ISOLATION AND CHARACTERIZATION OF GLYCOPROTEINS FROM THE MILK FAT GLOBULE MEMBRANE

By

LLOYD DALE SNOW

Bachelor of Science
Arkansas State University
Jonesboro, Arkansas
1969

Master of Science
Arkansas State University
Jonesboro, Arkansas
1972

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
December, 1976

ISOLATION AND CHARACTERIZATION OF

GLYCOPROTEINS FROM THE MILK FAT

GLOBULE MEMBRANE

Thesis Approved:

Kenneth Carraway
Thesis Adviser

Richard C. Essenberg

E. M. Hodnett

Roger E. Koeppe

Donald C Abbott

Norman N. Durham
Dean of the Graduate College

ACKNOWLEDGEMENTS

The author gratefully acknowledges the advice, understanding and patience of his major advisor, Dr. Kermit L. Carraway, during the course of the investigations and in the preparation of this thesis. He also wants to acknowledge and thank Drs. Roger E. Koeppe, Ernest M. Hodnett, Richard C. Essenberg, and Donald C. Abbott for their valuable time spent as members of the advisory committee and for their contributions towards the preparation of this thesis. A very special thanks is extended to Dr. George V. Odell who served as an advisor during Dr. Carraway's absence.

The technical assistance provided by Robert Doss, Keith Hodges, and Gordon McCraw during portions of this study is appreciated. Gratitude is expressed to Kevin Castleberry for preparing many of the figures in this thesis and to Dr. George Odell for making this service available. Thanks are extended to Dr. Douglas Colton for performing the carbohydrate analyses and to Dr. Robert Chesnut and Ms. Sandy Cantwell for performing the lectin agglutination assays. Thanks are extended to Dr. T. H. Liao for obtaining amino acid analyses. Financial assistance and facilities provided by the Oklahoma State University Biochemistry Department are gratefully acknowledged.

The author is grateful to Dr. John Kenneth Beadles for his constant faith, support and encouragement of the author during the early portion of his graduate career.

Special appreciation is expressed to my parents for their continual encouragement, understanding, patience, and financial support during the course of this study.

TABLE OF CONTENTS

Chapter		Page
I.	INTRODUCTION	1
II.	EXPERIMENTAL PROCEDURE	12
	Materials	12
	Methods	12
	Preparation of Milk Fat Globule Membranes	12
	Selective Extraction of Glycoproteins with Chloroform/Methanol/Water	13
	Labeling of Glycoprotein Sialic Acid Using ^{3}H-KBH_4	14
	SDS-Hydroxylapatite Chromatography of MFGM Glycoproteins	14
	SDS Gel Filtration of MFGM Glycoprotein-2	15
	SDS Acrylamide Gel Electrophoresis	16
	Solubilization of 5'-Nucleotidase	16
	Preparation of Divalent Concanavalin A	17
	Affinity Chromatography of Glycoproteins on Con A-Agarose	17
	Enzyme Purification	18
	Acrylamide Gel Electrophoresis without SDS	19
	Enzymatic Assays	19
	Analytical Procedures	20
III.	RESULTS	21
	Purification of the Major Membrane Glycoproteins	21
	Chromatography of Membrane Preparations in SDS	21
	Chromatography of Selectively Extracted Glycoproteins	29
	Characterization and Purification of 5'-Nucleotidase	52
	Assay Conditions	52
	Effects of Detergent and Lectin Perturbation on 5'-Nucleotidase	54
	Effect of Temperature on Enzyme Activity and Lectin Perturbation	77
	Affinity Chromatography of 5'-Nucleotidase on Con A-Agarose	89
	Purification of 5'-Nucleotidase	95
IV.	DISCUSSION	111

Chapter Page

V. SUMMARY 121

A SELECTED BIBLIOGRAPHY 123

LIST OF TABLES

Table		Page
I.	Comparison of Amino Acid Composition of MFGM, RBC Membranes, and Isolated Glycoproteins[a]	49
II.	Carbohydrate Composition of Glycoprotein-2	51
III.	Effect of Incubation Time and Mg^{+2} Concentration upon 5'-Nucleotidase Activity	53
IV.	Comparison of the Biological Activities of Con A and Succinyl-Con A	80
V.	Effect of Temperature on Hill Coefficients of 5'-Nucleotidase	86
VI.	Purification of 5'-Nucleotidase from MFGM	98

LIST OF FIGURES

Figure		Page
1.	SDS Gel Electrophoresis of MFGM	6
2.	Chromatography of SDS-Solubilized MFGM on Sepharose 4B in 1% SDS	23
3.	Hydroxylapatite Chromatography of Solubilized MFGM in 0.1% Dodecyl Sulfate	26
4.	Acrylamide Gel Electrophoresis of Hydroxylapatite Fractions of Whole Membrane	28
5.	Acrylamide Gel Electrophoresis of Chloroform/Methanol/Water Extract	32
6.	SDS Acrylamide Gel Electrophoresis of Tritium Labeled Glycoprotein Extract	34
7.	Chromatography of Tritium Labeled Glycoprotein Extract on Hydroxylapatite	37
8.	Polyacrylamide Gel Electrophoresis of ^{3}H-labeled Glycoproteins Fractionated by Hydroxylapatite Chromatography	39
9.	Sephadex G-200 Chromatography of Chloroform/Methanol Extract in 1% Dodecyl Sulfate	41
10.	Acrylamide Gel Electrophoresis of Sephadex G-200 Fractions	43
11.	Acrylamide Gel Electrophoresis of Purified MFGM Glycoprotein-2	46
12.	SDS Acrylamide Gel Electrophoresis of Purified Glycoprotein-2 on Gels of Varied Acrylamide Concentration	48
13.	Effect of pH on the Activity of 5'-Nucleotidase	56
14.	Effect of Increasing Concentrations of Deoxycholate on 5'-Nucleotidase Activity	58
15.	Solubilization of Membrane Protein and 5'-Nucleotidase Activity with Deoxycholate	60

Figure Page

16. Acrylamide Gel Electrophoresis of Deoxycholate Solubilized Membranes 62

17. Concentration Dependence of Con A Inhibition of 5'-Nucleotidase Membrane-bound 65

18. Hill Plot for Con A Inactivation of Membrane-bound 5'-Nucleotidase 68

19. Lineweaver-Burke Plot of Velocity Versus Substrate Concentration for the Membrane-bound 5'-Nucleotidase 70

20. Lineweaver-Burke Plot of Velocity Versus Substrate Concentration for the DOC-solubilized 5'-Nucleotidase 72

21. Concentration Dependence of Con A Inhibition of DOC-solubilized 5'-Nucleotidase 74

22. Hill Plot for Con A Inactivation of DOC-solubilized 5'-Nucleotidase 76

23. Effect of WGA and SBA on 5'-Nucleotidase Activity 79

24. Changes of 5'-Nucleotidase Activity with Temperature 82

25. Arrhenius Plots of 5'-Nucleotidase Activity 85

26. Alteration of Hill Coefficient for 5'-Nucleotidase-Con A Interaction by Temperature 88

27. Affinity Chromatography of 5'-Nucleotidase 91

28. Acrylamide Gel Electrophoresis of Con A-Agarose Chromatography Fractions 94

29. Sucrose Density Gradient Centrifugation of Solubilized 5'-Nucleotidase 97

30. SDS Gel Electrophoresis of Fractions from a Sucrose Gradient Containing Detergent 100

31. Sepharose 4B Gel Filtration of Enzyme Peaks from Sucrose Gradients 103

32. Gel Filtration of Partially Purified 5'-Nucleotidase on Sephadex G-200 105

33. SDS Gel Electrophoresis of 5'-Nucleotidase Fractions from a G-200 Column 107

34. Distribution of Protein Staining and Enzymic Activity in a Polyacrylamide Gel in DOC-Tris Medium 110

NOMENCLATURE

5'-AMP	- adenosine 5'-monophosphoric acid
ATP	- adenosine triphosphate
BSA	- bovine serum albumin
Con A	- Concanavalin A
CB	- Coomassie Blue
CPM	- counts per minute
DOC	- deoxycholate
EDTA	- ethylenediaminetetraacetic acid
GLC	- gal-liquid chromatography
GP	- glycoprotein
13762 MAT-A	- 13762 mammary ascites adenocarcinoma cells
MFGM	- milk fat globule membrane
α-MM	- α-methylmannoside
μM	- micromolar
mM	- millimolar
MW	- molecular weight
PAS	- periodate-Schiff
SA-180	- Sarcoma 180 ascites tumor cells
SDS	- sodium dodecyl sulfate
SBA	- soy bean agglutinin
Tris	- tris-(hydroxymethyl)-aminomethane
UDP	- uridine diphosphate
WGA	- wheat germ agglutinin

CHAPTER I

INTRODUCTION

Biological membranes have a crucial role in almost all cellular phenomena. An understanding of the structural attributes of the proteins of the mammalian plasma membrane is essential to comprehending their roles in membrane phenomena, including the control of many cellular activities (1). Only in the case of the erythrocyte membrane have such structural studies received significant attention. Glycoproteins are of great interest as integral components of mammalian membranes because of their suspected role in several functions of the cell surface such as cell association, contact phenomena and growth control, recognition of foreign or tumor cells and a number of other biological properties (1, 2). The fat globules of bovine milk are surrounded by a membrane (MFGM) derived primarily from plasma membranes of the secretory mammary cells (3). The ready availability of cream and the ease of membrane preparations makes this membrane an excellent source of material for investigations of plasma membrane protein and glycoprotein components.

Formation of milk fat droplets begins at the endoplasmic reticulum in the basal region of the alveolar cell (4, 5). The droplets increase in size as they move toward the apical end of the cell (6). The droplet surface becomes enveloped by apical plasma membrane which causes the droplet to protrude from the cell surface and into the lumen of the

mammary duct system. Droplet secretion from the cell is accomplished by severing the plasma membrane connection between the droplet and the cell surface (7). In bovine milk, the free milk fat globules range in diameter from 1 to 10 μ. Although general physical forces have been considered responsible for promoting the secretion process (3), a possible role of microtubules in mediating this process has been suggested by the observation that colchicine and vincristine which disassemble microtubules will also inhibit fat globule and aqueous phase secretions of the lactating goat (8).

The evidence for this secretory process has been obtained from electron microscopy and by biochemical comparison of MFGM with isolated mammary plasma membrane. The envelopment of fat globules in plasma membrane and their extrusion from the cell has been well documented by electron microscopy (7). The surface of the intracellular fat droplet may possess adsorbed proteins, phospholipids, and gangliosides which may adhere to isolated MFGM (9, 10). Little entrainment of cytoplasmic organelles or intracellular membrane fragments occurs in bovine MFGM secretion since characteristic constituents or enzyme activities of these components are absent or present in low levels. However this property varies between species, since in rat and human MFGM secretion, intracellular membrane inclusions may occur in significant amounts (11, 12). Sucrose density centrifugation has been used to separate three fractions of MFGM with identical SDS gel electrophoresis profiles (13). These results suggest that there is apparently one MFGM type which varies in lipid content due to varying retention of lipid.

MFGM and plasma membranes of mammary and liver tissue have identical phospholipid distribution patterns (3). The phospholipid distribu-

tion patterns of intracellular membranes differ significantly from plasma membrane and MFGM. Similarity of mammary plasma membrane and MFGM electrophoresis profiles have also been noted (14). In spite of biochemical and morphological similarities, isolated MFGM differ structurally from plasma membrane in that they fail to form vesicles and have a rigid plate-like structure in contrast to the vesicle-forming ability of plasma membranes (14).

Wooding (15) examined the structure of MFGM in whole milk by thin sectioning techniques and proposed that portions of the unit membrane are gradually lost after secretion through vesiculation and fragmentation leaving blebs of plasma membrane scattered across the globule surface. Using thin sections and freeze etching, Hensen et al. (16) proposed a rearrangement of the MFGM after secretion. Some biochemical studies have supported Wooding's blebbing theory. Radioactive labelling of intact and isolated MFGM suggested that all protein groups were partially exposed at the membrane surface (17). Proteolytic digestion of MFGM with trypsin or pronase resulted in cleavage of all the major membrane proteins as determined by SDS gel electrophoresis (18).

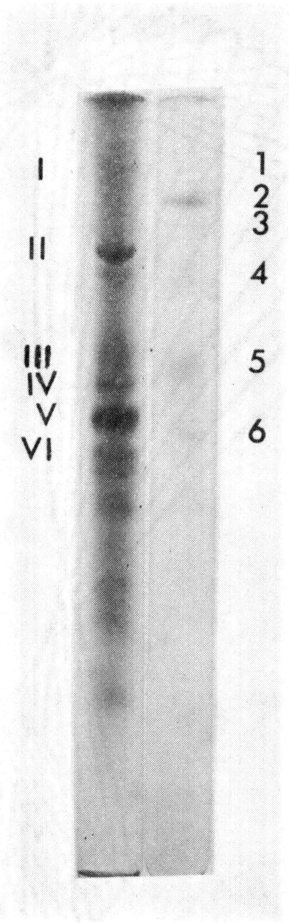
Recently new proposals have been made concerning MFGM structural features which suggest that some element of protein asymmetry may be retained by MFGM. Mather and Keenan (19) reported differences in trypsin catalyzed rates of hydrolysis of protein in intact and isolated MFGM. Some membrane proteins also seemed more accessible to lactoperoxidase-catalyzed iodination in isolated membranes than in intact globules. Isolated membranes and intact globules react to the same extent with Concanavalin A (20) and neuraminidase (19) which suggests that glycoprotein carbohydrate moieties are externally disposed.

Braumrucker and Keenan (21) followed the distribution of 5'-nucleotidase, adenosine triphosphatase, and phospholipid between fat and skim milk at intervals over 96 hours after milking. There was little change in enzyme distribution, but phospholipid in skim milk increased with time.

The contribution of intracellular constituents to the surface layer of milk fat droplets before secretion has been examined. Wooding (22) perfused caprine udder with glutaraldehyde and found intracellular vacuoles containing both lipid droplets and casein. The structural appearance of the membrane was identical to MFGM and lead him to conclude that MFGM could be formed without plasma membrane participation. Direct fixation with OsO_4^- of lactating rat mammary tissue reveals a noncontiguous thin limiting membrane around intracellular droplets. The membrane appears to represent one-half of a cisternal bilayer of agranular endoplasmic reticulum which may serve as a site for nucleation of droplet development (3). It was estimated on the basis of b-type cytochrome distribution that this membrane could contribute no more than 10% of the protein in MFGM fractions. Hood and Patton (9) isolated fat droplets from mammary tissue but found no evidence of ordered structure on the surface. They concluded that intracellular fat droplets were stabilized by a phospholipid-cholesterol layer similar to that of chylomicra.

