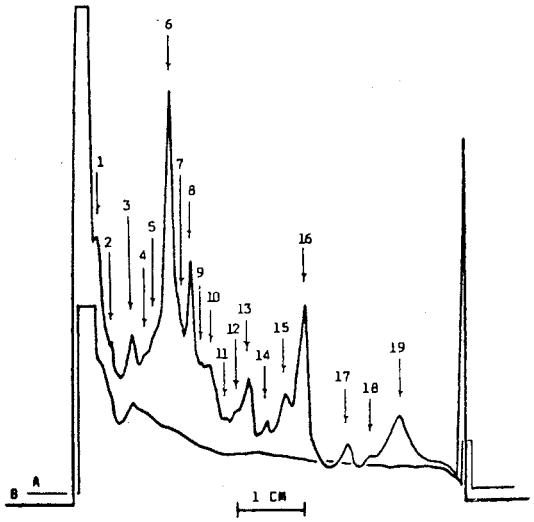
A. CBB stain (150 ug membranes).
B. PAS reagent (150 ug membranes).
C. CBB stain after pronase digestion of membranes.

gure 3. Electropherogram Component Patterns (Non-Detergent Gel System) of Stripped M. lysodeikticus Membranes Treated with:

A. PAS reagent (240 ug membranes).
B. Buffalo Black NBR stain (240 ug membranes).


20
(55,000 MW)
17
16
23
28 (42,000 MW)
11
36 (28,000 MW)
5
26
30
(16,000 MW)
40
43
A
B
1 cm
C

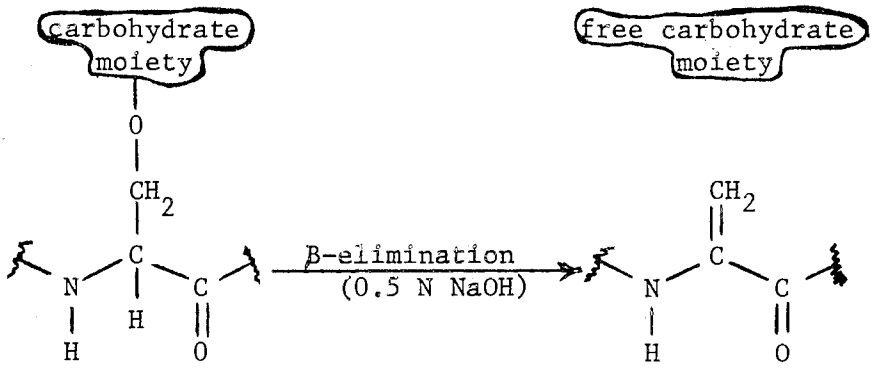
2


6
1
7
8
3
5
2
4
16
9
10
13
12
15
11
14
17
18
19
B
A
1 CM

3

rtifacts cannot occur. The results shown in Figure 3A reveal that on- y one component reacts with the carbohydrate stain; this component mi- rates to approximately the same place on the gel as protein species mber 3 (Fig. 3B). These data can be taken as further evidence that ıe PAS-positive component in the membrane of M. lysodeikticus is a lycoprotein.

In many mammalian glycoprotein species, carbohydrate moieties are tached to the protein via an O-glycosidic bond involving either ser- ıe or threonine (95). Such bonding exhibits the property of being olit by a β-elimination process under mild alkaline conditions. This sults in the conversion of serine and threonine to the respective de- ydroamino acids (71). These absorb strongly at 240 nm (36), and are nverted by acid hydrolysis (107) to the keto acid form (pyruvate from erine and α-ketobutyrate from threonine). The reaction and resulting oducts are shown below.

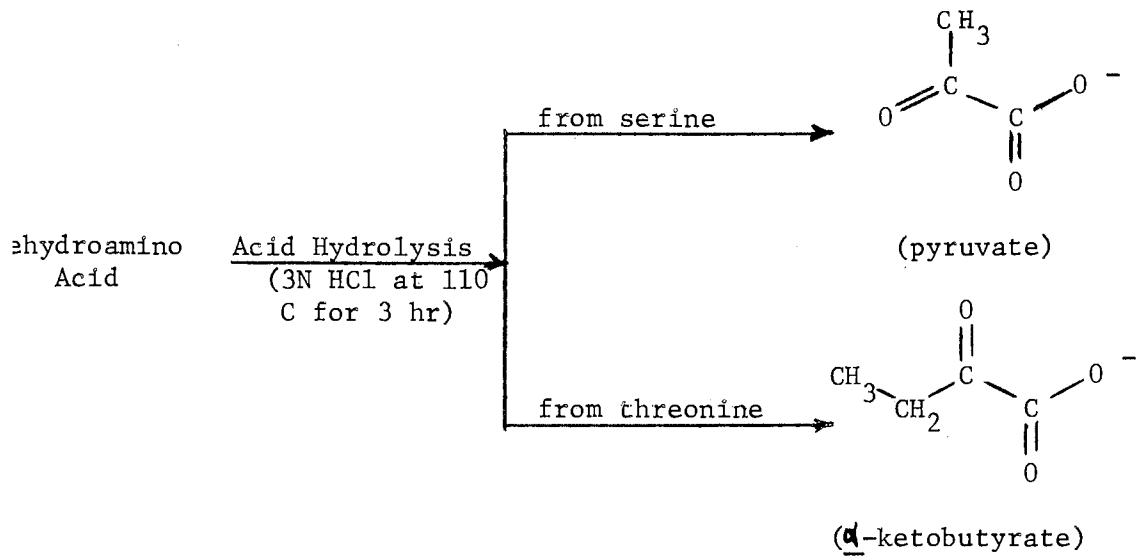


Serine in Peptide Chain

Dehydro Amino Acid (absorbs light at 240 nm)

(continued on next page)

(β-elimination Continued)



The demonstration of such O-glycosidic linkages in the membrane of . lysodeikticus would provide additional proof of a membrane component ontaining carbohydrate attached to protein. As shown in Figure 4, al- aline degradation results in a rapid increase in absorption at 240 nm. nis indicates the presence of glycosidic linkages which involve the ydroxyl groups of serine and/or threonine. It is also important to oint out that, after alkaline hydrolysis of membranes, the PAS-positive omponent cannot be detected in gel columns.

To determine which amino acid (serine or threonine) is involved in ne glycosidic linkage, two further types of analyses were performed on ne resulting keto acid product(s). One type was an endpoint enzymatic ssay (70) utilizing lactic acid dehydrogenase (LDH) and nicotinamide lenine dinucleotide (NADH). Pyruvate can be reduced readily by a low l unit) level of enzyme, while α-ketobutyrate requires a much higher evel (45 units). Therefore, when low levels of enzyme are present, ne early rapid decrease in absorbance can be used to determine the

ıount of pyruvate present. After completion of lactic acid formation, ıe further addition of large amounts of LDH (45 units) allows determiıtion of the amount of α-ketobutyrate present. The data obtained in ıis experiment are shown in Figure 5. There was an initial decrease ı absorption which was complete in approximately 10 seconds. Addition : more enzyme after 60 sec did not bring about any further decrease in ›sorption other than that due to dilution. Based on information obıined from this experiment, it appears likely that pyruvate was preənt in the acid hydrolyzate. This information further indicates that ı the undegraded glycoprotein, the carbohydrate moiety is attached to ıe protein through the hydroxyl group of serine.

The other type of analysis carried out involved preparation and ıromatography of the 2,4-dinitrophenylhydrazine derivative from the ɛto acid product. As can be seen from the data given in Figure 6, ʏruvic but not α-ketobutyric acid was the only keto acid product found. ɛcause pyruvic acid was not found in untreated membranes, it arose as result of alkaline β-elimination and the subsequent acid hydrolysis. ıese results substantiate those obtained using enzymatic endpoint an-ysis, and also strongly suggest that the carbohydrate is attached to ıe protein through serine.

Although the results of the two preceding experiments clearly reɛal that pyruvate is the keto acid product formed after β-elimination ıd subsequent acid hydrolysis, it cannot be concluded that the car›hydrate-protein linkage occurs through serine without further investiıtion. It has been reported (19) that the amino acids cysteine and ʏstine give rise to keto acid residues by β-elimination under alkaline ›nditions. Like serine, the product formed after acid hydrolysis is

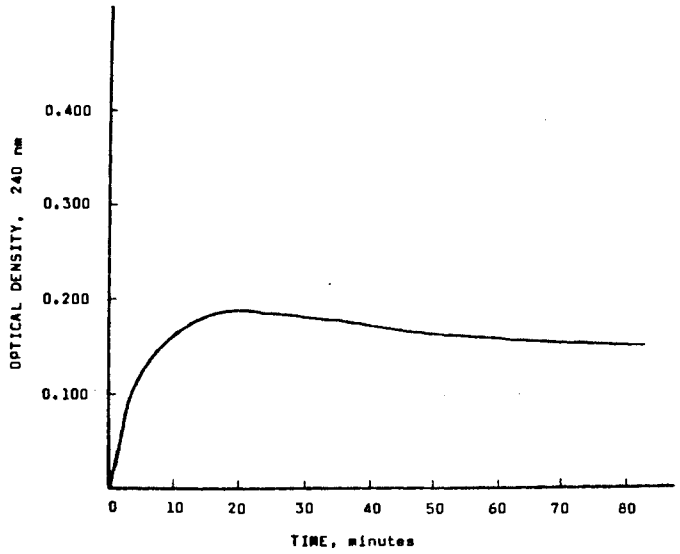
.gure 4. Alkaline Degradation of M. lysodeikticus Membranes. Stripped and Solubilized Membrane (0.94 mg/ml) was Exposed to 0.5 N NaOH at 50 C.