In the molecular weight range of 15,000 to 240,000, SDS gel electrophoresis permits the identification in the MFGM (Figure 1) of at least six major Coomassie blue staining polypeptides (13, 19, 23, 24) and at least six major periodate-Schiff (PAS) staining glycoprotein bands (13, 23, 25, 26). Anderson *et al.* (23) employed SDS gradient gels

Figure 1. SDS Gel Electrophoresis of MFGM. MFGM were solubilized for SDS gel electrophoresis as described in Experimental Procedure. The gels were stained for protein (left) and carbohydrate (right). Protein and glycoprotein components of MFGM are labeled by roman and arabic numerals, respectively.


I
II
III
IV
V
VI
1
2
3
4
5
6

to obtain more reliable estimates of MFGM glycoprotein molecular weights. Three apparently high molecular weight MFGM glycoproteins behaved anomalously on SDS gels and did not stain with Coomassie blue while three lower molecular weight glycoproteins were apparently the same as three of the six major Coomassie blue staining protein bands in their preparation.

Selective solubilization of MFGM components has been limited since all MFGM components appear to be solubilized to the same extent by most methods. Some selectivity in extraction of protein by sodium deoxycholate occurs since 82% of the 5'-nucleotidase activity is extracted under conditions which keep the MFGM intact as determined by electron microscopy (27). The Mg^{++}-stimulated adenosine triphosphatase (Mg-ATPase) was also selectively solubilized but its recovery was low (27). Extraction experiments with EDTA, NaCl, and guanidine hydrochloride led to some discrimination in solubilization of membrane proteins (13). Two membrane components of approximately 43,000 and 48,000 daltons were selectively extracted from MFGM with 1.5 M $MgCl_2$ (19). Allen and Humphries adapted gradient isoelectric focusing for membrane component isolation by incorporating zwitterionic surfactants in the gradient (28). Previously this technique was of little use since many membrane components tended to precipitate.

Several attempts have been made to characterize glycoprotein containing fractions derived from MFGM. Coulson and Jackson (29) recovered a water-soluble glycoprotein by precipitation of buttermilk from washed cream with ammonium sulfate, removal of fatty and phospholipid components with alcohol and ether, dispersion in water, dialysis, and lyophilization. The isolated material contained a mucoprotein of

123,000 daltons which contained two contaminants. Swope (30) and Cawston (31) have reported the isolation of MFGM glycoprotein species. Swope (30) prepared colloidally dispersed membrane fragments from washed cream and classified them by differential ultracentrifugation into three pellet fractions. Each pellet fraction was lyophilized and extracted with clororform/methanol (2:1). The residue proteins were suspended in 1 M KCl, centrifuged, and the clear supernatants were saved. A glycoprotein fraction appeared to be selectively associated in one of the pellet fractions. It remains uncertain if the isolated MFGM glycoproteins represent the same species or 2-3 individual glycoproteins.

The enzymes with highest specific activity and associated with MFGM are those characteristic of plasma membrane (3, 31). Golgi apparatus, endoplasmic reticulum, and mitochondrial enzyme markers are absent or present in low specific activity. Studies of the MFGM enzyme complement have been limited to measurement of activity in most cases. The enzymes xanthine oxidase and alkaline phosphatase have been purified from milk and may also be associated loosely with the MFGM (32, 33). Four washes with water of MFGM released 85% of the xanthine oxidase and alkaline phosphatase activities (34). Alkaline phosphatase of MFGM is unusually stable in SDS and can be located in SDS gels (35). Assay of 5'-nucleotidase before and after membrane release suggests that all the enzyme is located on the outer MFGM surface (36). Nucleotide pyrophosphatase and Mg-ATPase may be at least partially located on the inner membrane surface. Extensive shedding of 5'-nucleotidase and Mg-ATPase has not been observed (21). An MFGM preparation fractionated by isoelectric focusing contained two peaks of 5'-nucleotidase activity with

isoelectric points of pH 4.9 and 5.9 (28). Huang and Keenan (37) solubilized 5'-nucleotidase activity from MFGM with deoxycholate and resolved two fractions of activity on gel filtration after employing the isolation technique of Widnell and Unkeless (38). Based on substrate specificity, K_m, and metal ion effects, they concluded that there were two forms of the enzyme in MFGM. The ATPase activity of MFGM has been characterized (39). The activity is stimulated by Mg^{++}, slightly stimulated by K^+, not stimulated by Ca^{++}, and not affected by Na^+ or ouabain.

The enzyme 5'-nucleotidase is located primarily in the plasma membrane of mammalian cells and is used as a marker enzyme in membrane isolation (40). The physiological function of the enzyme is not clear. Adenosine produced by the enzyme is vasodilatory, and the regulation of the enzyme by adenine nucleotides is postulated to be a mechanism for the autoregulation of blood flow in myocardial and skeletal muscle (41). Adenosine has also been implicated as a neurohumoral agent in brain (42). Adenosine has been examined for possible involvement in the process of hormonal stimulation of lipolysis in rat fat-cells (43). Kim et al. (44) suggested that the activity of the enzyme may vary as a function of the metastasizing ability of tumors. Enzyme activity may be altered in some neoplastic tissues (45). Partially purified preparations of the enzyme have been characterized from bull seminal plasma, rat liver, rat heart, porcine smooth muscle and brain (43), rat fat-cells (42), MFGM (37), rat mammary gland (46), and 13762 mammary ascites tumor cells.

Widnell and Unkeless (38) purified the enzyme from rat liver plasma membrane by means of detergent solubilization followed by

ammonium sulfate fractionations, heat and pH denaturation steps, and gel filtration. Evans and Gurd (40) solubilized the enzyme from mouse liver plasma membrane with detergent and purified the enzyme by sucrose gradients and gel filtration. 5'-nucleotidase was a glycoprotein of 140,000 molecular weight and composed of two active and similar subunits.

Concanavalin A (Con A), wheat germ agglutinin (WGA), and soy bean agglutinin (SBA) are plant proteins called lectins which can bind to specific carbohydrate determinants on the surface of mammalian cells (2, 47). They can agglutinate various normal and neoplastic animal cells. Con A can stimulate mitosis and blastogenic transformation of lymphocytes, inhibit phagocytosis by polymorphonuclear leukocytes, prevent lymphocyte cap and patch formation induced by anti-immunoglobulin, and produce insulin-like effects in isolated adipocytes (48). These effects are probably initiated by Con A binding to an oligosaccharide chain at the cell surface. The mechanisms of the subsequent effects of Con A interaction are largely obscure.

Lectins are currently being extensively used as tools for the investigation of a wide variety of phenomena at cell surfaces as well as many properties of the surface plasma membrane (2). Con A has been shown to inhibit 5'-nucleotidase of plasma membrane from rat liver (49), rat mammary gland (46), 13762 rat ascites tumor, and C6 glioma cells (50). There are apparently two forms of 5'-nucleotidase in rat mammary gland plasma membrane with K_m of 30 μM and 2 mM (46). The enzyme is inhibited by Con A in a process that is apparently noncompetitive and reversible by incubation with α-methylmannoside. The inhibition exhibits no cooperativity in the intact 13762 ascites cell or its

membrane envelopes but shows substantial cooperativity when the membranes are in vesicular form or are solubilized in deoxycholate. In contrast, normal rat mammary gland plasma membrane enzyme exhibited cooperativity in the inhibitory process in both membrane-bound and solubilized forms.

The object of this study was to develop techniques for isolation of MFGM glycoprotein components and characterize them by biochemical methods. This information may reveal features of polypeptide or carbohydrate structure which are significant to their incorporation into the membrane or to their function. One of the glycoproteins studied is 5'-nucleotidase. Con A has been utilized as a probe to investigate the constraints imposed on the enzyme by its association with the membrane, the molecular requirements for enzyme-lectin interaction, and the mechanism of the perturbation involved.

CHAPTER II

EXPERIMENTAL PROCEDURE

Materials

Raw cream was obtained from the Oklahoma State University Dairy. Radioactive potassium borohydride (^3H-KBH_4) was obtained from New England Nuclear. All enzymes and chemicals for enzyme assays were products of Sigma Chemical Company. Highly purified lectins were obtained from Sigma Chemical Company or Miles-Yeda, Ltd. Chemicals for electrophoresis were obtained from Eastman or Bio-Rad. Columns and chromatographic supports were obtained from Pharmacia. Other chemicals were reagent grade or highest purity available. Bio-Gel HT (hydroxylapatite) was a product of Bio-Rad. Glycoselyx-A (Con A-Agarose) was purchased from Miles-Yeda, Ltd.

Methods

Preparation of Milk Fat Globule Membranes

Chilled milk was passed through a DeLeval separator at the Oklahoma State University Dairy, and a raw cream fraction was collected. The raw cream fraction was washed by three suspensions in 0.25 M sucrose, 10 mM imidazole, 2 mM $MgCl_2$ (pH 7.0) at room temperature (13). The washed cream was suspended to a concentration of 33 per cent cream in the same buffer. The 33 per cent cream suspension was subjected to

one cycle of freezing at -20° C for 20 hr and thawing to a temperature of 37° C. Membranes were isolated from the thawed 33 per cent cream suspension by centrifugation at 40,000 x g for 1 hr at 5° C and washed twice by centrifugation. Membranes were dialyzed overnight against water at 4° C before use for protein and enzyme assays. In some experiments noted below, the washed cream was utilized for extraction without preparing membranes.

Selective Extraction of Glycoproteins with Chloroform/Methanol/Water

One volume of washed cream or MFGM (10 mg protein/ml) was mixed with 9 volumes of chloroform/methanol (2:1) for 30 min at room temperature. Two volumes of water were added and mixing continued for 15 min (51). Using separatory funnels and centrifugation at 4000 x g for 15 min, the upper aqueous phase was recovered. A second cycle of chloroform/methanol extraction was included for most cream extractions. The aqueous phase, which contained the solubilized glycoproteins, was concentrated by rotary evaporation to 50 to 150 ml and centrifuged at 100,000 x g for 1 hr. The supernatant was dialyzed exhaustively against cold distilled water. The extract was concentrated by covering the dialysis bag with Aquacide flakes (Calbiochem). The concentrated extracts were characterized by SDS gel electrophoresis and assays for sialic acid, neutral sugar, and protein. Extracts were stored at -20° C.

Labeling of Glycoprotein Sialic Acid Using ^3H-KBH_4

Glycoproteins were suspended in 10 mM phosphate, 0.1% SDS (pH 8.0) at a concentration of 1 mg sialic acid/5 ml buffer (52). Sialic acid residues were modified by oxidation in 0.1 M sodium metaperiodate with a two-fold molar excess (IO_4^-/sialic acid). The solution was agitated frequently at room temperature for 10 min. Oxidation was stopped by the addition of 0.1 M glucose to consume excess periodate. The sample was dialyzed against distilled water and 8 mM phosphate, 0.1% SDS (pH 8.0). Modified sialic acid residues were reduced by incubating with 1 μCi ^3H-KBH_4/0.1 mg sialic acid at room temperature for 30 min with frequent agitation. After 30 min, enough $NaBH_4$ in one ml of buffer was added to give a 30:1 $NaBH_4$/sialic acid ratio, and the solution was agitated frequently at room temperature for 10 min. After 10 min, the pH was adjusted to 4.0 with 2 N HCl and kept cold for 10 min or until gas evolution stopped. The pH was then raised to 6.0 with 2 N NaOH, and the sample dialyzed against water at 4° C.

SDS-Hydroxylapatite Chromatography of MFGM Glycoproteins

Membrane samples or extracted glycoprotein samples were solubilized in 1.0% SDS, 10 mM phosphate (pH 6.4) and 1% mercaptoethanol. Solubilized protein samples were dialyzed overnight against 0.1% SDS, 10 mM phosphate (pH 6.4), and 2 mM mercaptoethanol. Chromatography was performed on Bio-Gel HT (1.5 x 20 cm) at room temperature. The column was eluted with increasing concentrations of sodium phosphate buffer at

pH 6.4 by means of a continuous gradient formed from mixing 10 mM phosphate and 0.5 M phosphate, both containing 0.1% SDS and 2 mM mercaptoethanol (53). Columns were regenerated after use by removing the top layer of the bed and washing the column with one bed volume of 1 N NaCl followed by at least four column volumes of starting buffer.

SDS Gel Filtration of MFGM Glycoprotein-2

Frozen chloroform/methanol glycoprotein extracts of washed cream were thawed and solubilized by bringing the solution to 2% SDS, 50 mM Tris (pH 7.4), 1% mercaptoethanol and incubating for 2 hr at 37° C. The SDS solubilized glycoproteins were chromatographed on Sephadex G-200 (2.5 x 90 cm; 1.5 x 90 cm) or Sepharose 4B (2.5 x 90 cm) at room temperature using 1% SDS, 50 mM Tris (pH 7.4) and 0.02% sodium azide as the elution buffer. Samples of fractions from protein peaks determined by absorbance at 280 nm were electrophoresed on polyacrylamide (5%) gels in the presence of 0.1% SDS. Fractions enriched in MFGM glycoprotein-2 were pooled, concentrated and rechromatographed in a similar fashion. After the second chromatographic separation, the forepart of the glycoprotein-2 peak was pooled and characterized by SDS gel electrophoresis. Rechromatographed fractions which gave one band on SDS gel electrophoresis were pooled and used for amino acid and carbohydrate analysis.

SDS gel filtration molecular weight determinations for glycoprotein-2 were by using β-galactosidase, bovine serum albumin (BSA) monomer and dimer, α-chymotrypsinogen and cytochrome C as molecular weight standards.

SDS Acrylamide Gel Electrophoresis

Protein samples were dissolved by boiling for 5 min in 4% SDS, 1% mercaptoethanol, 5 mM EDTA, 20% sucrose, 0.01 mg/ml Bromophenol Blue, 50 mM Tris (pH 7.4) at a protein concentration of 2 mg/ml. Ten cm gels were prepared by the method of Weber and Osborn (54) to give a 5% acrylamide concentration (crosslinker/monomer ration, 0.026). After a 30 min prerun, 50-300 µg of protein was applied to the gels and the electrophoresis continued for 3-4 hours at 8 mAmp/gel. Gels were stained for protein and carbohydrate by the method of Fairbanks et al. (55).

Glycoprotein molecular weights were estimated by the method of Segrest and Jackson (56). Plots of the logarithm of molecular weight versus migration distance for a series of standard proteins (β-galactosidase, BSA monomer and dimer, α-chymotrypsinogen, and cytochrome C) at acrylamide gel concentrations of 5, 7.5, 10, and 12.5% were used to determine the apparent molecular weight of glycoproteins at each gel concentration. From the curves obtained by plotting apparent molecular weight versus per cent acrylamide, an estimate of an asymptotic minimal molecular weight was obtained.

Solubilization of 5'-Nucleotidase

In a typical enzyme preparation, 17.5 mg of membrane protein was suspended at 37° C for two hours in 42 ml of 0.25% (w/v) DOC, 50 mM Tris (pH 7.9). The membrane suspension was centrifuged at 100,000 x g for 1 hr and supernatant collected. Around 85% of the 5'-nucleotidase activity was present in the supernatant. The 5'-nucleotidase activity

could also be extracted directly from washed cream by suspending 14 gm of washed cream (28-35 mg protein) in 42 ml of 1.0% DOC, 50 mM Tris (pH 7.9). After centrifugation at 100,000 x g for 1 hr, around 95% of the enzyme activity remained in the supernatant.

Preparation of Divalent Concanavalin A

A dimeric Con A derivative, succinyl-Con A, was prepared by modification of the method of Gunther et al., (57). At room temperature, 20 mg of Con A was dissolved in 4 ml of saturated sodium acetate. The protein solution was added to 1 ml of saturated sodium acetate containing 3 mg of succinic anhydride. The solution was stirred at 4° C for 1 hr, dialyzed overnight against water, and lyophilized. The lyophilized protein was subjected to a second derivatization at room temperature for 90 min. The succinyl-Con A was then dialyzed exhaustively against water and lyophilized.

Affinity Chromatography of Glycoproteins on Con A-Agarose

Glycosylex-A (Miles Laboratories) was thoroughly washed and equilibrated with 0.25% DOC, 50 mM Tris (pH 7.9). Samples of DOC-solubilized MFGM proteins (1.5 mg in 10 ml of the same buffer) were applied to 1.5 ml columns of Glycosylex-A contained within a 2 ml glass hypodermic syringe. Following sample application, columns were thoroughly washed with the same DOC-Tris buffer. Bound glycoproteins were eluted with 0.1 M α-methylmannoside in 0.25% DOC, 50 mM Tris (pH 7.9). Fractions were monitored for protein by A_{280} and for 5'-nucleotidase by the spectrophotometric assay. Fractions were dialyzed

against 40% methanol at room temperature and against several changes of water at 4° C, lyophilized, and electrophoresed on SDS gels.

Enzyme Purification

Membranes were obtained from raw cream in the usual manner described under "Preparation of Milk Fat Globule Membranes". MFGM were suspended in 50 mM Tris, pH 7.9. Sodium deoxycholate (10%) was added to a final concentration of 6 mg detergent/mg of membrane protein. The suspension was incubated for 2 hr at 37° C and then centrifuged at 100,000 x g for 1 hr and the supernatant collected.

In initial experiments which employed small volumes, the supernatant was concentrated 10-fold by pressure filtration in a stirred Amicon ultrafiltration cell using a Diaflo PM 30 ultrafilter. All of the enzyme activity was retained by the PM 30 ultrafilter. After concentration, 0.5 ml of the concentrated DOC-Tris extract was layered on a linear gradient made by mixing 2.3 ml of 5% (w/w) and 2.3 ml of 20% (w/w) sucrose. Sucrose solutions were prepared in 0.25% DOC, 50 mM Tris (pH 7.9). Several such gradients were centrifuged at 200,000 x g for 9.2 hr in a Beckman SW 50.1 rotor. The gradient contents were unloaded by pumping 60% (w/v) sucrose into each tube, and 5 drop fractions were collected. To each fraction one ml of buffer was added, and those fractions containing 5'-nucleotidase activity were pooled.

After concentration by pressure filtration as described above, the enzyme was further purified by gel filtration through columns of Sepharose 4B, Sephadex G-100, G-150, and G-200 equilibrated with 0.25% DOC, 50 mM Tris (pH 7.9).

Acrylamide Gel Electrophoresis without SDS

Enzyme samples were also run on polyacrylamide gels in DOC-Tris medium to correlate enzyme activity with protein bands. Samples (0.1-0.2 ml) containing enzyme activity in 0.25% DOC, 50 mM Tris were added to 50 µl of 40% sucrose containing Bromophenol blue (5 mg/ml). Electrophoresis was carried out on 5% polyacrylamide gels in 30 mM Tris, 30 mM glycine, 0.25% DOC (pH 7.4) (40). Companion gels were stained for protein with Coomassie blue and for carbohydrate by the periodate-Schiff procedure used with SDS gels. Before staining, DOC was removed from the gels by soaking overnight in 40% methanol. A third gel was sliced into 2 mm sections and gel portions were incubated in media used for the 5'-nucleotidase assay measuring the release of inorganic phosphate (58, 59).

Enzymatic Assays

5'-Nucleotidase activity for isolated membranes and DOC-solubilized enzyme was determined spectrophotometrically by coupling the reaction of 5'-nucleotidase to the deamination of adenosine as described by Ipata (60). Except where noted, the assay medium included 0.1 mM 5'-AMP, 1 mM $MgCl_2$, 0.1 mM β-glycerophosphate, 2 units adenosine deaminase, 50 mM Tris (pH 7.9) and enzyme sample in a final volume of 3.0 ml.

In some experiments, especially those involving cream samples, 5'-nucleotidase activity was assayed by a modification of the method of Morre (58) in which the reaction mixture (final volume of 0.4 ml) consisted of 50 mM Tris (pH 7.9), 10 mM AMP, 1 mM $MgCl_2$, and appropriate enzyme sample. The reaction mixture was incubated at 37° C for 15 min, and the reaction terminated by addition of 0.2 ml of 0.32% perchloric

acid. Liberated phosphate was determined by the method of Lazarus (59).

In enzyme assays at different temperature (5-40° C), the temperature of the sample compartment of the spectrophotometer and the incubating sample tubes were controlled by a circulating constant temperature bath. Reaction temperatures were measured by insertion of a thermocouple directly into the cuvettes. The initial rates of reaction were linear for several minutes and proportional to the amount of protein used in all measurements reported. The pH of a typical reaction mixture was measured at 5 and 40° C. A change of 0.5 pH units, which would not significantly affect the activity of the enzyme, was observed in changing the temperature through this range.

Analytical Procedures

Protein (61), neutral sugar (62), and sialic acid (63) were determined by standard procedures.

Amino acids were determined by the procedure of Spackman *et al.* (64), on a microanalyser designed and built by Dr. T. H. Liao. Determinations were made after hydrolysis for 20 hr *in vacuo* at 110° C in 6 N hydrochloric acid.

The conditions used for cleavage, derivatization, and gas-liquid chromatography (GLC) of carbohydrates were described by Reinhold (67).

CHAPTER III

RESULTS

Purification of the Major Membrane Glycoproteins

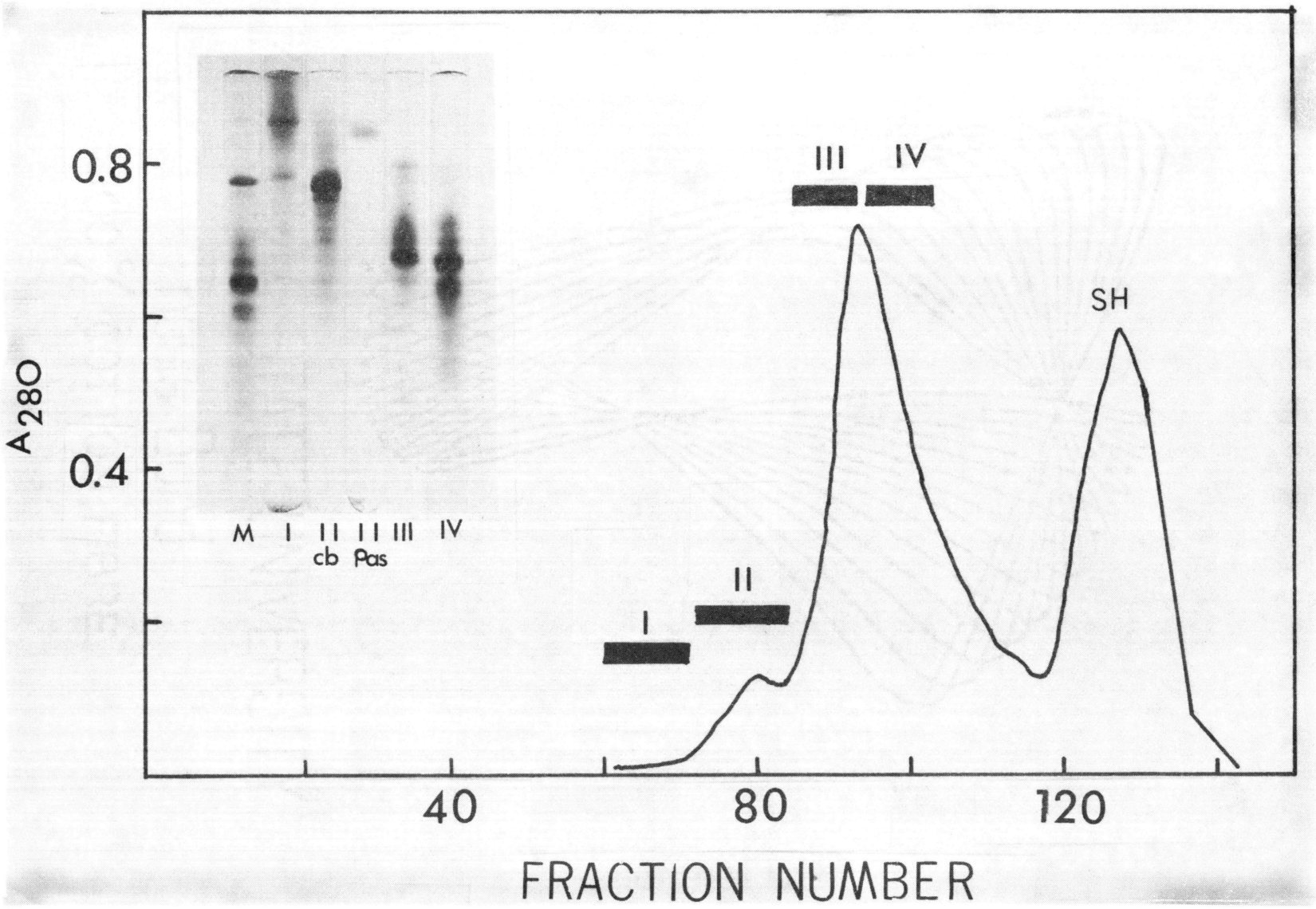
Several techniques were examined for their potential in separating MFGM glycoproteins from other membrane components. Glycoproteins were released from the membrane by either detergent solubilization or chemical extraction. The solubilized glycoproteins were fractionated by column chromatographic techniques employing different chromatographic mechanisms. The systems investigated were gel filtration, adsorption and affinity chromatography.

Chromatography of Membrane Preparations in SDS

SDS gel electrophoresis permits the identification in the MFGM of at least 6 major Coomassie blue staining polypeptides and at least 6 major periodate-Schiff staining glycoprotein bands (Figure 1). Molecular weights for the protein bands are approximately: I, 240,000; II, 155,000; III, 92,000; IV, 80,000; V, 65,000; VI, 53,000 (13).

Initial attempts at isolating MFGM glycoproteins involved solubilizing isolated membranes in 1% SDS, 50 mM Tris and chromatographing over Sepharose 4B in the same buffer. In a typical separation (Figure 2), four major fractions were noted. The last peak in the profile (SH) contained only mercaptoethanol. Analysis of protein distribution by

Figure 2. Chromatography of SDS-Solubilized MFGM on Sepharose 4B in 1% SDS. Membranes were solubilized in 5% SDS in 50 mM Tris (pH 7.4) containing 1% mercaptoethanol. After incubation at 37° C for 2 hr, the sample (10 mg protein/ml) was brought to 10% sucrose and applied to a 2.5 x 90 cm column. Fractions of 3.7 ml were collected and monitored for absorbance at 280 nm. Tubes were combined to yield the fractions indicated by the dark bars. Fractions denoted by the black bars were prepared for electrophoresis as described in Experimental Procedure. The peak contained in fractions 120-140 (SH) is mercaptoethanol. Results of acrylamide gel electrophoresis are shown in the inset. The number at the bottom is the fraction number for the column fractions. M indicates control membrane preparation. The gels were stained for protein (cb) and carbohydrate (pas).


0.8
0.4
A_{280}
M
I
II cb
II Pas
III
IV
I
II
III
IV
SH
40
80
120
FRACTION NUMBER

SDS gel electrophoresis shows that fractions I, II, and III each contain a unique distribution of proteins (Figure 2, inset). The major glycoprotein (GP-2) elutes primarily the fraction II. Also present in this fraction is a major membrane protein (P-II). Fraction I appears to be enriched in protein I with little PAS staining glycoprotein present. Fractions III and IV contain 92,000 through 53,000 molecular weight protein components III, IV, V, and VI as well as several glycoprotein components (GP-3, 4, 5, 6) of the membrane. Hence, this technique as a preliminary bulking step provides effective separation of high molecular weight proteins (P-I, II) and glycoproteins (GP-2) from the pool of solubilized membrane components. The primary limitation of this procedure is the cross contamination of Coomassie blue staining proteins with PAS staining glycoprotein components that fall within overlapping molecular weight ranges.