.gure 5. Endpoint Assay for Pyruvate and α-ketobutyrate Utilizing LDH and NADH. Solubilized Stripped Samples of M. lysodeikticus Membrane Exposed to: Mild Acid Hydrolysis only (dashed line); Alkaline-Catalyzed Degradation Followed by Mild Acid Hydrolysis (solid line).

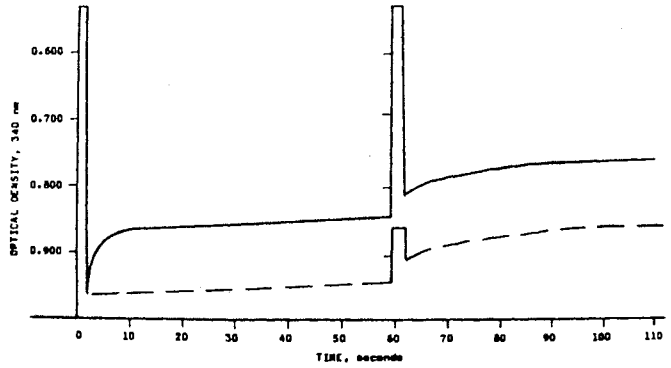
.gure 6. Chromatographic Patterns of Various 2,4-dinitrophenylhydrazine Derivatives. Solvent System: N-butyl Alcohol, 0.5N NH_4OH and 95% Ethyl Alcohol (70/20/10).

A. Pyruvate.
B. α-ketobutyrate.
C. Untreated Stripped Whole Membranes.
D. Stripped and Solubilized Membranes Exposed to 0.5 N NaOH at 50 C for 1 hr prior to Formation of the Derivative.

4


OPTICAL DENSITY, 240 nm
0.400
0.300
0.200
0.100
0
10
20
30
40
50
60
70
80
TIME, minutes

5


OPTICAL DENSITY, 340 nm
0.600
0.700
0.800
0.900
0
10
20
30
40
50
60
70
80
90
100
110
TIME, seconds

6

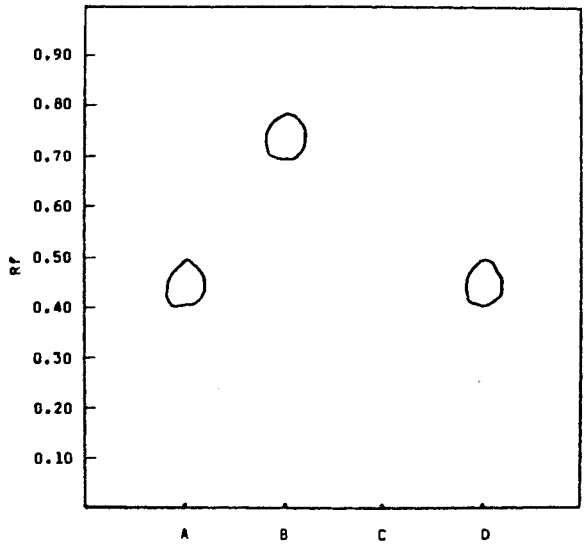

Rf
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
A
B
C
D

TABLE III

AMINO ACID AND CARBOHYDRATE COMPOSITION OF M. LYSODEIKTICUS STRIPPED MEMBRANES

›mponent	Residues per 1000 Amino Acid Residues
ıino Acid	
Alanine	146.4
Glycine	107.3
Leucine	101.8
Aspartic Acid	82.4
Glutamic Acid	80.1
Valine	76.1
Threonine	64.9
Arginine	61.6
Serine	55.1
Proline	47.8
Isoleucine	41.4
Phenylalanine	37.2
Lysine	32.7
Methionine	20.3
Tyrosine	18.8
Histidine	16.8
Half-Cystine	9.9
›nosaccharide*	
Mannose	78.6
Glucose	3.9

*Hexosamines and sialic acid could not be detected.

ruvate. Recently, cysteine has been shown to be S-glycosidically nked to carbohydrate in glycopeptides isolated from human urine (62) d human erythrocyte membrane (108). Since cysteine, although present a minor component (Table III), can be demonstrated in stripped membranes of M. lysodeikticus, data obtained from all the B-elimination experiments using whole or stripped membranes are not completely reliable. r this reason, it appeared advisable to isolate in pure form the glyprotein component and directly characterize the covalent attachment carbohydrate.

Strength of association of the component with the membrane was dermined prior to attempting isolation. This was accomplished by exnsive washing (10x) in Tris buffer of freshly isolated membranes. ectrophoretic analysis showed that the component is not removed by ch treatment. Since stripping or extensive washing does not remove e glycoprotein from the M. lysodeikticus membrane, it appears to be strongly associated molecule.

Isolation and Purification of The M. lysodeikticus Membrane Glycoprotein Component

Proteins which have the unique ability to agglutinate red blood lls are widely distributed in the plant kingdom, and are called phytoemagglutinins or lectins (for recent review see ref. 32). Concanavan A (Con A), isolated from the jack bean (Canavalia enisformis), is e most extensively investigated lectin. Like most lectins, it reires manganese and calcium ions for binding carbohydrate (45), but is like most in that covalently attached carbohydrate is not present

thin the molecule (60). The residues of the α-D-manno- or α-D-glucoranosyl type are most complementary to the binding sites of Con A (83, , 75, 93, 82); however, precipitation evidently requires binding of lecules containing branched saccharide moieties (75). Interaction y be prevented (and Con A-receptor complexes can be dissociated) by hibitors such as α-methyl-D-glucoside or α-methyl-D-mannoside (82).

In light of this information, it was thought that Con A could posbly be utilized as a tool in isolating the glycoprotein component from e membrane of M. lysodeikticus. A study was made to determine if Con could react with membrane materials (whole, stripped and reaggregated mbranes) from this organism. These experiments revealed that regardss of the type of membrane tested, agglutination with Con A occurred. was also observed that the resulting precipitated complexes dissocied in the presence of inhibitor (Table IV). In this experiment, the n A was not pretreated with either manganese or calcium, yet complete glutination was observed within 1 hr in all situations studied. Alough the results are not shown, pretreatment of lectin with calcium d manganese did not enhance agglutination, but did increase rate of e overall process. Evidently the commercial product (Sigma) contains fficient amounts of these metals for activity.

In the test wherein stripped membranes were utilized, agglutinaon occurred quickly and required only about 10 min. The fact that ripping enhances agglutination indicates several possibilities: 1) ne receptor components may be partially buried within the untreated mbrane and are not readily accessible for interaction with Con A; d/or 2) Stripping allows the individual receptors to lie in closer oximity, thereby allowing a more intimate interaction with concomitant

TABLE IV

INTERACTION OF M. LYSODEIKTICUS MEMBRANES WITH CON A

nditions and Type of Sample	O.D. at 660 nm (1 hr)
ole Membranes	
Without Con A	0.18
With Con A	0.29
Con A + inhibitor*	0.17
ripped Whole Membranes**	
Without Con A	0.29
With Con A	0.55
Con A + inhibitor	0.31
aggregated Membranes***	
Without Con A	0.16
With Con A	0.20
Con A + inhibitor	0.15

*Inhibitors utilized were either α-methyl-D-mannoside or the glucoside form (0.01 M final concentration). The above data were obtained using the mannoside. Inhibitor was added after 1 hr and the optical density read immediately after mixing.

**Removal of phospholipids and carotenoid pigments (stripping) was accomplished utilizing 90% acetone containing 50 ul NH_4OH per 100 ml reagent (30 min at 4 C).

***Aggregates formed during dialysis in Tris buffer (0.0025 M; pH 7.4) containing 0.01 M Mg^{++} at 25 C for 24 hr from detergent-solubilized whole membranes (85).