SDS-solubilized protein separations upon hydroxylapatite chromatography are different from those obtained by gel filtration in SDS since the proteins do not all elute in order of molecular weight (53). This chromatographic method was applied to SDS solubilized MFGM. The membrane protein was completely adsorbed to the hydroxylapatite column when applied in 0.1% SDS, 2 mM mercaptoethanol, 10 mM phosphate (pH 6.4) (Figure 3). The proteins eluted at phosphate concentrations between 0.1 and 0.3 M. Three peaks were obtained with elution concentrations of 0.14 M, 0.2 M, and 0.29 M phosphate, respectively. The peaks were divided into fractions and analyzed by SDS gel electrophoresis (Figure 4). The glycoproteins and low molecular weight acidic polypeptides eluted overwhelmingly as peak one. Polypeptides with intermediate SDS gel mobilities (P-IV, V, and VI) eluted in fractions 2, 3, and 4. MFGM

Figure 3. Hydroxylapatite chromatography of solubilized MFGM in 0.1% dodecyl sulfate. Membrane proteins, solubilized and prepared as described in Experimental Procedure, were adsorbed to hydroxylapatite columns and eluted with linear gradients made from 0.01 M phosphate and 0.5 M phosphate (pH 6.4), both containing 0.1% SDS and 2 mM mercaptoethanol. Absorbance (●--●) was measured at 280 nm. The molarities (▲--▲) were determined from conductivity measurements. Pooled fractions are denoted by dark bars.

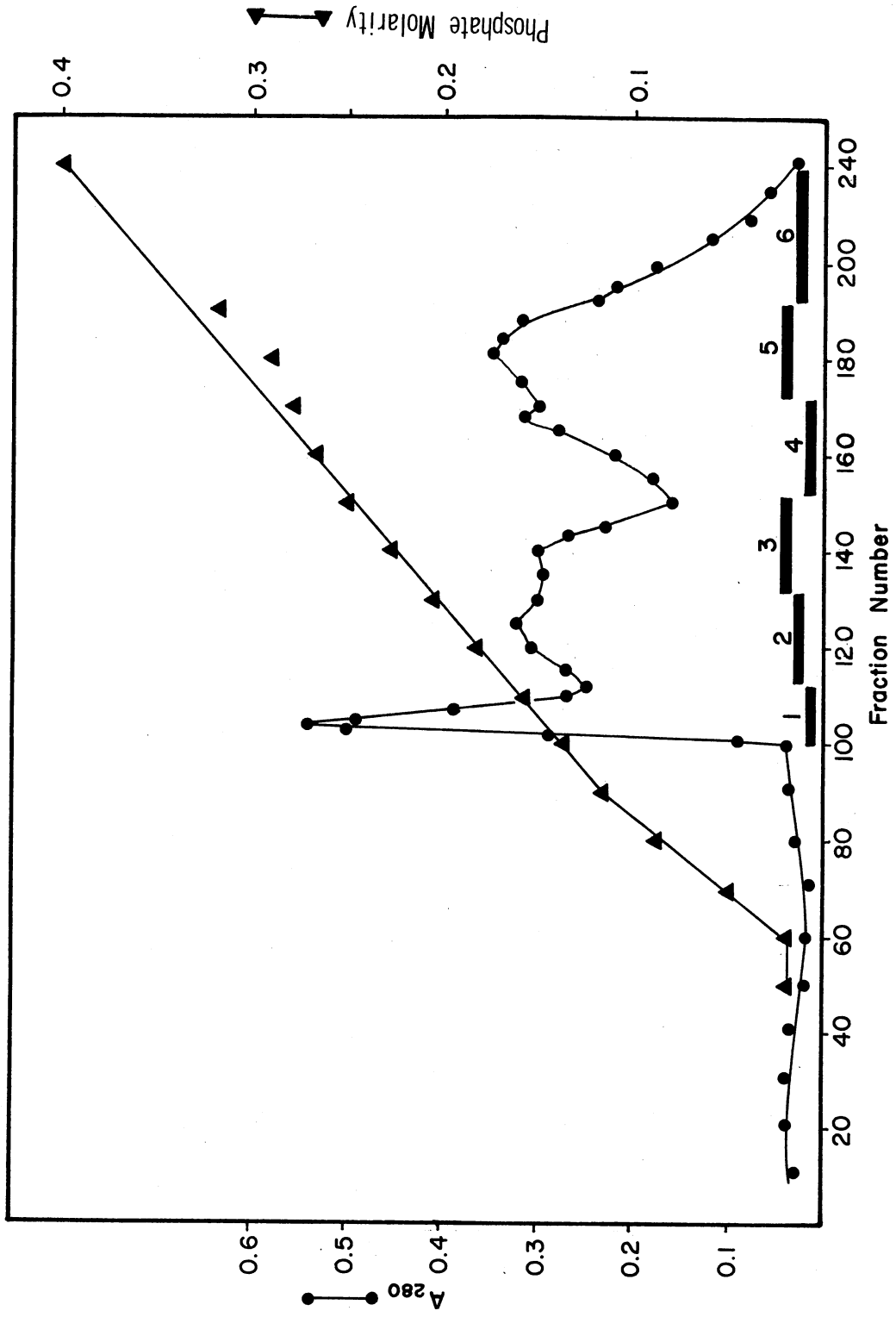
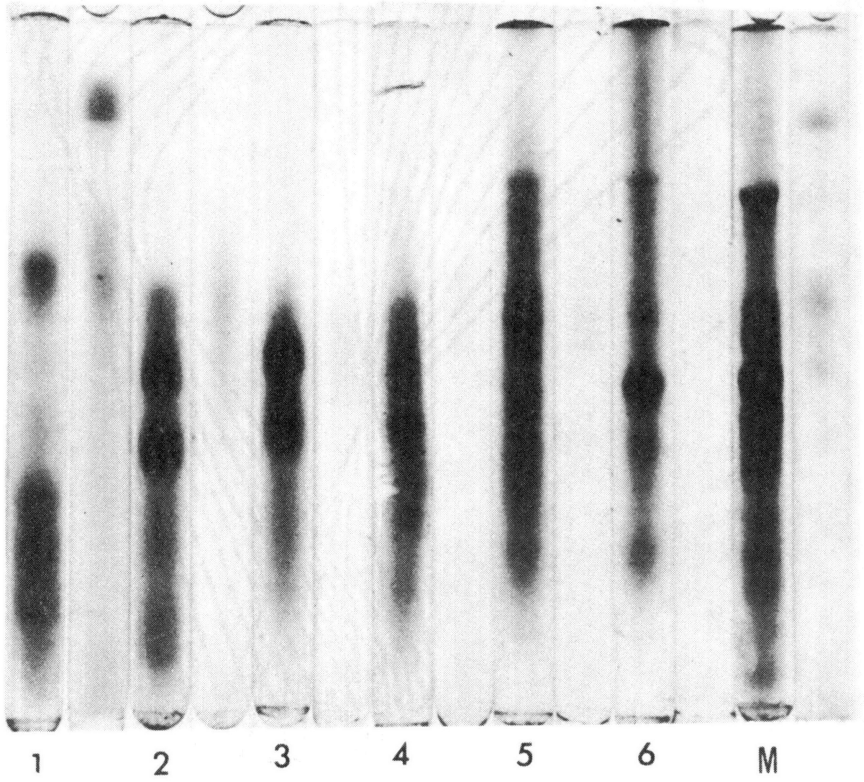

Phosphate Molarity
0.4
0.3
0.2
0.1
A_{280}
0.6
0.5
0.4
0.3
0.2
0.1
20
40
60
80
100
120
140
160
180
200
240
Fraction Number
1
2
3
4
5
6

Figure 4. Acrylamide gel electrophoresis of hydroxylapatite fractions of whole membrane. Samples are arranged (left to right) with alternating gels stained for protein and carbohydrate. Pooled fractions from Figure 3 were dialyzed against distilled water and lyophilized. Fractions were prepared for SDS gel electrophoresis as described in Experimental Procedure. M indicates control membrane preparation. The number at the bottom is the fraction number for the column fraction.


1
2
3
4
5
6
M

polypeptides with slower SDS gel mobilities (P-II, III, and IV) eluted as fractions 4 and 5. Each elution peak apparently contained a unique group of MFGM polypeptides.

In this technique glycoproteins may be separated from most Coomassie blue staining proteins of similar electrophoretic mobility. This resolving capability of hydroxylapatite chromatography may be profitably incorporated as a preparative separation of glycoproteins from solubilized membranes. However less complicated techniques permitting selective solubilization by incubation in an appropriate medium were considered more desirable for the present study.

Chromatography of Selectively Extracted Glycoproteins

Selective methods of chemical extraction employing such agents as phenol, lithium diiodosalicylate, chloroform/methanol, or aqueous pyridine have been used to separate glycoproteins in water soluble form from membranes and intact cells (65). Chemical extraction was investigated as an alternative to detergent solubilization since the former offered the opportunity to selectively solubilize glycoproteins without contaminating Coomassie blue staining proteins of similar molecular weights. The chloroform/methanol/water extraction procedure developed by Hamaguchi and Cleve (51) for extraction of erythrocyte glycoproteins was applied to MFGM and also directly to washed cream.

Although yields varied considerably between preparations, on the average 50 mg protein, 10 mg sialic acid, and 30 mg neutral sugar were extracted from 500 ml of 33% washed cream. The glycoprotein solution obtained by chloroform/methanol extraction contained all the major

glycoprotein components of the membrane plus small amounts of low molecular weight Coomassie blue staining material (Figure 5). These contaminants were removed by gel filtration in Sephadex G-200 or Sepharose 4B. The qualitative yield of glycoprotein components was similar for both membranes and washed cream extractions. Hence washed cream was used as the starting material for all subsequent experiments. After extraction of the glycoproteins, column chromatographic procedures were employed in attempts to isolate the major glycoprotein of the membrane, GP-2.

Chromatography involving extracted glycoproteins introduced the problem of locating μg quantities of the components upon sample elution. To aid in monitoring column eluates for glycoprotein, chloroform/methanol extracts were modified by sequential sodium periodate oxidation and tritiated sodium borohydride reduction by the method of Liao et al. (52). This procedure selectively modifies sialyl residues and leads to incorporation of tritium into the modified product, 5-acetamido-3,5-dideoxy-L-arabino-2-heptulosonic acid.

Figure 6 shows the radioactivity profile and PAS bands separated by SDS gel electrophoresis from an aliquot of tritium labeled glycoproteins. In this preparation the yield of GP-1 and 5 was very low; however, these components also incorporated label in subsequent experiments.

Since sample load may affect the resolving capability of hydroxylapatite columns, extracted glycoproteins were chromatographed on this medium to determine if the removal of the major Coomassie blue staining proteins altered the elution behavior of the glycoproteins. The glycoproteins were eluted at phosphate concentrations between 0.2 M and 0.3

Figure 5. Acrylamide gel electrophoresis of chloroform/methanol/water extract. Samples are arranged (left to right) with alternating gels stained for protein and carbohydrate. MFGM (10 mg protein/ml) were extracted with chloroform/methanol at 25° C as described in Experimental Procedure. The solubilized glycoproteins contained in the aqueous phase were lyophilized and solubilized for SDS gel electrophoresis. M indicates membrane control. E represents extract sample.

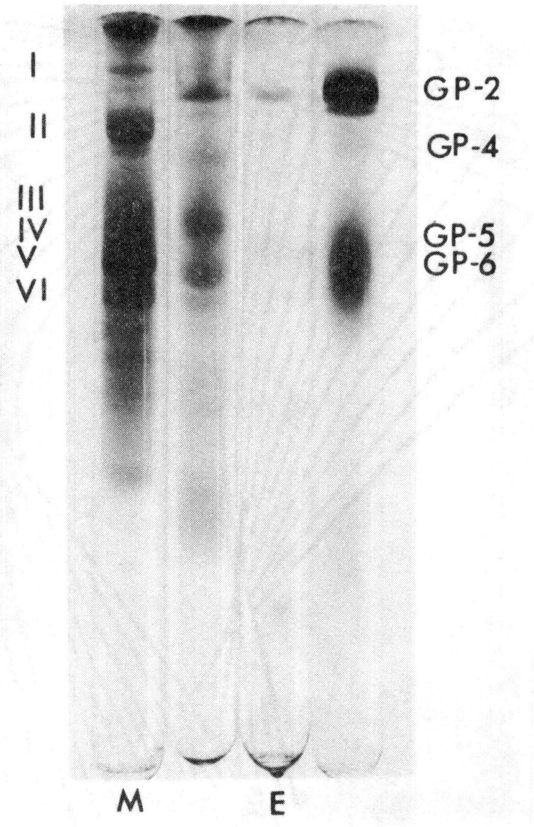
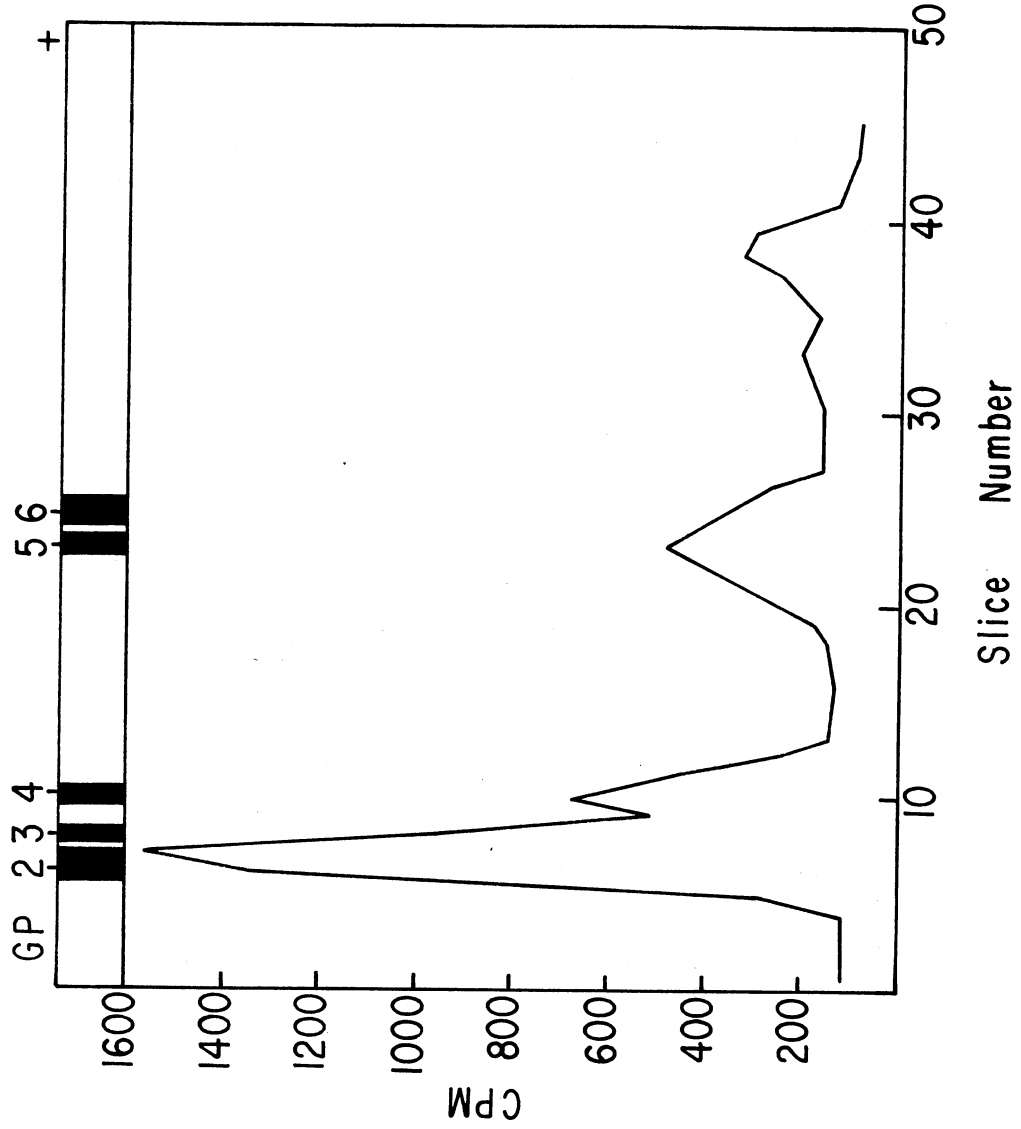

I
II
III
IV
V
VI
GP-2
GP-4
GP-5
GP-6
M
E

Figure 6. SDS acrylamide gel electrophoresis of tritium labeled glycoprotein extract. Chloroform/methanol glycoprotein extracts of washed cream were labeled by oxidation of sialic acid residues with 0.1 M sodium metaperiodate and reduction with ^{3}H-KBH_4. Aliquots were prepared and subjected to SDS acrylamide gel electrophoresis as described in Experimental Procedure. Companion gels were stained for carbohydrate and sliced into 2 mm segments and counted for radioactivity. The diagram at the top shows the PAS staining pattern. The radioactive peaks corresponded to the migration positions of MFGM glycoproteins.


GP
2
3
4
5
6
+
1600
1400
1200
1000
800
600
400
200
CPM
10
20
30
40
50
Slice Number

M phosphate (Figure 7). Three major peaks eluting at 0.24 M, 0.25 M, and 0.265 M phosphate were resolved and analyzed by SDS gel electrophoresis (Figure 8). Fraction A contained a minimal amount of unbound glycoprotein and may also represent unbound glycolipid material. Fraction B included glycoproteins 1, 2, and 3. Fraction D included predominately lower molecular weight glycoprotein components. Fraction C included all of the glycoprotein components due to overlapping of the peaks. Fractions B and D apparently contained unique groups of MFGM glycoproteins. When compared to the hydroxylapatite chromatography of whole MFGM, the experiment demonstrates that the precise elution molarity of protein appears to depend on sample load. Since steepness of the gradient and column length influence elution, it is possible that further manipulation of conditions could enhance the resolution of glycoprotein separation either by less steep gradients or selective stepwise elutions.

Although hydroxylapatite chromatography seemed promising as a potential purification method, separation of extracted-solubilized glycoproteins by gel filtration in Sephadex G-200 was undertaken to evaluate the relative resolution of the two techniques. Concentrates of chloroform/methanol extracted glycoprotein were solubilized in 1.0% SDS and fractionated on Sephadex G-200 (Figure 9). Fractions were monitored by SDS gel electrophoresis (Figure 10). The first major peak contained primarily glycoprotein-2 with a broad trailing shoulder containing GP-3. The foreportion of the major peak of two such chromatographic separations were pooled, concentrated with Aquacide, and refractionated on the same column. The forepart of the major peak obtained upon the second chromatographic cycle yielded one band upon

Figure 7. Chromatography of tritium labeled glycoprotein extract on hydroxylapatite. Glycoproteins were extracted from washed cream and labeled with tritium as described in Experimental Procedure. Labeled glycoproteins were solubilized in 1.0% SDS, 0.01 M phosphate (pH 6.4) and 1% mercaptoethanol. Samples were prepared for chromatography, adsorbed and eluted from a (1.5 x 20 cm) hydroxylapatite column as described in Figure 3. Radioactivity of fractions (●--●) was monitored, and molarity (▲--▲) was determined from conductivity measurements. Fractions denoted by bars were combined.

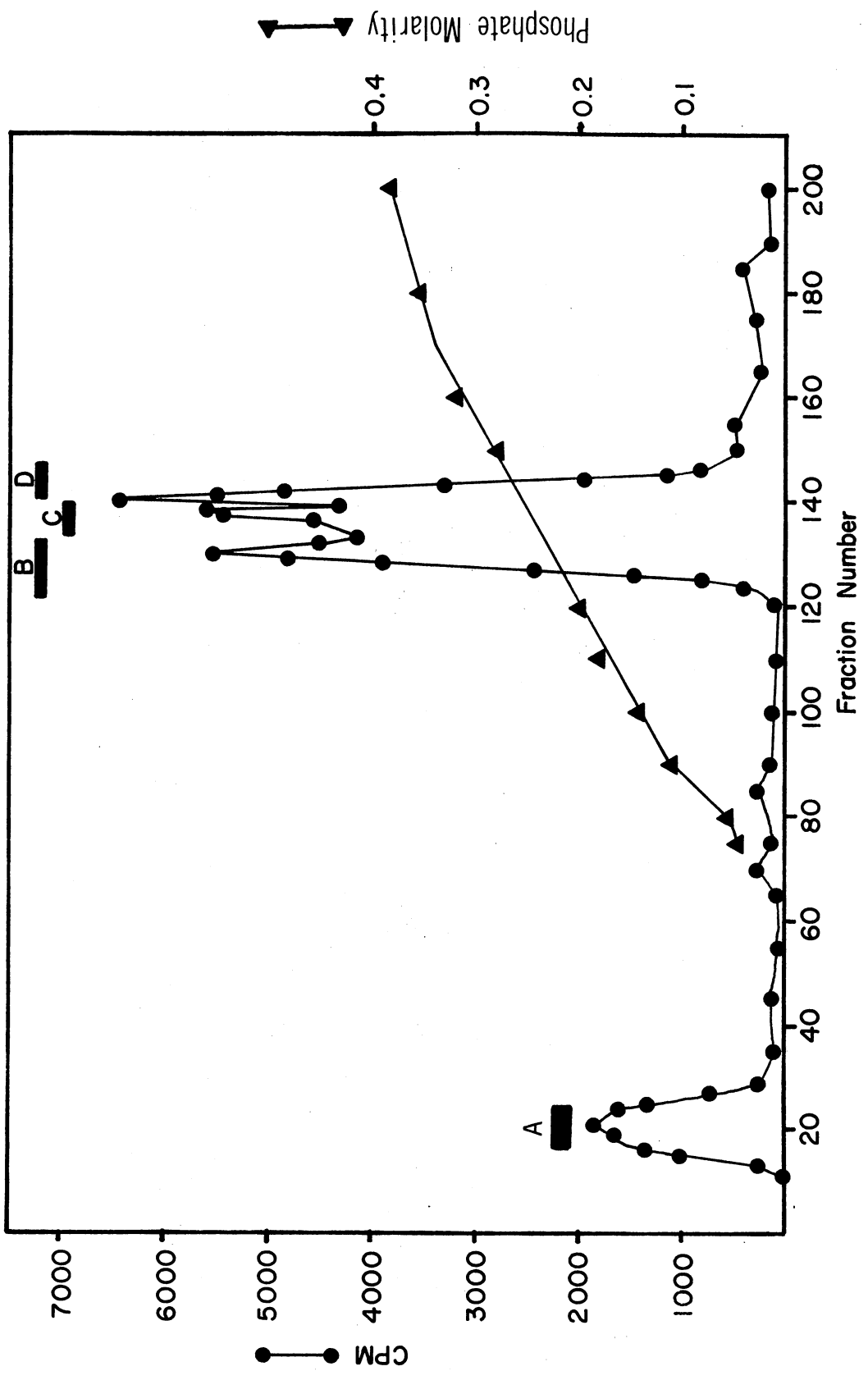

Phosphate Molarity
0.4
0.3
0.2
0.1
CPM
7000
6000
5000
4000
3000
2000
1000
A
B
C
D
20
40
60
80
100
120
140
160
180
200
Fraction Number

Figure 8. Polyacrylamide gel electrophoresis of ^{3}H-labeled glycoproteins fractionated by hydroxylapatite chromatography. Pooled fractions from Figure 7 were dialyzed against water, lyophilized, dissolved in SDS and mercaptoethanol and subjected to electrophoresis on 5% acrylamide gels in dodecyl sulfate. The letter at the bottom is the fraction designated for the column fractions of Figure 7. Samples are arranged (left to right) with alternating gels stained for protein and carbohydrate.

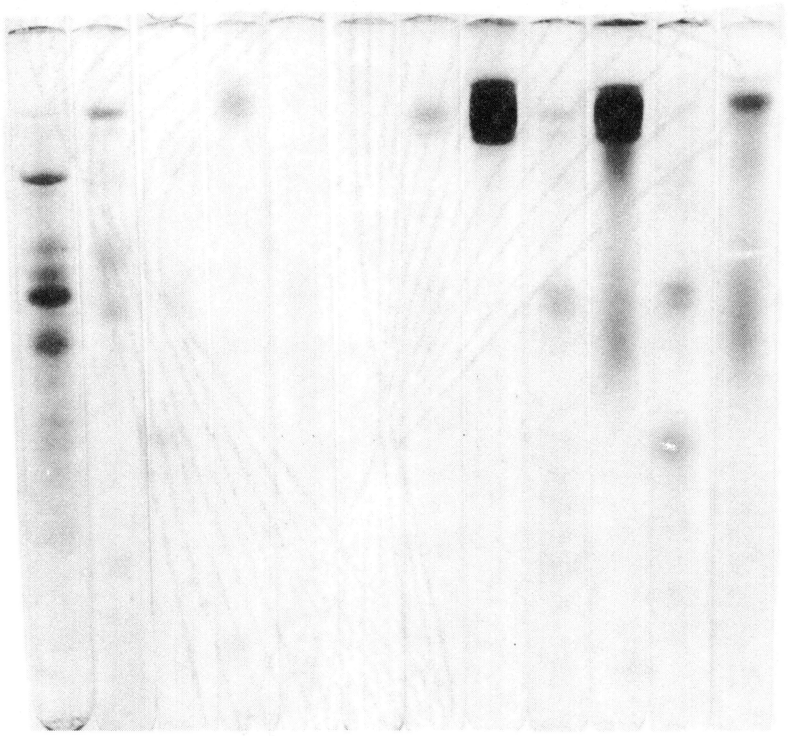

M
A
A
B
C
D

Figure 9. Sephadex G-200 chromatography of chloroform/methanol extract in 1% dodecyl sulfate. Chloroform/methanol extractions were obtained and prepared for G-200 chromatography as described in Experimental Procedure. Samples typically contained (2 mg sialic acid) and were applied to 2.5 x 90 cm columns equilibrated in 50 mM Tris, 1% SDS (pH 7.4). Fractions of 3.7 ml were collected and monitored for absorbance at 280 nm. Individual tubes were analyzed by SDS gel electrophoresis. The black bar denotes the GP-2 containing fraction which was pooled and rechromatographed over the same column to yield one band on SDS gel electrophoresis.