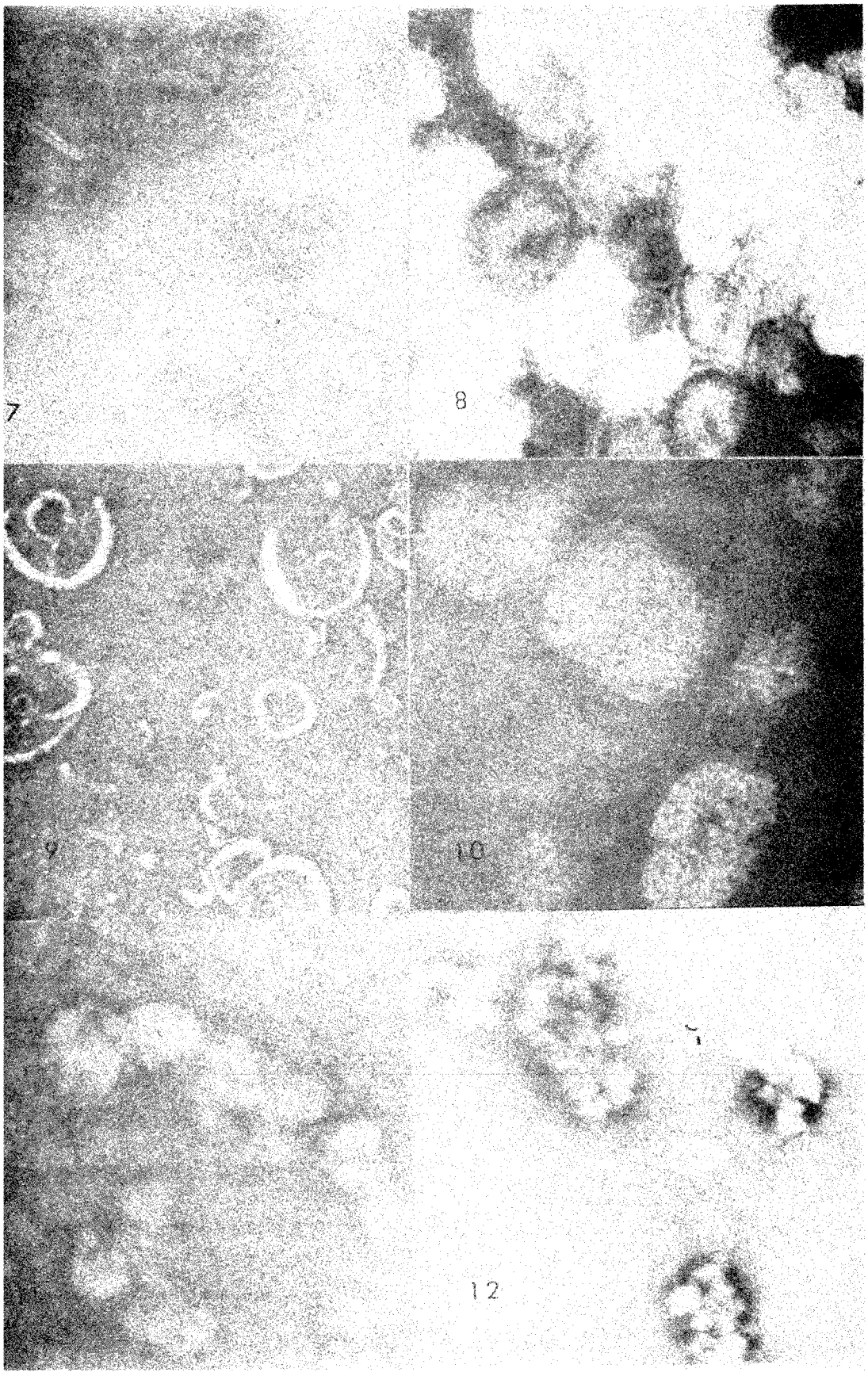
crease in agglutination rate (92).

Electron microscopic examination of the membrane samples, before d after Con A agglutination, revealed several easily discernible difrences. Before Con A was added, membrane sheets as well as fragments

re observed to exist primarily as separate structures. Afterwards, ese were piled and interacted together; they also appeared more elec- on dense and smaller in diameter (compare Fig 7 and 8). When α-meth- -D-mannoside was added to agglutinated membranes, dissociation occur- d, but the resulting separated sheets appeared to be slightly rolled the edges (Fig. 9). This indicates that there is interaction be- een Con A and receptors located on the same membrane sheet, and this tramembrane association may be less susceptible to dissociation than termembrane associations. Although not shown, similar results were served using stripped and also reaggregated membranes.

Addition of SLS (0.02 M final concentration) to membranes previous- reacted for 1 hr with Con A does not completely disaggregate the com- ex. The remaining insoluble materials appear as small (compared to e average diameter of the three separate whole membrane sheets shown a straight line in Fig. 7), globular, porous-like structures (Fig.). Addition of inhibitor further solubilizes these detergent-resis- ıt structures. This information indicates that incomplete solubili- tion by detergent is due to Con A and receptor interactions. It may so be suggested that the Con A receptors (glycoprotein) are fairly enly spaced and distributed over the surface of the membrane.

To gain further insight relating to positioning of the glycoprotein, litional experimentation was performed. Whole membranes were first saggregated by adding SLS (0.02 M final concentration) for 30 min at C and all insoluble materials were removed by centrifugation (54,500 g for 30 min at 4 C). Con A (600 ug/ml final concentration, adjusted lue) was then added to determine if interaction and precipitation ıld occur. After several hr, a precipitate was observed which had the


7
8
9
10
12

)earance of a globular, porous-like form (Fig. 11). These precipi-
:es were much smaller, however, than the limit structures shown in
;ure 10. If stripped membranes were used instead of whole membranes,
;imilar result and product was obtained (Fig. 12). Since receptor
ıponents were precipitated from clean, disaggregated membrane prepa-
:ions (all insoluble material had been previously removed), it can be
ıcluded that the receptor component is not of cell wall origin, but,
:her, is in the cytoplasmic membrane.

In summary, the preceding data reveal that specific Con A recep-
:s are present in the membrane of M. lysodeikticus. The data also re-
ıl that these receptors are integral components in that they are not
ıoved or denatured by stripping of lipoidal materials and that they
: capable of entering into reaggregated membrane sheet structures.
'ther, the carbohydrate moiety (ies) of the receptor component is ap-
'ently oriented extrinsically to the membrane continuum, otherwise
:eraction with Con A could not occur. In addition, it is evident that
: A-receptor complexes are insoluble in SLS, whereas the unreacted
ıbrane is completely disaggregated by the detergent.

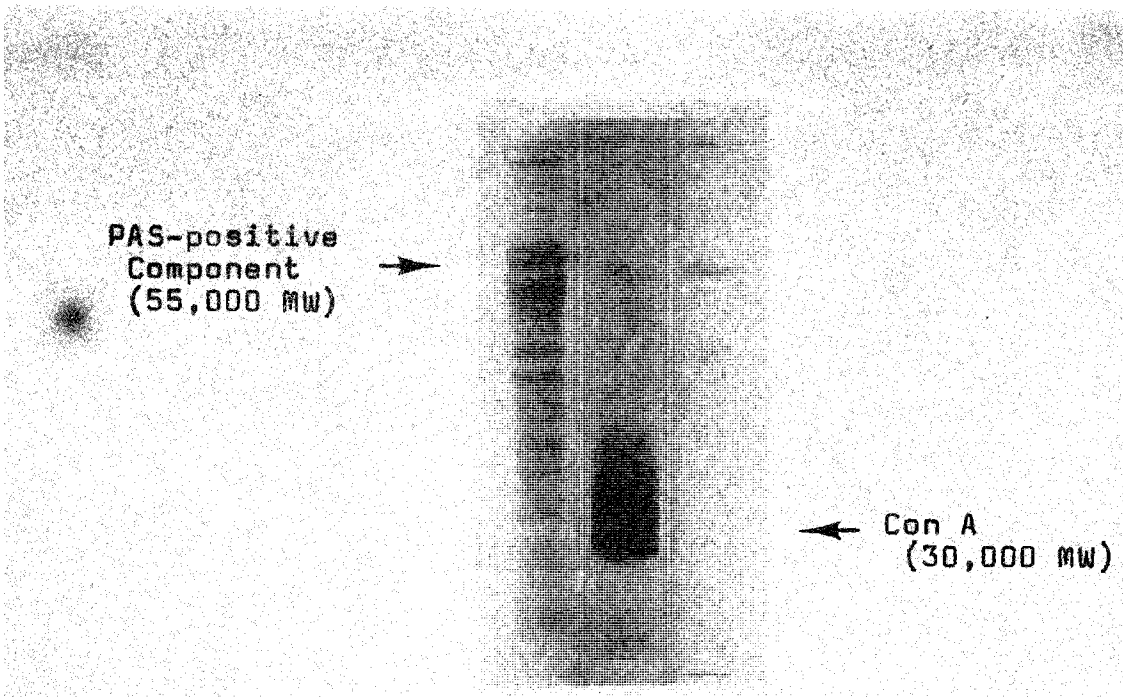
The above information was utilized to aid in purification of the
lysodeikticus membrane glycoprotein component (flow chart shown in
;ure 1). In step four, Con A-receptor complexes result and polyacryl-
de gel electrophoretic analysis (detergent system with 0.01 M α-meth-
D-glucoside added to the solvent) of this material reveals only one
ıbrane component, in addition to Con A, to be present (Fig. 13). This
ıponent has a mobility rate similar to that of the PAS-positive compo-
.t (glycoprotein) present in the intact membrane. In addition, it was
erved that this component reacted with both the protein stain (CBB)

re 13. SLS Electrophoretic Gel Columns Containing:

A. Isolated Whole Membrane Stained for Protein by CBB (120 ug).
B. Resulting Con A-Receptor Complex Formed after Addition of Con A to Disaggregated Whole Membrane Material (CBB).
C. The same as above (B), but Stained for Carbohydrate (PAS procedure).