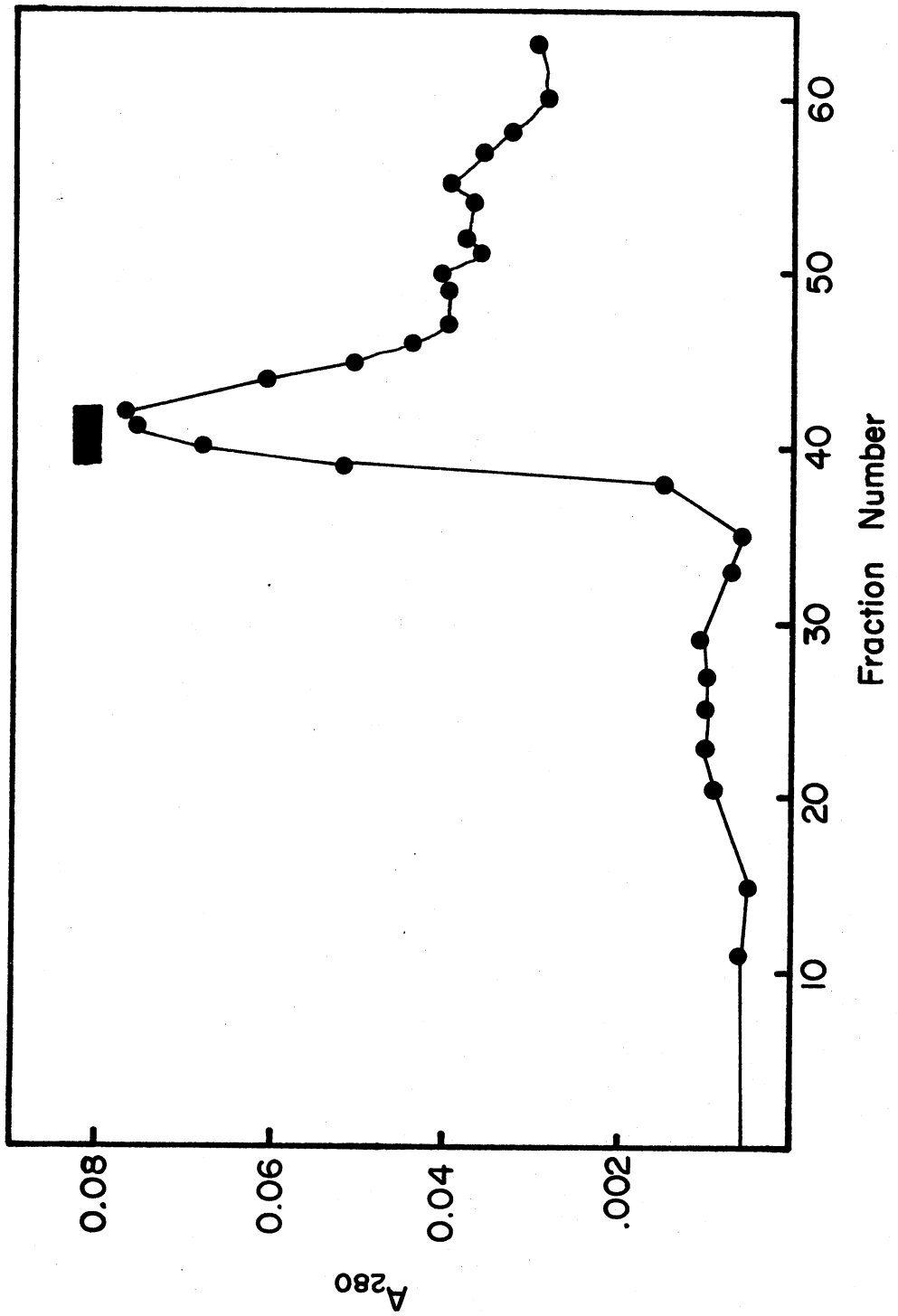
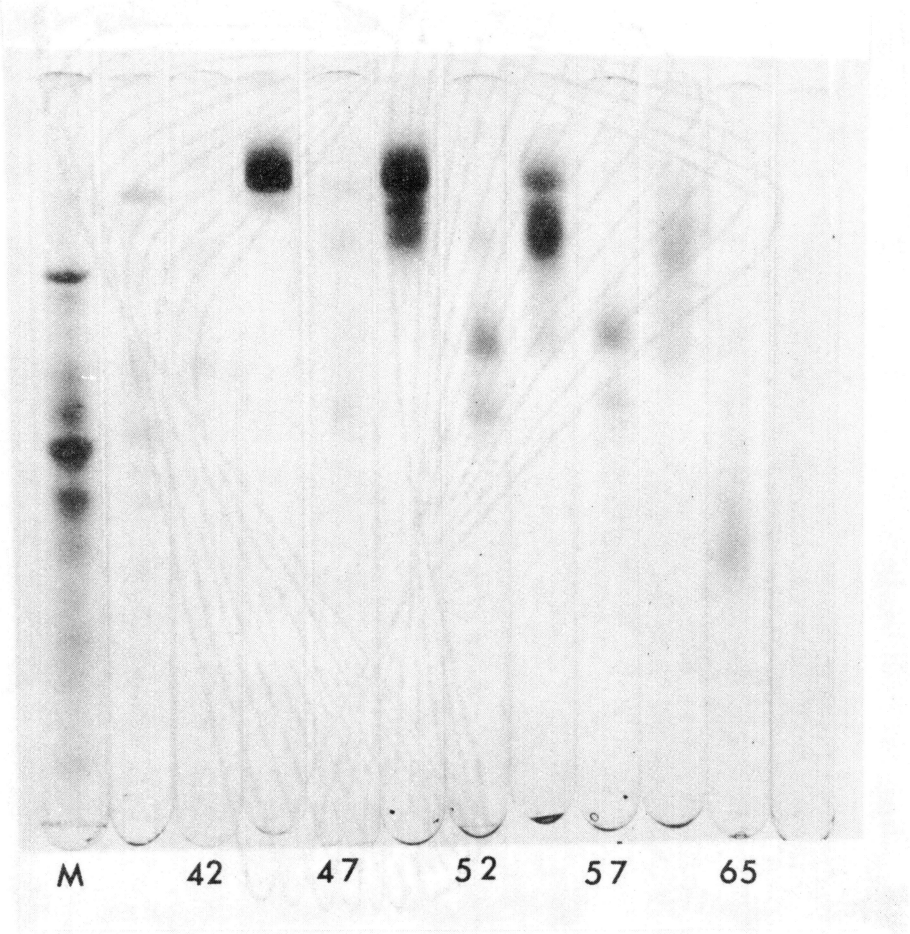

0.08
0.06
0.04
.002
A_{280}
10
20
30
40
50
60
Fraction Number

Figure 10. Acrylamide gel electrophoresis of Sephadex G-200 fractions. Individual tubes obtained from the G-200 columns described in Figure 9 were dialyzed, lyophilized and prepared for SDS gel electrophoresis as described in Experimental Procedure. Numbers at the bottom represent fraction numbers from the column. M indicates control membrane preparation. Samples are arranged (left to right) with alternating gels stained for protein and carbohydrate.


M
42
47
52
57
65

SDS gel electrophoresis (Figure 11). The purified glycoprotein was isolated from G-200 without tritium labelling since it was found that A_{280} profiles permitting glycoprotein location could be obtained by monitoring eluates with a Gilford Model 240 spectrophotometer.

The glycoprotein prepared by this procedure appears to be a single molecular species as judged by acrylamide gel electrophoresis at gel concentrations ranging from 5 to 12.5 per cent acrylamide. The monomeric unit has an apparent molecular weight of 185,000 on 5% gels which changes to 70,000 on 12.5% gels (Figure 12). A molecular weight value may be estimated by extrapolation of the electrophoresis results to high acrylamide percentages (Figure 12). By column chromatography in Sepharose 4B in 1% dodecyl sulfate the same glycoprotein gave a molecular weight value of 105,000.

SDS gel electrophoresis and gel filtration techniques are subject to considerable inaccuracies for glycoprotein molecular weight determinations. Thus an estimate of molecular weight and ratio of carbohydrate/protein for the purified glycoprotein was made by correlating quantitative amino acid analysis data and quantitative carbohydrate data from GLC carbohydrate analysis from the same purified glycoprotein sample. The amino acid distribution for the glycoprotein is shown in Table I.

There are marked differences between the major MFGM glycoprotein and the major bovine RBC glycoprotein amino acid composition as reported by Hudson *et al*. (66). The significance of the amino acid variations between the two glycoproteins is difficult to evaluate. In both glycoproteins, serine plus threonine residues account for roughly 20% of the amino acid distribution. However in MFGM glycoprotein the serine/

Figure 11. Acrylamide gel electrophoresis of purified MFGM glycoprotein-2. Samples are arranged (left to right) with alternating gels stained for protein and carbohydrate. M indicates control membrane preparation. Repetitive chromatography of SDS solubilized chloroform/methanol extracts on Sephadex G-200 permitted resolution of glycoprotein fractions which gave one band on 5% acrylamide gels in SDS.

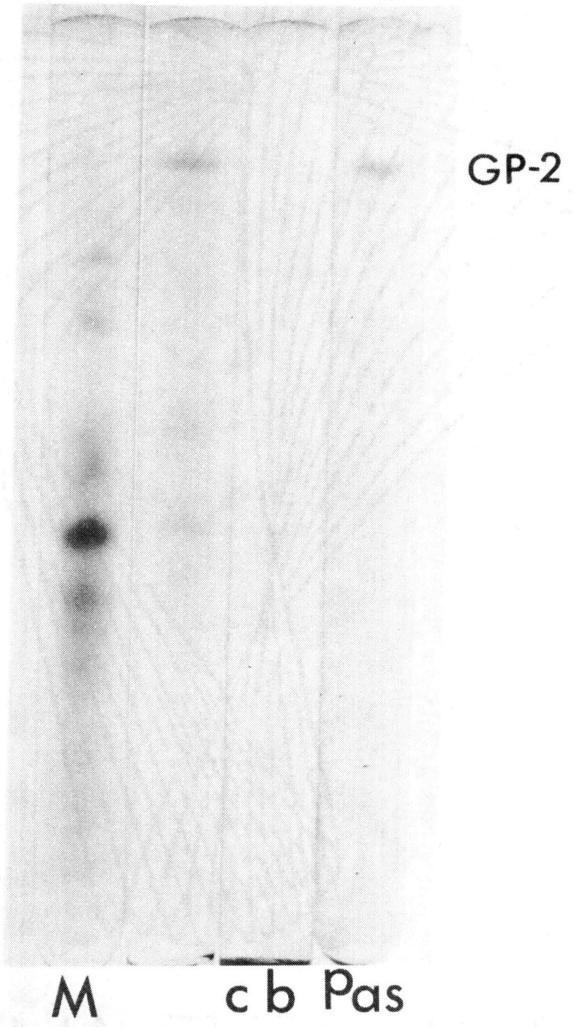

GP-2
M
c b
Pas

Figure 12. SDS acrylamide gel electrophoresis of purified glycoprotein-2 on gels of varied acrylamide concentration. Plot gives molecular weight versus per cent gel utilized. The purified glycoprotein was prepared for electrophoresis as described in Experimental Procedure. Electrophoresis on SDS gels of varied acrylamide concentrations by the methods of Segrest and Jackson (56) was performed. Molecular weights were calculated from plots of relative mobility versus log molecular weight of standard proteins at each gel concentration.

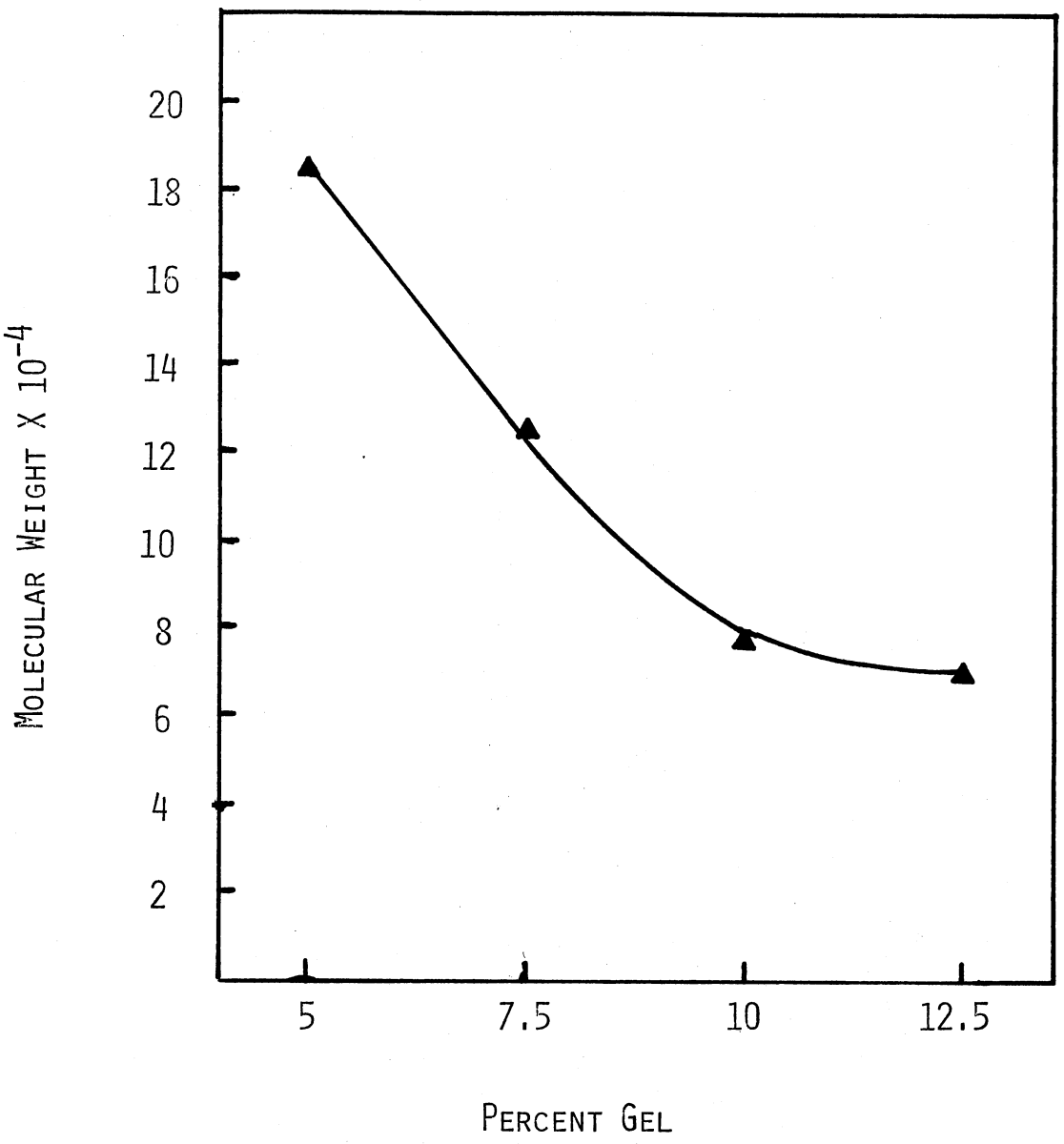

Molecular Weight X 10^{-4}
20
18
16
14
12
10
8
6
4
2
5
7.5
10
12.5
Percent Gel

TABLE I

COMPARISON OF AMINO ACID COMPOSITION OF MFGM, RBC MEMBRANES, AND ISOLATED GLYCOPROTEINS[a]

Amino Acid	Mole %			
	MFGM[b]	RBC[b]	RBC Glycoproteins[c]	MFGM Purified Glycoprotein
Lysine	5.6	5.1	3.0	4.3
Histidine	1.8	2.1	1.2	3.6
Arginine	4.6	4.9	6.8	3.7
Aspartic Acid	9.6	9.6	5.4	6.3
Threonine	6.0	3.5	12.1	8.5
Serine	8.5	8.0	10.2	15.6
Glutamic Acid	12.0	13.6	13.5	12.6
Proline	6.5	6.1	11.8	7.2
Glycine	7.4	6.1	7.0	11.2
Alanine	7.2	7.6	6.5	9.2
Valine	6.8	5.8	6.4	3.9
Isoleucine	4.2	4.3	4.5	3.1
Leucine	9.3	11.6	5.3	6.0
Tyrosine	2.4	2.2	1.2	1.4
Phenylalanine	4.1	3.6	1.7	2.5
Methionine				0.83

[a]Values are expressed as mole % of amino acids. Tryptophan and half-cysteine were not determined.

[b]Kobylka and Carraway (13).

[c]Hudson et al. (66).

threonine residue ratio is 1.8 whereas this ratio is only 0.84 for the RBC glycoprotein. Other notable differences include higher relative MFGM mole percentages for glycine and alanine and lower MFGM mole percentages values for proline and arginine.