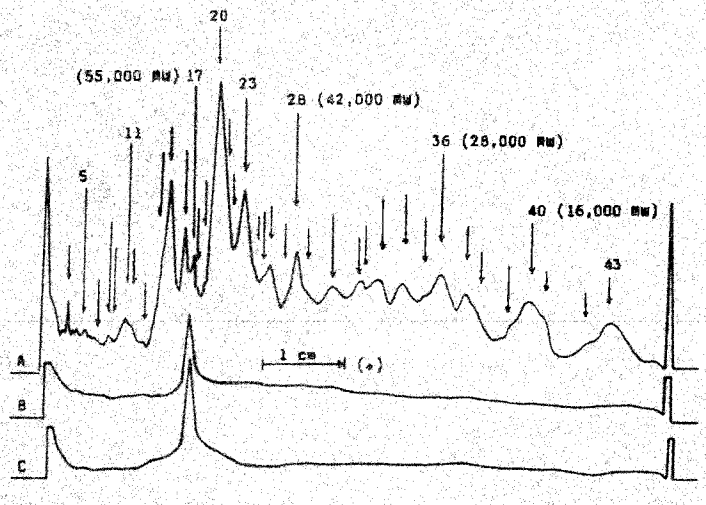
re 14. Electropherogram Component Patterns (Detergent Gel System) of:

A. Isolated Whole Membrane Stained for Protein by CBB (120 ug).
B. The Con A Receptor (Glycoprotein) Isolated from M. lysodeikticus Membrane; treated with CBB.
C. The Same as above (B), but Stained for Carbohydrate (PAS).


PAS-positive
Component
(55,000 MW)
Con A
(30,000 MW)

13

A B C


20
(55,000 MW) 17
23
28 (42,000 MW)
11
36 (28,000 MW)
5
40 (16,000 MW)
43
1 cm
(+)
A
B
C

14

and carbohydrate detecting reagent (PAS), whereas Con A, even though obviously present in large quantity, only reacted with CBB. This observation attests further to the validity of the carbohydrate staining procedure utilized, and allows the conclusion that both protein and carbohydrate are present in the M. lysodeikticus membrane component which interacts with Con A.

Addition of hot phenol to precipitated and resolubilized glycoprotein (steps 5 - 7 in the flow chart) results in a partitioning of the glycoprotein into the aqueous phase after centrifugation in the cold (111). As expected, Con A remains in the phenol phase. Further purification of the glycoprotein component originally consisted of simple dialysis of the aqueous phase to remove α-methyl-D-glucoside and residual phenol, followed by lyophilization to concentrate the material. This was an undesirable procedure since many tedious manipulations were involved and the glycoprotein component had a tendency to disappear, perhaps by sticking to the dialysis tubing. Consequently, this step was eventually replaced by employing acetone precipitation and washing since both α-methyl-D-glucoside and phenol are soluble in acetone, whereas the glycoprotein component is not. It is interesting that the glycoprotein material prepared in this manner sticks readily to glass tubing and remains behind as the acetone is poured off; yet, appears to be very soluble in distilled water.

Electrophoretic characterization (detergent system) of the acetone precipitated material reveals only one component to be present (Fig. 14). It reacts positively with both protein (CBB) and carbohydrate (PAS) staining reagents, and possesses a mobility rate identical to that of the PAS-positive component (glycoprotein) previously shown to be present

in the intact membrane of M. lysodeikticus. Therefore, these data allow the conclusion that the membrane glycoprotein of this organism can be separated from the other membrane components by the procedure outlined in Figure 1. Even though both protein and carbohydrate are presentin the isolated molecule, it is interesting that the color intensity appears greater when carbohydrate staining is employed. This probably indicates that the bulk of the glycoprotein consists of carbohydrate.

Chemical Characterization of the M. lysodeikticus Membrane Glycoprotein

The glycoprotein material obtained by the procedure outline in Figure 1 (page 12) was initially analyzed for amino acids and carbohydrate in the Department of Biochemistry under the supervision of Dr. B. G. Hudson. Mannose was the only carbohydrate detected.

Preliminary protein analysis (63) to determine the amount necessary for amino acid analysis (utilizing a Beckman Model 120C Analyzer operated manually) revealed that the sample contained a significant amount of protein; however, upon analysis for amino acids, it was observed that concentrations were insufficient for computation. Similar results were obtained when the analysis was repeated even though sample concentration was substantially increased.

After discussion of these findings, it was thought that amino acid analysis could possibly be obtained on another analyzer if it was more sensitive to amino acid detection than the Beckman Model utilized. At this time, I was located in another university and laboratory, and had access to a new automatic amino acid analyzer (Jeolco JLC-6AH), considered by many to be one of the best available on the current market. In

addition to the chromatogram recorder usually present on all analyzers, this unit is also equipped with an integrating computer (Spectra-Physics Autolab System AA) to enhance determination of amino acid concentrations. One of the primary advantages of an integrating computer is that it is more sensitive to optical density changes than a chromatogram recorder, and allows computation of much lower concentrations (less than 8 nM).

Amino acid analysis of the glycoprotein material utilizing the Jeolco Analyzer revealed that amino acids were present. In estimated order of decreasing concentration, they are: glycine, glutamic acid, serine, alanine, aspartic acid, leucine, isoleucine, threonine, lysine, histidine, tyrosine, valine and methionine. Even though sufficient material was used for analysis (approximately 100 ug), none of the amino acids was present in sufficiently large enough concentration to be computed. In essence, this conclusion is the same as that reached by Dr. Hudson.

An in-depth review of the literature concerning amino acid analysis of various types of proteins revealed that presence of large amounts of carbohydrate, as in some glycoprotein molecules, leads to excessive losses of the amino acids during hydrochloric acid (6N) hydrolysis (6). This loss is especially severe in carbohydrate-protein complexes, wherein carbohydrate to protein ratios are greater than one; as the ratio increases, so does the loss.

In light of this information and the occurrence of a carbohydrate to protein ratio near one in the native glycoprotein of the M. lysodeikticus membrane, a modification of the normal acid hydrolysis procedure was planned. It was reasoned that possible amino acid degradation due to presence of carbohydrate during hydrolysis could be circumvented by

decreasing either the time allowed for hydrolysis or the normality of the hydrochloric acid utilized. It was decided to reduce the time from the previously used twenty-four to four hours.

Unfortunately, another problem arose; available glycoprotein material was not depleted, and facilities to isolate more are not available at my new location. As a result, we decided to take the polyacrylamide gel which was scanned for the densitometer chromatogram shown in Figure 14C, macerate and hydrolyze the gel segment containing the glycoprotein material, and, after centrifuging (25,000 x g for 30 min) to remove any gel fragments, analyze this material using the Jeolco Analyzer. It was reasoned that if there were at least 5 ug of glycoprotein on the gel (10 ug were estimated), this would allow a concentration of approximately 250 ng per amino acid or a concentration of between 2 - 4 nM, which is sufficient to be detected by the integrating computer. Data from this experiment are given in Table V.

Amino acid analysis of the glycoprotein molecule reveals it to have several compositional similarities to the delipidized (acetone procedure) membrane of this organism. The amino acids which are present in greatest quantities in the intact membrane (alanine, glycine, leucine, glutamic and aspartic acids) are also those present in greatest quantity in the glycoprotein; however, concentration of alanine and leucine is reduced in the glycoprotein. The most noticeable difference regarding major amino acids is that serine constitutes approximately thirteen percent in the glycoprotein, but less than six percent in the membrane. The increased amount of serine in the glycoprotein might be due to attachment of the carbohydrate residues to the polypeptide backbone via the hydroxyl of serine, not at one site, but at several. Multi-attach-

TABLE V

AMINO ACID COMPOSITIONS OF M. LYSODEIKTICUS STRIPPED MEMBRANE, THE MEMBRANE GLYCOPROTEIN AND ANOTHER HIGH MOLECULAR WEIGHT MEMBRANE COMPONENT

Amino Acid	Molar Ratios*		
	Stripped Membranes**	Glycoprotein***	Material Isolated by the Marchesi procedure (66)***
Glycine	10 (2)	44	49
Glutamic Acid	8 (4)	32	42
Serine	6 (8)	28	25
Aspartic Acid	8 (4)	24	22
Alanine	15 (1)	18	16
Leucine	10 (2)	12	9
Phenylalanine	4 (11)	10	8
Lysine	3 (13)	10	8
Tyrosine	2 (14)	8	7
Valine	8 (4)	6	-
Threonine	7 (7)	4	4
Proline	5 (10)	4	4
Isoleucine	4 (11)	4	3
Histidine	2 (14)	4	2
Methionine	2 (14)	2	2
Arginine	6 (8)	-	-
½ Cystine	1 (17)	-	-
Ratio Basic/Acidic Amino Acids****	7 - 10	1 - 4	1 - 6
% Hydrophobic Amino Acids Present*****	60	53	51

*Tryptophan not determined. Methionine set at two.

**Number in parenthesis is rank of the amino acids, with low number signifying greater concentration.

***Cysteine/cystine not determined.

****Basic amino acids: histidine, lysine, and arginine (arginine content determined only in the stripped membrane sample). Acidic amino acids: aspartic and glutamic acids.

*****Hydrophobic amino acids: tryptophan, tyrosine, phenylalanine, methionine, proline, isoleucine, leucine, valine, alanine and glycine.

ment would certainly not be unexpected since this has been shown to be characteristic of the major glycoprotein in the erythrocyte membrane (54). Unfortunately, presence of cysteine in the M. lysodeikticus membrane glycoprotein was not determined. If significant amounts of cysteine are present, the increase in 240 nm absorbing material (dehydro amino acids, page 26) could be due to alterations of cysteine rather than cleavage of serine to carbohydrate bonds.

It can be noted that relatively large amounts of glutamic and aspartic acids are released from the glycoprotein and intact membrane proteins by acid hydrolysis. This indicates that the proteins are acidic; however, many of these molecules may exist as glutamine and asparagine (neutral forms) within the molecules.

Presence of arginine in the glycoprotein molecule could not be determined (due to masking by the large amount of ammonia present, which in turn was due to ammonium persulfate in the polyacrylamide gel and from buffer contamination).

It can also be noted that both the stripped membrane and the glycoprotein appear to be hydrophobic. Although additional data will be helpful, it is possible to tentatively conclude from these preliminary data that the glycoprotein possesses significant areas of hydrophobicity and is probably acidic in character.

In an early phase of this investigation, the procedure described by Marchesi and Andrews to isolate glycoprotein from human red blood cell membranes using lithium diiodosalicylate (66) was used in attempts to isolate glycoprotein from the M. lysodeikticus membrane. Although the electrophoretic data are not given, the material isolated by this process was demonstrated to have essentially the same polyacrylamide gel

electrophoretic characteristics as commercial mannan (see Table II, page 19). There was, however, one slight difference, i.e., this material could be lightly stained by the protein detecting reagent, CBB. When the gels were thoroughly destained, however, all color was removed. Because of this type reaction, it was surmised that this material was most likely the mannose homopolymer described by MacFarlane to be present in the membrane of this organism (65).

An amino acid analysis of the gel segment containing this material was also performed (data included in Table V). Surprisingly, it appears that this high molecular weight membrane component (near 100,000 daltons using SLS gel electrophoresis) is also a glycoprotein. In the report of MacFarlane, there is no mention of whether testing was performed to determine if protein was present in the so-called mannose polymer she isolated. It is quite possible that the high molecular weight glycoprotein I have purified using the procedure of Marchesi and the mannose polymer described by MacFarlane are the same component.

At present, these findings are not well understood. The fact that the amino acid compositions of the two M. lysodeikticus membrane components (100,000 and 55,000 daltons) are very similar suggests that the two molecules may be related. Further study will be necessary before we can arrive at any definitive conclusions.

Although amino acid decomposition due to presence of mannose during hydrolysis has not been studied further, the data obtained permit an approximation of the chemical residue ratio of the 55,000 dalton glycoprotein to be made (Table VI). The molecular weight is considered only an estimate for several reasons. First, it was estimated by polyacrylamide gel electrophoresis in a detergent (SLS) system. Previous inves-

tigations of erythrocyte membrane glycoproteins have shown that such calculations have little theoretical basis because of the carbohydrate, and should be considered as rough approximations (9, 88). Migration of a protein on a polyacrylamide gel during electrophoresis (SLS system) is partially dependent on its negative charge. The overall charge will be smaller for a glycoprotein compared to a polypeptide of the same mass because carbohydrate binds SLS only poorly. If migration of a glycoprotein molecule in gels of increasing acrylamide concentration is not altered appreciably with respect to other high molecular weight peptides, then calculations are regarded to have a greater degree of validity. Although such data are not shown, testing of the M. lysodeikticus membrane glycoprotein in gels of increasing acrylamide concentrations (5, 10 and 15%) did not noticeably affect the migration rate in this respect. Therefore, it is felt that the molecular weight (55,000 daltons) is a fair estimation. Of interest are the reported molecular weights for the monomeric unit of the major glycoprotein of the human red blood cell membrane (88). These range from 31,000 (now considered to be the weight) to 160,000 daltons. With this in mind, it seems reasonable to consider that the weight of the M. lysodeikticus membrane glycoprotein may not be exactly 55,000 daltons.

Of importance in any chemical residue ratio determination is the percentage of carbohydrate present. Because the analyses were of a preliminary type, only rough mannose percent (50) was ascertained by Dr. Hudson. To obtain an estimate, the areas under the curves on the electropherograms shown in Figures 14B and C were integrated. In this way, it was judged that the glycoprotein molecule is composed of about fifty-five percent mannose. To further aid in making calculations, it can be

TABLE VI

APPROXIMATED COMPOSITION RATIO OF THE M. LYSODEIKTICUS MEMBRANE GLYCOPROTEIN*

Component	Number of Residues per Molecule (55,000 MW**)	Daltons
Amino Acid		
Glycine	37	2113.0
Glutamic Acid	27	3490.0
Serine	24	2090.0
Aspartic Acid	20	2303.0
Alanine	16	1140.0
Leucine	11	1247.0
Phenylalanine	9	1325.0
Lysine	9	1154.0
Tyrosine	7	1142.0
Valine	5	496.0
Threonine	5	906.0
Proline	4	404.0
Isoleucine	4	453.0
Histidine	3	412.0
Methionine	2	262.0
	183	18,536.0
Carbohydrate***		
Mannose	185	29,970.0 (55%)
Water Displaced in Bonding	365	6,570.0
		55,076.0 Daltons

*Composition ratio only approximated because amino acid decomposition due to presence of mannose was not determined; neither were cysteine/cystine, arginine and tryptophan.

**Molecular weight estimated by SLS gel electrophoresis.

***Percent carbohydrate estimated by integrating the areas under the curves on the electropherograms shown in Figures 14B and C. Presence of mannose as the only carbohydrate ascertained by Dr. Hudson.

****Calculations based on linear moiety models.

noted that the degree of branching of the peptide and carbohydrate moieties would alter the amount of water displaced by bonding, with less displacement the greater the branching. The approximation given in Table VI is the best that can now be made considering the limited data available.

It is of some interest to compare characteristics of the M. lysodeikticus membrane glycoprotein (Table VII) to those of the much investigated major glycoprotein in the erythrocyte membrane (for recent report, see ref. 10). With the exception of molecular weight, both molecules seem to possess much the same characteristics. Other then a great-

TABLE VII

CHARACTERIZATION OF THE MAJOR ERYTHROCYTE* AND M. LYSODEIKTICUS MEMBRANE GLYCOPROTEINS

Characteristics	Erythrocyte Glycoprotein	M. lysodeikticus Membrane Glycoprotein
Molecular Weight (daltons)	31,000	55,000
Percent Carbohydrate	66	55
Percent Serine Composition	14	13
Ratio Basic/Acidic Amino Acids	0.64	0.25
Percent Hydrophobic Amino Acids	47	53

*Reported by Winzler and his colleagues (54, 55, 109).

er concentration of carbohydrate present in the erythrocyte membrane glycoprotein, the only large difference in the figures is that between the ratio of basic to acidic amino acids. Considering that recovery of arginine in the M. lysodeikticus membrane glycoprotein was not accomplished, even this difference would actually be less then shown.

The general similarity between the two glycoprotein molecules may be noteworthy; it is felt that the chemical characteristics shown in Tables V and VI are a good basis for any future chemical investigations.