The purified glycoprotein contained 0.927 μmoles of sialic acid per mg protein. Analysis of hexose content by gas-liquid chromatography (GLC) showed that almost 50 percent of the hexose portion of the sample was glucose. Glucose is not a common component of membrane glycoproteins, and it apparently occurs only in glycoproteins of the collagen type (65). Harrison (68) has shown that the sialoglycopeptides obtained by Pronase treatment of MFGM contain no glucose. The glucose fraction in the GP-2 preparation apparently represents glucose derived from the chromatographic medium, Sephadex G-200, or from sucrose included in solubilizing buffers for sample application to chromatography columns. A remedy for this problem would involve assaying blank tubes off the column and filtering samples by Millipore before analysis. Alternatively, Bio-Gel chromatographic columns could be used for glycoprotein isolation. In reporting the results, the glucose fraction was not considered as a significant GP-2 component.

Overall, 50 per cent by dry weight of the molecule was carbohydrate and 50 per cent was protein. The carbohydrate portion of GP-2 consisted of hexosamine (20% of the total GP), hexose (16%), and sialic acid (13%). The analysis for individual sugars by GLC is given in Table II. The mole percentages of total carbohydrate of galactose (15.9), mannose (11.1), frucose (5.84), N-acetylgalactosamine (14.0), N-acetylglucosamine (22.3), and sialic acid (30.5) correspond to a carbohydrate unit ratio of 3:2:1:2.5:4:5, respectively. Assuming an asymptotic molecular

TABLE II

CARBOHYDRATE COMPOSITION OF GLYCOPROTEIN-2

Monosaccharide	μmol/mg protein	Mole%	Moles per Mole of Protein[a]
Galactose	0.484	15.9	34
Mannose	0.338	11.1	24
Fucose	0.177	5.84	13
Glucose[b]	3.54	-	-
N-acetylgalactosamine	0.427	14.0	22
N-acetylglucosamine	0.677	22.3	35
Sialic Acid	0.927	30.5	34

[a]Based on SDS gel electrophoresis asymptotic molecular weight of 70,000 and 50% by weight carbohydrate content of GP-2.

[b]Apparently derived from Sephadex chromatographic medium and not included in calculations.

weight of 70,000 from SDS gel electrophoresis and total carbohydrate content of 50 per cent by weight, the molar content of carbohydrates could be galactose (34.3 mol), mannose (23.9), frucose (12.6), N-acetylgalactosamine (22.1), N-acetylglucosamine (35.3), and sialic acid (34.5).

Characterization and Purification of 5'-Nucleotidase

The enzyme 5'-nucleotidase is apparently a glycoprotein component of the MFGM. In this study the effect of detergents, lectin treatments, and temperature on enzyme activity were characterized. Purification and analysis of the enzyme were undertaken to aid in explaining the effect of membrane perturbations on 5'-nucleotidase activity.

Assay Conditions

Table III shows the effect of incubation time and Mg^{++} concentration on MFGM 5'-nucleotidase activity. Preincubation in the assay medium for periods up to 1 hr did not alter enzyme activity. As obtained in our membrane preparation procedure which included $MgCl_2$ in the isolating medium, 5'-nucleotidase activity was not enhanced by the addition of 1 mM Mg^{++} to the assay medium. Higher concentrations of magnesium seemed to prove slightly inhibitory with prolonged incubation. In all subsequent experiments a 15 min preincubation in assay medium without substrate was employed. To insure that adequate Mg^{++} ion was retained in any subsequent enzyme evaluation after various treatments, each assay was subsequently made 1 mM in Mg^{++}.

The effect of pH on the hydrolysis of AMP is illustrated in Figure

TABLE III

EFFECT OF INCUBATION TIME AND Mg^{+2} CONCENTRATION UPON 5'-NUCLEOTIDASE ACTIVITY

Preincubation Time (min)	Specific Activity (μmol/hr/mg protein)		
	Control	1 mM Mg	10 mM Mg
10	66.6	67.4	64.6
40	70.0	70.8	65.7
60	68.8	68.8	56.9

[a]Spectrophotometric assays were at 37° C with varied preincubation times before addition of substrate. Reaction mixtures contained 0.1 mM AMP, 0.1 mM β-glycerophosphate, 50 mM Tris (pH 7.9), and 2 units of adenosine deaminase in a final volume of 1 ml.

13. In the presence of 1 mM $MgCl_2$, the reaction proceeded at maximal velocity at pH 7.5 for both membrane-bound and solubilized forms of the enzyme.

Effects of Detergent and Lectin Perturbations on 5'-Nucleotidase

The effect of increasing concentrations of DOC on enzyme activity is shown in Figure 14. The 5'-nucleotidase activity increased to about 110% of its initial value in 0.05% DOC and then decreased gradually to 80% of its original value in 1% DOC. In contrast, dodecylsarcosinate and SDS produced substantial inactivation of the enzyme at concentrations as low as 0.1 and 0.2%, respectively.

Figure 15 shows the degree of solubilization of membrane protein and 5'-nucleotidase activity with increasing concentrations of DOC at 37° C for 2 hr. The DOC concentration which gave maximum enzyme solubilization with minimum solubilization of membrane protein was 0.25% DOC (6 mg detergent/mg protein). At lower temperatures the yield of solubilized enzyme was reduced. Following treatment with 0.25% DOC at 37° C, at least 95% of the enzyme activity and only 20% of the total membrane protein were solubilized. These conditions were subsequently used routinely to solubilize the enzyme. The above procedure permitted an almost complete separation of the enzyme from the membrane.

SDS gel electrophoresis revealed some selectivity by DOC in membrane protein and glycoprotein extraction. For the gel pattern shown in Figure 16, all Coomassie blue gels received 50 µg of supernatant or pellet protein whereas PAS gels all received 150 µg of supernatant or pellet protein. Based on staining intensity of the gels, the super-

Figure 13. Effect of pH on the activity of 5'-nucleotidase. The spectrophotometric assay was employed. Reaction mixtures contained 0.1 mM AMP, 1 mM $MgCl_2$, 0.1 mM β-glycerophosphate, 50 mM Tris and 2-4 units of adenosine deaminase in a final volume of 3 ml. Homogenized membrane samples (▲--▲) and DOC-solubilized enzyme (●--●) samples were assayed at varied pH values. Assays were at 37° C with 15 min preincubation before the addition of substrate.

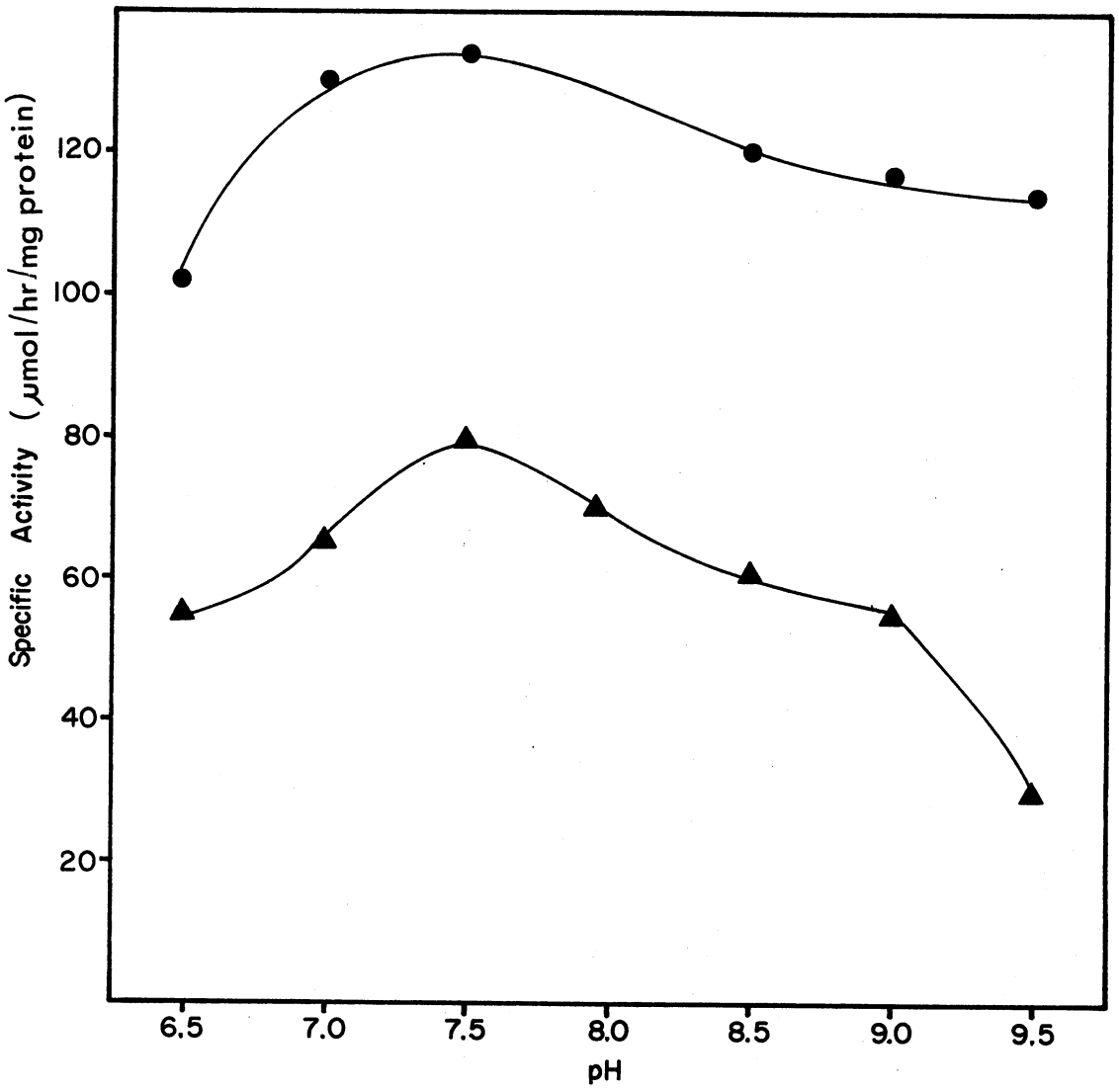

Specific Activity (μmol/hr/mg protein)
120
100
80
60
40
20
6.5
7.0
7.5
8.0
8.5
9.0
9.5
pH

Figure 14. Effect of increasing concentrations of deoxycholate on 5'-nucleotidase activity. Membrane samples were suspended in 50 mM Tris (pH 7.9) containing varied concentrations of DOC. Incubation was for 2 hr at 37° C. Assays of the suspensions were performed by the spectrophotometric method with the medium containing 0.1 mM AMP, 0.1 mM β-glycerophosphate, 1 mM $MgCl_2$, 4 units of adenosine deaminase, and 50 mM Tris (pH 7.9).

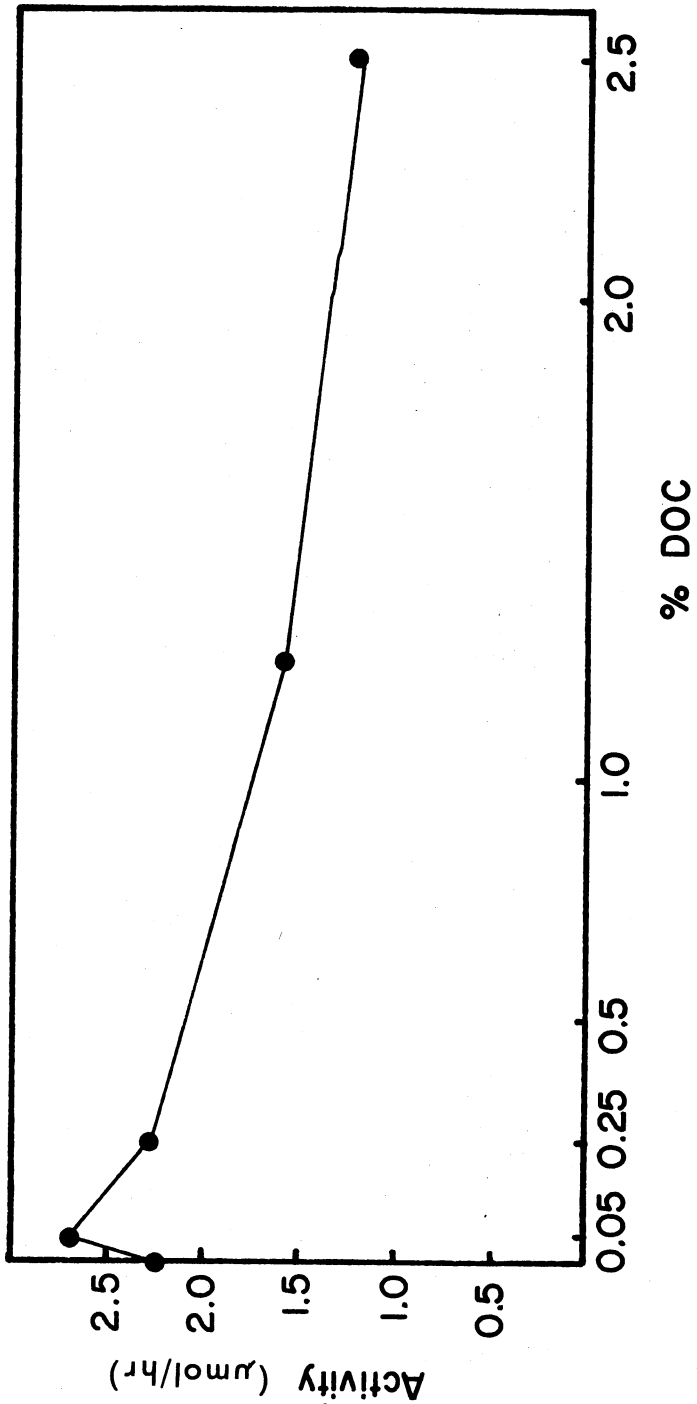

Activity (μmol/hr)
2.5
2.0
1.5
1.0
0.5
0.05 0.25 0.5
1.0
2.0
2.5
% DOC

Figure 15. Solubilization of membrane protein and 5'-nucleotidase activity with deoxycholate. Membrane samples were suspended in 50 mM Tris (pH 7.9) containing varied concentrations of DOC. After 2 hr incubation at 37° C, the suspensions were centrifuged at 100,000 x g for 1 hr. The supernatant protein (O--O) was determined by Lowry and solubilized enzyme activity (Δ--Δ) was measured by the spectrophotometric method described in Figure 14. Similarly, the pellet protein (●--●) and enzyme activity (▲--▲) were determined.