Some Physical Properties of the M. lysodeikticus Membrane Glycoprotein

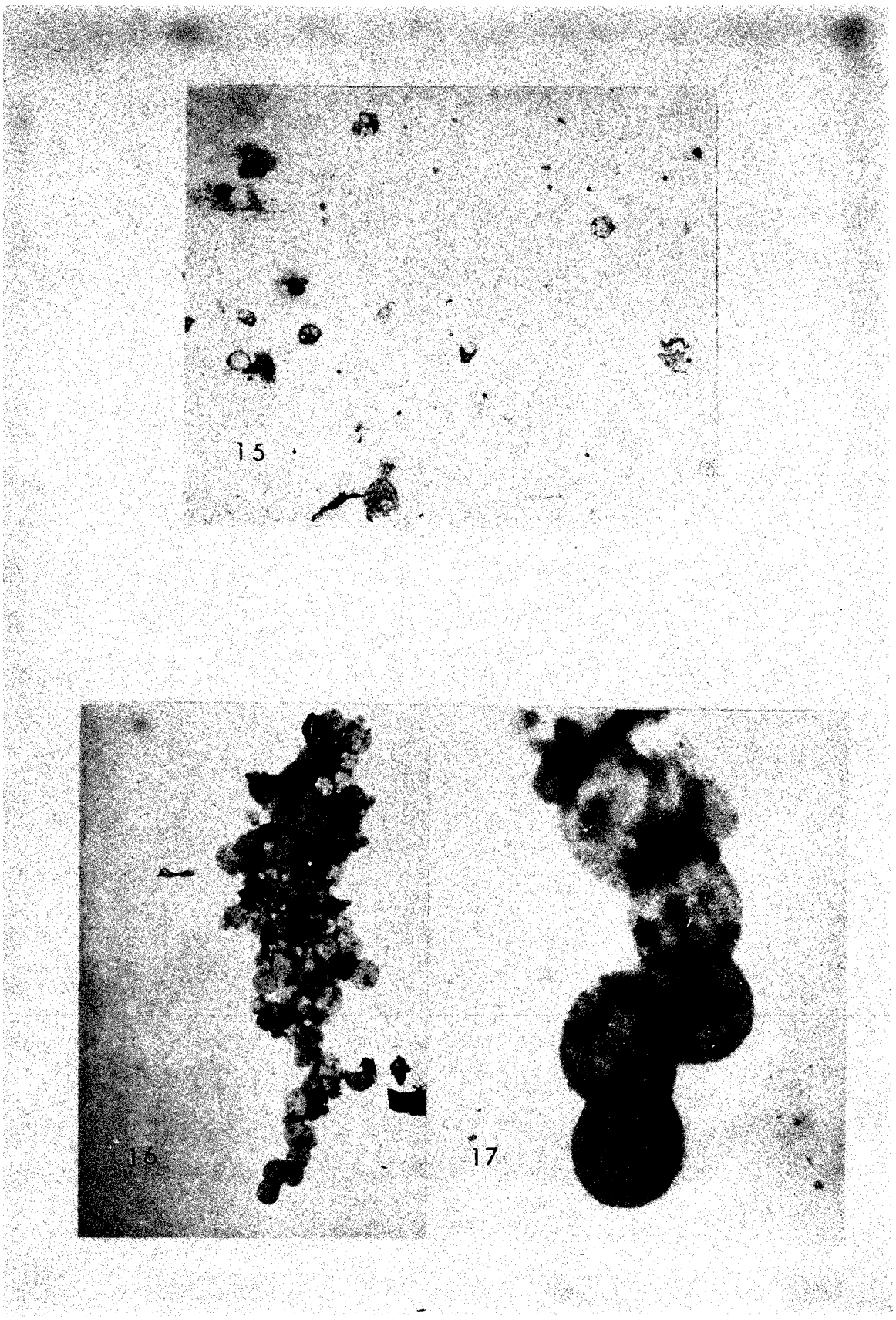
In an earlier section, it was pointed out that the glycoprotein component in the membrane of M. lysodeikticus is oriented such that the carbohydrate moiety, at least that part involved in interaction with Con A, is extrinsic to the membrane continuum. Using isolated membranes, it was not possible to determine if the carbohydrate is exposed on the outer or inner (cytoplasmic) surface. To distinguish between the two possibilities, protoplasts were prepared from M. lysodeikticus and interacted with Con A. It was reasoned that if the carbohydrate moiety is on the outer surface, Con A should cause agglutination of protoplasts.

Electron microscopic examination revealed that untreated protoplasts exist as separate entities, whereas protoplasts treated with Con A are agglutinated (Figs. 15, 16, 17). Furthermore, it seems that untreated protoplasts are readily lysed during preparation for electron microscopy (Fig. 15). On the other hand, treatment with Con A appeared to stabilize the protoplasts in that few ghosts were observed (Figs. 16 and 17). Overall, these data allow the conclusion that Con A binding

igure 15. Protoplast Preparation Made From M. lysodeikticus Cells (5,000X).

igure 16. Protoplasts (M. lysodeikticus) Exposed and Interacted With Con A (5,000X).

igure 17. Same as Shown in Figure 16 (28,000X).


15
16
17

carbohydrate units are present on the external surface of the membrane. Although the results do not eliminate the possibility of an additional internal location, this may be unlikely since carbohydrate is probably not present on both ends of the glycoprotein.

In summary, it seems that a major amount of the carbohydrate portion of the glycoprotein molecule is present on the outer surface of the membrane of M. lysodeikticus. The associated peptide penetrates into and may (perhaps) extend across the entire membrane continuum since the molecule cannot be removed by extensive washing (page 29).

To better understand possible association of the glycoprotein with other proteins in the membrane of M. lysodeikticus, a cross-linking study utilizing glutaraldehyde was undertaken.

Glutaraldehyde has been reported to be an effective protein cross-linking reagent in that this bifunctional compound reacts readily with free amino groups (84). Recently it has been utilized by Steck (96) and Capaldi (18) in cross-linking studies of isolated erythrocyte membranes. Steck suggested that some of the major protein components of the human erythrocyte membrane exist in specific oligomeric associations rather than in a random array, and can be fixed by a cross-linking reagent. Capaldi reports that glutaraldehyde cross-links the bulk of the beef erythrocyte membrane proteins with the exception of the glycoproteins, which are described as 'freely floating'.

In this investigation, extensive cross-linking of the proteins in the membrane of M. lysodeikticus was observed in that less than twenty percent of the protein was soluble after prior exposure of membrane material to four percent glutaraldehyde (Table VIII). Heating to 100 C for 15 min does not appreciably increase solubility. In fact, very

TABLE VIII

AMOUNT OF PROTEIN SOLUBILIZED FROM WHOLE AND GLUTARALDEHYDE TREATED MEMBRANE OF M. LYSODEIKTICUS

Treatment	% of Dry Wt*	% Total Protein
Control Whole Membranes**		
1 N NaOH	58 - 68	100
0.02 M SLS	59 - 64	100
Membranes Treated With 4% Glutaraldehyde**		
1 N NaOH	13	19
0.02 M SLS	12	19
Membranes Treated With 4% Glutaraldehyde***		
1 N NaOH	14	20
0.02 M SLS	12	19

*Protein was estimated by the method of Lowry et al. (67) using bovine serum albumin as a standard.

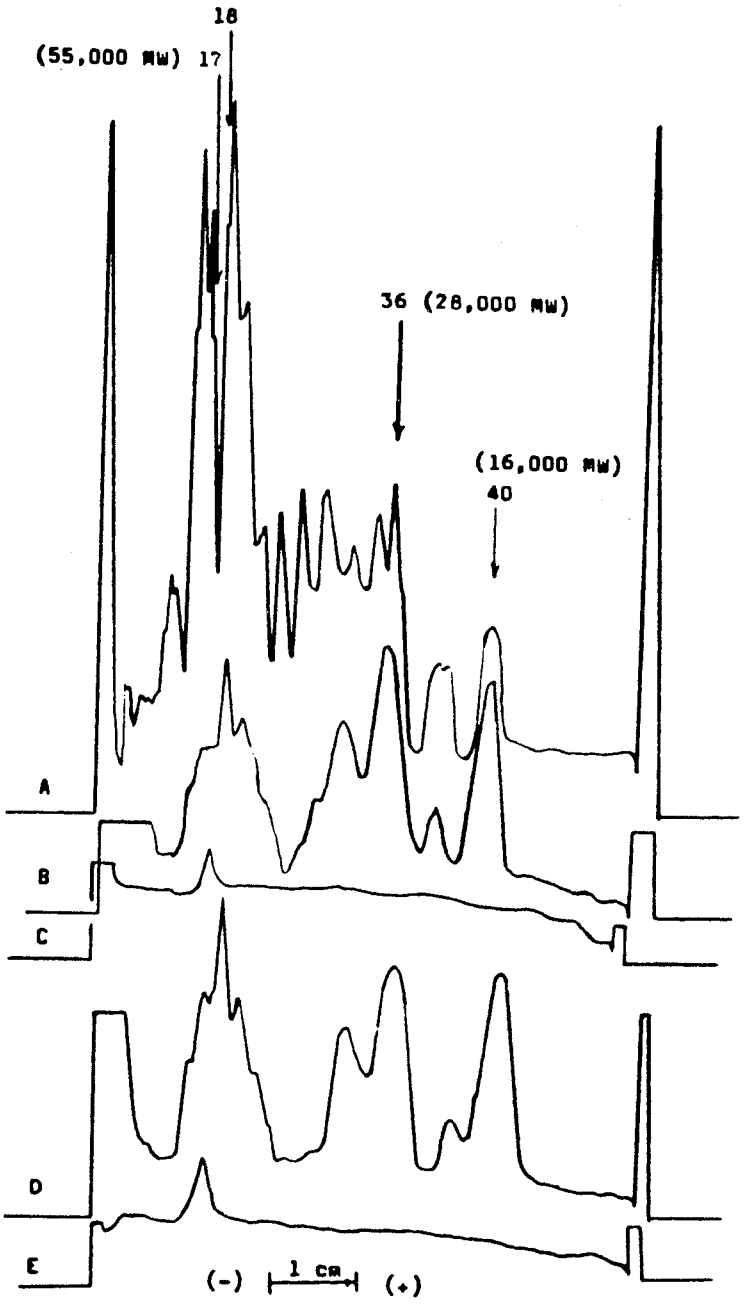
**Incubation in solvent at 37 C for 2 hr prior to protein analysis.

***Heated in solvent to 100 C for 15 min prior to protein analysis.

large amounts of membrane protein never enter the polyacrylamide gel, but rather 'stack' on top (cathode end) even after heating to 100 C in electrophoretic solvent (compare Fig. 18A to 18B and 18D). It is apparent that the solubility of all or most of the components are significantly effected by the reagent, with the exception of the glycoprotein number 17), component 18 and several lower MW (10,000-40,000 dalton range) protein components. It can readily be seen that not all the components that react with the reagent are proportionally effected; some

Figure 18. Electropherogram Component Patterns (Detergent Gel System) of Membrane From M. lysodeikticus Treated With CBB Stain (A, B and D) or PAS Reagent (C and E).

A. Whole Membranes (150 ug).
B. Whole Membranes Treated With 4% Glutaraldehyde, and Incubated at 37 C for 2 hr in Electrophoretic Solvent Prior to Analysis (Stained for Protein).
C. Duplicate Gel of B Sample, but Stained for Carbohydrate.
D. Whole Membranes Treated With 4% Glutaraldehyde, and Heated to 100 C for 15 min in Electrophoretic Solvent (Stained for Protein).
E. Duplicate Gel of D Sample, but Stained for Carbohydrate.


18
(55,000 MW) 17
36 (28,000 MW)
(16,000 MW)
40
A
B
C
D
E
(-) 1 cm (+)

re completely removed like the higher MW components (above 70,000 dal-ons), whereas others are only partially removed. Heating to 100 C for 5 min appeared to decrease the amount of material 'stacked' on the gel op and slightly increased the amount of solubilized components describ-d above (compare Fig. 18B to 18D). This information indicates that hese components are not significantly cross-linked to the other mem-rane components by glutaraldehyde, but rather appear to be trapped up-n membrane treatment and fixation.

Although not shown, it was found that removal of phospholipids and arotenoid pigments from membranes prior to glutaraldehyde treatment oes not significantly affect the electropherogram component patterns. his information indicates that lipid may not be responsible for pre-enting reaction between glutaraldehyde and those components which do ot cross-link. It would seem that these data are not in agreement with he fluid mosaic model (92) in that the non-cross-linked components do ot appear to be embedded in a matrix of phospholipid. It is entirely ossible that these components may not cross-link because they do not ossess available free amino groups to react with the glutaraldehyde.

The above data indicate that there is close association between ost of the protein components in the membrane of M. lysodeikticus since hey are readily cross-linked with glutaraldehyde. A few components including the glycoprotein molecule) do not appear to be in close asso-iation with the majority of the protein components or, else, they do ot contain available free amino groups to allow cross-linking. Overall, hese data are in good agreement with the findings of Steck (28, 96) and apaldi (18), and are consistant with previous reports demonstrating the reat mobility (translational diffusion) of glyroprotein components (29,

.00, 73, 110), Con A binding sites (4, 53) and antigens (25, 61) in nembranes.

In an attempt to better understand topographical relationships of :he glycoprotein component, further Con A agglutination studies were)erformed in the presence of pantoyl lactone (PL). A major site of ac-:ion of this compound has been shown to be the cell membrane (40, 39) /herein it appears to cause alteration(s) or perturbation(s) which de-:rease fluidity within the structure (49).

The data shown in Table IX reveal that presence of PL definitely iffects the membrane agglutination reaction; however, unexpected results /ere obtained. At a concentration of 0.11 M or less, PL stimulates ag-;lutination; whereas above this concentration, PL exerts an inhibiting :ffect. Because agglutination is eventually complete at all concentra-:ions of PL tested, the overall effect of PL on the Con A-membrane ag-;lutination process is on rate of the reaction. Since varying levels of 'L cause an increase or decrease in rate of agglutination response, it ippears that the carbohydrate portion of the glycoprotein may move about)r become partially covered or uncovered by other membrane components. /hatever the mechanism, it is evident that the environment of the gly-:oprotein is not a static one.

Overall, the data presented do not agree with information previous-.y reported by Hopfer (49). He reported that agglutination of horse :rythrocytes was reduced in the presence of 0.077 M PL. For several :easons, it is difficult to compare results directly. The most signif-.cant is that different type membranes were utilized, and PL might af-:ect each uniquely. In addition, different concentrations of PL were :ested; Hopfer used only one, and only the endpoint was read.

TABLE IX

EFFECT OF PANTOYL LACTONE ON AGGLUTINATION OF *M. LYSODEIKTICUS* MEMBRANES BY CON A*

Final Concentration (Molar) of Pantoyl Lactone in Agglutination Reaction Mixture**	Time (hr) for Precipitant Front to Reach Bottom of Tube	Optical Density at 660 nm Time (hr)						
		0.25	0.50	0.75	1.00	1.25	6.50	24.0
0.00	1.00	0.39	0.42	0.48	0.10	0.06	0.02	0.02
0.028	0.25	0.22	0.20	0.18	0.16	0.14	0.05	0.07
0.055	0.50	0.28	0.20	0.18	0.15	0.13	0.05	0.02
0.11	0.75	0.39	0.40	0.19	0.16	0.15	0.07	0.02
0.22	7.00	0.36	0.39	0.41	0.44	0.46	0.20	0.05
0.30	7.00	0.35	0.37	0.39	0.41	0.42	0.19	0.05

*Con A (600 ug/ml final concentration) was reacted with freshly isolated whole membranes (2.0 mg/ml final concentration) in Tris buffer (0.0025 M, pH 6.8) at 25 C.

**Whole membranes were exposed to pantoyl lactone 10 min prior to addition of Con A.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

The major objective of this study was to determine if glycoprotein s present in the membrane of M. lysodeikticus. All proteins that con-ain covalently attached carbohydrate, regardless the extent of glycosy-ation, are defined as glycoproteins. If definitive evidence for such component could be obtained, it was hoped that isolation and purifi-ation could be accomplished, and further studies regarding chemical and hysical characteristics made.

In an earlier investigation (85), whole membranes of this organism ere found to contain a component which could be resolved by polyacryl-mide gel electrophoresis and yielded positive reactions upon treatment ith both protein (Coomassie Brilliant Blue, CBB) and carbohydrate (Per-odic Acid-Schiff, PAS) detecting reagents. This information indicated hat the component could be a glycoprotein; however, because anomalies ill occur in detergent (sodium lauryl sulfate, SLS) electrophoretic ystems unless proper PAS-staining procedures are followed (31), the onclusion was not made at that time.

In light of this information, the first test performed to deter-ine if the membrane component is a glycoprotein involved re-examination f the electrophoretic characteristics. This time, however, the recom-ended PAS staining procedure of Glossman and Neville (31) was utilized. urthermore, characterization was enhanced by use of a non-detergent

el system (72, 41, 85); thereby, eliminating any possible effects of etergent on the carbohydrate detecting reaction.

In the detergent (SLS) system, only one component was found to re-ct positively with the CBB and PAS reagents. It can be resolved from ne other (forty-plus) membrane proteins, and possesses a molecular eight (MW) estimated to be 55,000 daltons. By integrating the areas nder the curves on densitometer tracings of polyacrylamide gels con-aining the component stained for protein and carbohydrate, the percent arbohydrate was judged to be about fifty-five percent.

In the non-detergent system, one component was found which also eacts with both protein (Buffalo Blue Black) and carbohydrate (PAS) eagents.

The data obtained from these two types of gel systems strongly in-icate the membrane molecule to be a glycoprotein; certainly, chance or erroneous interpretations due to detergent anomalies are lessened. ne data, however, do not allow us to ascertain whether or not carbo-ydrate exists covalently attached to protein. It should be pointed ut, though, that the amount and characteristics of migration during lectrophoresis of the component cannot be accounted for on a theoret-cal basis unless it is covalently attached to protein because carbo-ydrate binds SLS only poorly (9, 88).

If the PAS-positive component is not glycoprotein, the other alter-atives are that it is either mannan (a mannose homopolymer) or glyco-ipid. It has been reported that both components are present in the embrane of this organism (65, 59).

It is unlikely that the component is a glycolipid for several rea-ons. The membrane lipids of M. lysodeikticus have been shown (85) to

igrate near the electrophoretic gel front, far ahead of the 55,000 MW omponent. Carbohydrate can be detected in this area when resolved hole membrane components are treated with the PAS reagent; however, hese carbohydrate-containing lipids are most likely mannose-containing lycolipids since stripping (removal of phospholipid and carotenoid maerials) removes them. This information allows the conclusion that the AS-positive component remaining after stripping is not glycolipid, but oes not rule out mannan or glycoprotein.

To determine if the PAS-positive component is a homopolymer of manose or glycoprotein, other experiments were performed. Commercially repared mannan (Sigma) was electrophoretically characterized, and the esults were compared to those of the PAS-positive membrane component. he only similarity is that both molecules yield a positive reaction hen treated with the PAS reagent. This would be expected. The most ignificant difference is that mannan does not react with the protein etecting reagent (CBB). Overall, the fact that the two molecules poses, almost totally, dissimilar characteristics (Table II, page 19) upports the idea that the PAS-positive membrane molecule is a glycoprotein and not a homopolymer of mannose.

To further clarify the situation, another type electrophoretic exeriment involving proteolysis of intact membranes by pronase was perormed. It was reasoned that if the carbohydrate is attached to protein, roteolysis would change the electrophoretic migration rate of the comonent, with movement being faster due to a decrease in molecule mass. reatment resulted in disappearance of the PAS-positive component as ell as all other protein peaks; only a broad CBB-positive zone could be emonstrated near the leading edge of the gel. This appears to be small

V peptide fragments. Since the PAS-positive component could not be ɔund in the supernatant after proteolysis, the conclusion was made that : was degraded. Information from this experiment allows the conclusion ıat the PAS-positive membrane component consists of protein and carbo-ydrate moieties covalently linked together, and therefore most likely s not the mannan molecule described by MacFarlane (65).

Even though presence of glycoprotein in the membrane of M. lyso-eikticus appears to be a distinct possibility, demonstration of the ype linkage between the two moieties would provide additonal proof for xistence of this type molecule. In some glycoproteins, the carbohy-rate moiety is O-glycosidically bonded to the protein through either erine or thronine (95). Such bonding can be broken by a β-elimination rocess under mild alkaline conditions, and results in the conversion f serine and threonine to the respective dehydroamino acids (71). ıese absorb strongly at 240 nm (36), and are easily converted by acid ydrolysis (107) to the keto acid form (pyruvic from serine and α-keto-utyric from threonine).

Treatment of M. lysodeikticus membrane material with mild alkali esults in significant absorption at 240 nm; this indicates the possible resence of O-glycosidic linkages. To determine which amino acid might e involved, two additional analyses were performed on the resulting eto acid product. One was an endpoint enzymatic assay (70) utilizing DH and NADH; the other involved preparation and chromatography of ,4-dinitrophenylhydrazine derivatives. Results from these tests indi-ate the carbohydrate may be attached to protein through the hydroxyl f serine. Unfortunately, cysteine and cystine also yield keto acid esidues by β-elimination under alkaline conditions (19). Like serine,

e product formed after acid hydrolysis is pyruvic acid. Since cys-
ine, although a minor component, is present in the membrane of M.
sodeikticus, data obtained from β-elimination experiments using whole
stripped membranes must be cautiously evaluated. Thus, it appeared
cessary that the glycoprotein be obtained in pure form before the pro-
in to carbohydrate bonding could be correctly studied.

After much testing, a procedure was successfully developed to iso-
te the glycoprotein. Essentially, it involves interaction of Con A
th the glycoprotein, followed by isolation, dissociation and parti-
oning of the two molecules using hot phenol at 67 C. Con A is a phy-
haemagglutinin which does not have any covalently attached carbohy-
ate and selectively binds and precipitates branched residues of the
D-manno- and α-D-glucopyranosyl type (83, 64, 75, 93, 82).

Electrophoretic characterization (detergent system) of the materi-
finally isolated revealed only one component to be present, and it
ntains both protein and carbohydrate as determined by the CBB and PAS
actions. Furthermore, it possesses a mobility rate identical to that
the PAS-positive component (55,000 MW) present in the intact membrane
this organism, and found to consist of protein and mannose in prelim-
ary Lowry (63) and carbohydrate (89, 59, 95) tests. Overall, the data
dicate that this molecule is a glycoprotein which can be separated
om the other M. lysodeikticus membrane protein components.

Based upon amino acid analysis, the glycoprotein molecule appears
have a composition similar to that of the entire protein fraction of
e intact membrane. The most noticeable difference is that serine is
major amino acid in the glycoprotein (thirteen percent, but less than
x percent in the total membrane proteins). The increased ratio of

rine in the glycoprotein might be an indication that attachment of e carbohydrate residues to the polypeptide backbone occurs via the hyoxyl of serine, not at one site but at several. Multi-attachment uld not be unexpected since this is a characteristic of the major glyprotein in the erythrocyte membrane (54).

The amino acid, carbohydrate and electrophoretic information allow approximate chemical composition ratio to be determined. Surprising, the characteristics thus obtained are much like those of the major ycoprotein in the erythrocyte membrane (54, 55, 109). Because of this markable similarity, the M. lysodeikticus membrane glycoprotein comsition ratio can be discussed and studied in light of the information at the erythrocyte glycoprotein extends from one side of the membrane the other (reviewed in ref. 10).

The scheme reported by Bretscher (10) for fitting the major glycootein into the erythrocyte membrane is as follows: "The NH_2-terminal gion and associated carbohydrate are on one side (outer) of the memane, followed by a region of alpha-helix to pass through the membrane pproximately 25 amino acid residues necessary in length since each mino acid traverses about 1.5 angstroms in an alpha-helix), terminated the COOH-end of the molecule (inside) by a very hydrophilic tail".

Data are not available from this study to allow such a precise defnition of the M. lysodeiktieus membrane glycoprotein component. Data e available, however, to aid in determining the location of the manse moiety. Because Con A interacts with and aggregates stabilized otoplasts prepared from this organism, a peripheral positioning of the rbohydrate on the outside of the membrane seems likely. Since the ycoprotein molecule cannot be removed from the membrane by extensive

shing procedures, this could indicate that at least part of the asso-
ated peptide penetrates into and perhaps extends across the entire
mbrane bilayer. Such positioning would be aided by a hydrophobic
ore' within the glycoprotein molecule. Although presence of a 'core'
nnot be documented at this time, it is perhaps significant that a
rge amount (53%) of hydrophobic amino acid residues are present in the
lecule.

Extension of a protein across a membrane would probably be most
aracteristic of a molecule which is not in close association with the
jority of other proteins (18). It is noteworthy that the M. lysodeik-
cus membrane glycoprotein shows little tendency to cross-link to other
mbrane components upon treatment with glutaraldehyde.

In terms of membrane order (defined as being in an organized or
tural state), a molecule that crosses the membrane would probably be
pected to be present in the early phases of membrane fabrication. If
e disaggregation-reaggregation process of the membrane into ordered
eet structures can be likened to fabrication within the viable bacter-
m, then data are available (85) which reveal that the 55,000 MW gly-
protein component enters the reaggregated membrane structure well be-
re entry of the majority of other protein components.

During this study, the procedure described by Marchesi and Andrews
isolate glycoprotein from human erythrocyte membranes (66) was used
an attempt to isolate the 55,000 MW glycoprotein molecule from the
lysodeikticus membrane. The material obtained has been shown to
ve essentially the same polyacrylamide electrophoretic characteristics
commercial mannan (Table II, page 19), and it appeared possible that
is is the mannan described by MacFarlane. Both amino acids and carbo-

'drate were, however, detected in this component (MW approximately)0,000 daltons). Such information indicates that this molecule may al-) be a glycoprotein. Unfortunately, MacFarlane gave no information re- ırding possible amino acid content of the 'mannan' she isolated. Thus, .though it is only conjecture, the mannan she isolated and the compo- ent isolated during this study using lithium diiodosalycylate (and now ıought to be another glycoprotein) may be the same component. Further :sting is necessary, though, before any definitive conclusion can be :ached.

It should be pointed out that protein would not have been detected ı this large membrane molecule (100,000 MW) without an amino acid anal- ;is. Perhaps other membrane and envelope molecules, which are reported) be only carbohydrate polymers such as the hexose polymer reported 'esent in the A. laidlawii membrane (101) , should be re-examined in ıis respect.

The recent observations of Owen and Salton (76) are also pertinent) this discussion. These investigators report the presence of weakly ;sociated mannose-polymers in membrane fractions isolated from this :ganism, and these are removed by washing in low concentrations of buf- ;rs. A glycoprotein component is also present, but no information as) characteristics were made available (personal communication to Dr. E. Grula). The tentative information as to presence of at least one .ycoprotein supports the findings reported in this thesis. It would ; interesting if amino acids are present in the mannose polymers.

Another report published during the writing of this manuscript de- :ribes the presence of at least three glycoproteins in the membrane of lysodeikticus (27). Examination of the electrophoretic data pre-

ented indicates that the conclusions of these investigators are questionable. This is based on several points. First, stacking of sample n top of the gel (a result of incomplete membrane solubilization) is pparent in their pictorial data. Second, the pattern presented for ne whole membrane does not even resemble the patterns previously reorted for this organism (77, 41, 85). Third, nothing is mentioned as o whether measures were taken to eliminate any possible occurrence of nomalies due to presence of detergent in the electrophoretic system tilized.

In summary, one glycoprotein molecule (55,000 MW) and possibly nother (approximately 100,000 MW) are present in the membrane of M. ysodeikticus. The amino acid compositions of the peptide moieties of nese two molecules are similar and indicate that the peptides may be elated. The carbohydrate moiety of the 55,000 dalton component consists of mannose, and may be linked to the peptide through serine. rthermore, the mannose moiety is extrinsically located, and indirect ata indicate the peptide penetrates deep within and possibly across ne bilayer membrane continuum.

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