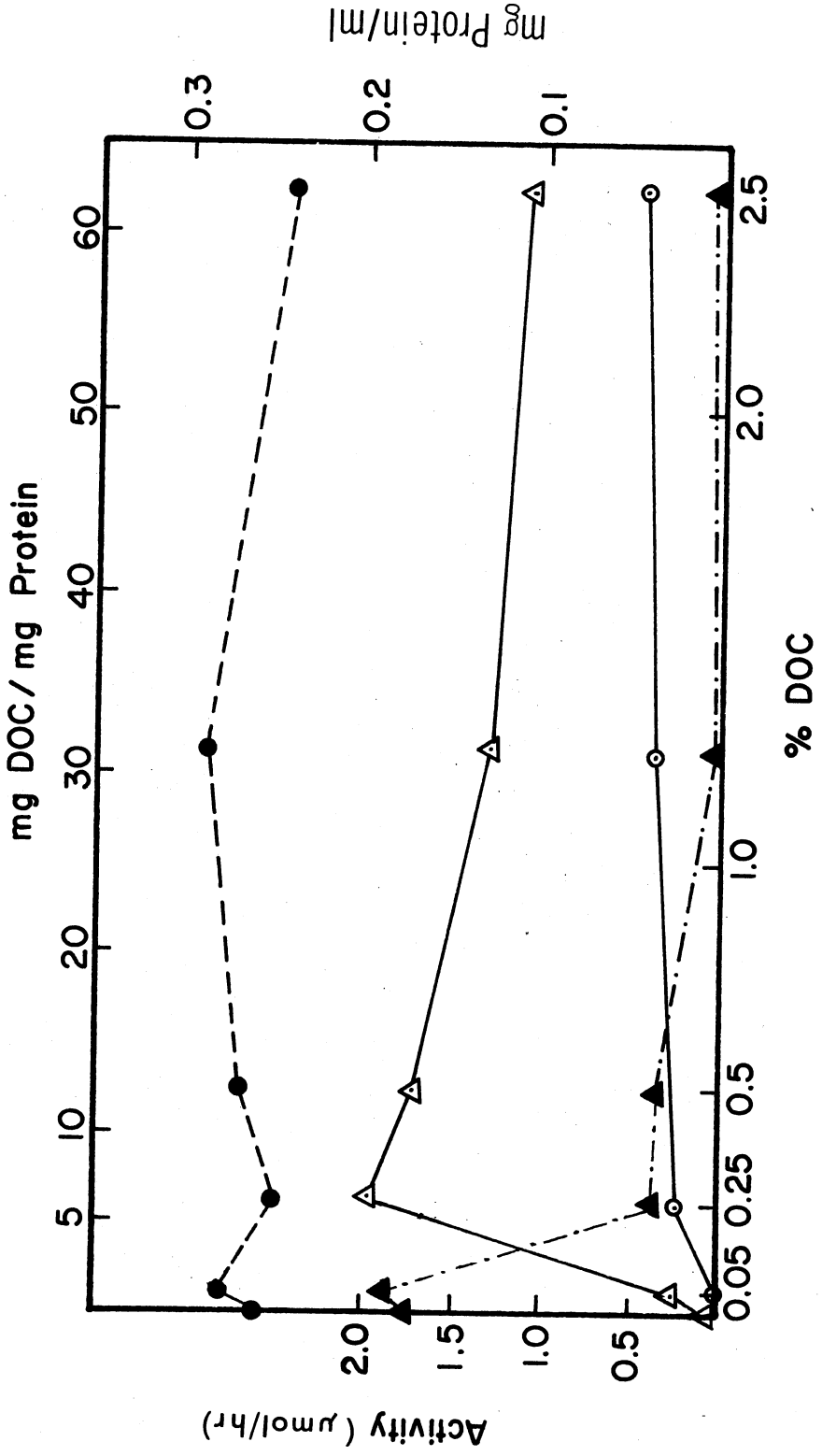
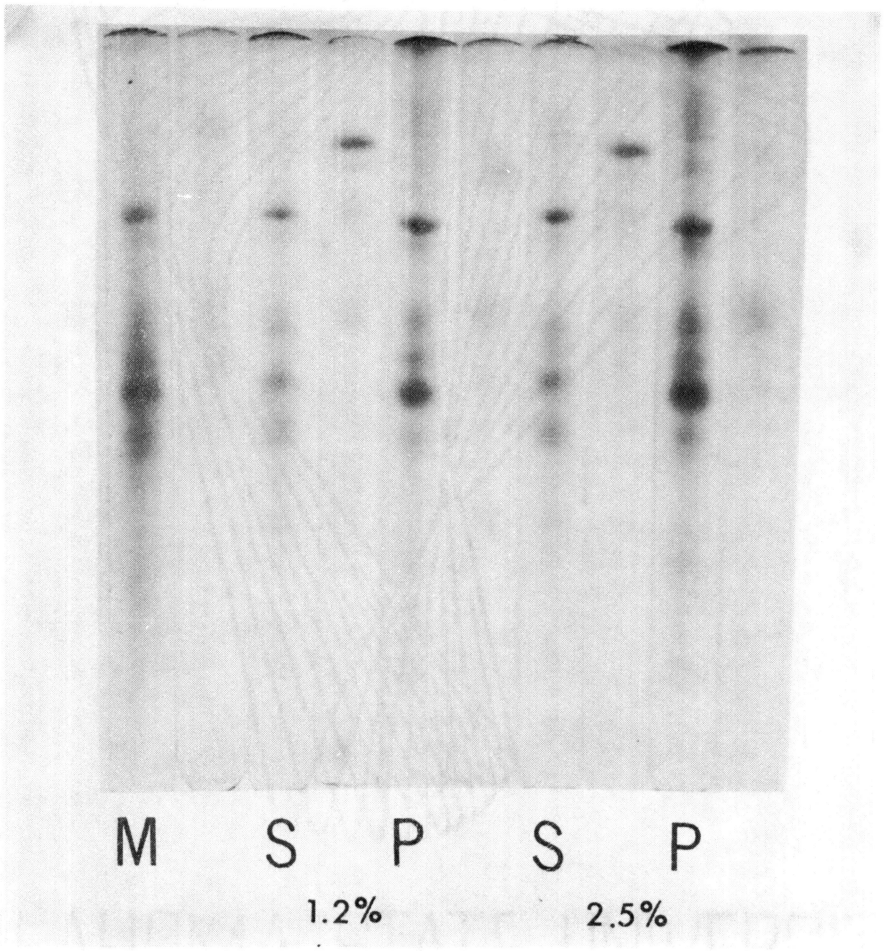

Activity (μmol/hr)
0.5
1.0
1.5
2.0
mg DOC/mg Protein
5
10
20
30
40
50
60
% DOC
0.05
0.25
0.5
1.0
2.0
2.5
mg Protein/ml
0.1
0.2
0.3

Figure 16. Acrylamide gel electrophoresis of deoxycholate solubilized membranes. Samples are arranged (left to right) with alternating gels stained for protein and carbohydrate. Supernatant fractions from the solubilization experiment described in Figure 15 were lyophilized. The samples were prepared for SDS gel electrophoresis as described in Experimental Procedure. S and P denote supernatant and pellet samples, respectively. Gels for 1.25% and 2.5% DOC incubations are shown.


M
S
P
S
P
1.2%
2.5%

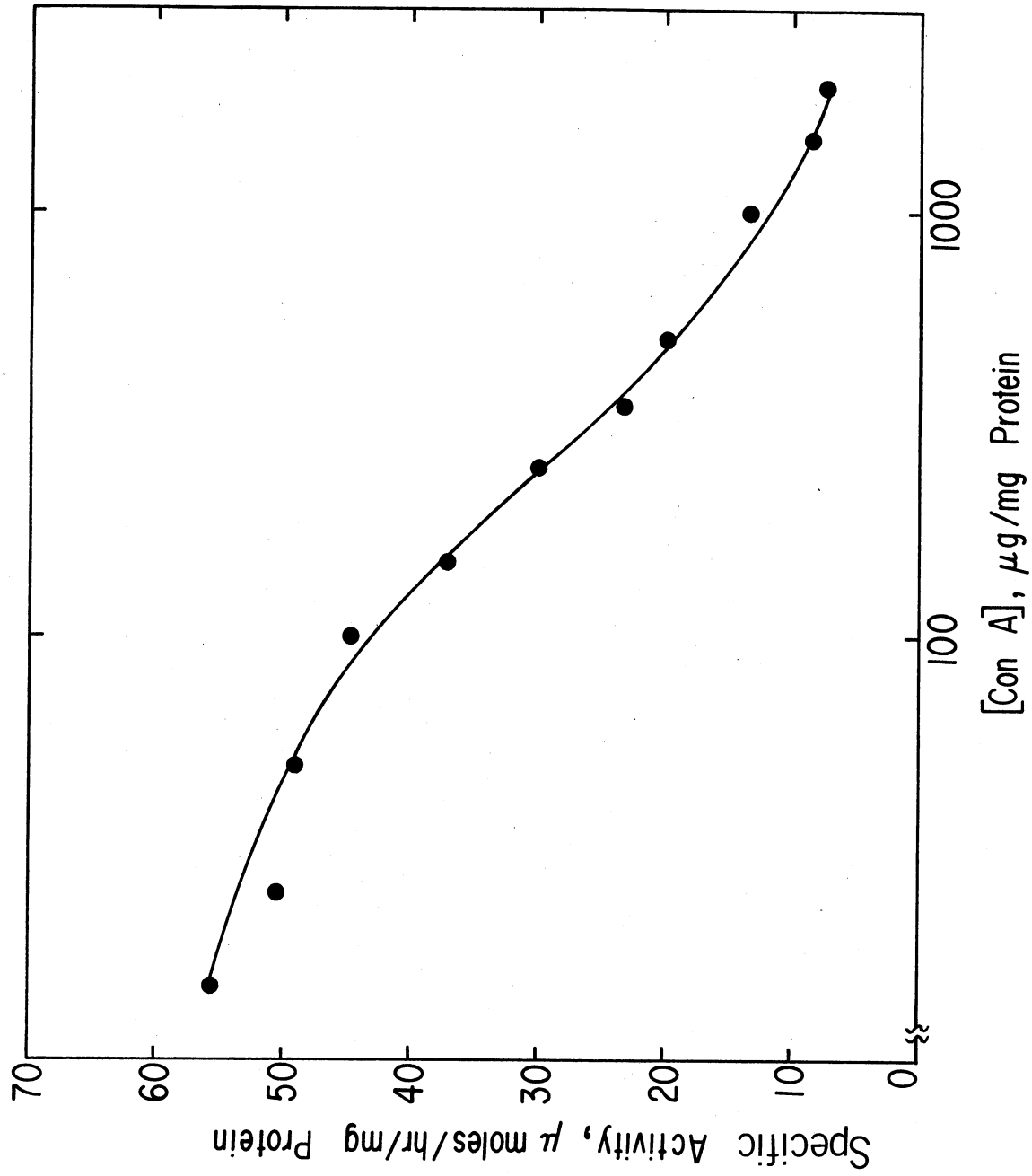
natant PAS gels reveal that GP-2, 3, 4, and 5 of the membrane are substantially enriched in DOC supernatants. In contrast the Coomassie blue gels demonstrate that the relative extraction of membrane protein components from the pellet material is less extensive and protein V is particularly resistant to extraction.

Similarly, 100% of the 5'-nucleotidase activity of washed cream could be solubilized. However, the 1% DOC concentration necessary for this step was four times that required for comparable solubilization from membranes. Since subsequent purification steps require concentration of the solubilized enzyme, the increased detergent procedure was not used for isolation purposes.

Figure 17 shows that the addition of relatively high concentrations of Con A to the assay medium caused considerable inhibition of the 5'-nucleotidase activity of isolated MFGM. The concentration required for half-maximal inhibition of the enzyme by Con A is 250 μg/mg membrane protein. Con A binds specifically to α-D-glucopyranosyl and α-D-mannopyranosyl residues of glycoproteins and polysaccharides. Enzyme samples were also incubated in the presence of Con A plus 50 mM α-methylmannoside. At the highest Con A concentrations tested, 90% of the inhibition was reversed by the addition of the α-methylmannoside which is a competitive inhibitor for the Con A saccharide binding site. Both the inhibition reaction and the reversal process proceeded rapidly and were virtually complete within 5 min.

The data of Figure 17 were used to prepare a Hill plot (69) for inactivation by Con A of membrane-bound 5'-nucleotidase. The slope gives a Hill coefficient of 1.0 which indicates no apparent cooperativity in the inhibitory process promoted by the interaction between the

Figure 17. Concentration dependence of Con A inhibition of 5'-nucleotidase membrane-bound. A 5'-nucleotidase-adenosine deaminase coupled spectrophotometric assay was used with homogenized membrane samples. The reaction mixture contained 0.1 mM AMP, 0.1 mM β-glycerophosphate, 50 mM Tris (pH 7.9), 1 mM $MgCl_2$, and 2-4 units adenosine deaminase in a final volume of 1 ml. Preincubation before lectin addition was for 15 min at 37° C. Upon lectin addition, an additional 15 min incubation at 37° C was included.


Specific Activity, μ moles/hr/mg Protein
70
60
50
40
30
20
10
0
100
1000
[Con A], μg/mg Protein

membrane-bound enzyme and the lectin (Figure 18).

The results of a fairly high concentration of Con A on 5'-nucleotidase activity at varied substrate concentrations in the range of 0.02 and 0.2 mM AMP yields a double reciprocal plot showing apparent noncompetitive inhibition (Figure 19). In the absence of Con A the apparent Km for the membrane-bound enzyme is 20 μM and V_{max} is 55 μmoles/hr/mg protein. In the presence of 500 μg Con A/mg protein the V_{max} changes to 13 μmoles/hr/mg protein.

Results of kinetic studies on DOC-solubilized enzyme in the presence of Con A were similar to those for membrane-bound enzyme. DOC-solubilized enzyme shows apparently noncompetitive inhibition of 5'-nucleotidase activity in the presence of substantial Con A (Figure 20). The apparent K_m is 15 μM, and V_{max} is 12 μmoles/hr/mg protein. Upon addition of 350 μg Con A/mg solubilized protein, the V_{max} changes to 6 μmoles/hr/mg protein.

Figure 21 shows that the DOC-solubilized enzyme is also inhibited by high concentrations of Con A with a concentration of 400 μg Con A/mg solubilized protein required to give 50% inhibition. The Hill plot for this data reveals a significant difference between membrane-bound and DOC-solubilized enzyme. The slope of the Hill plot for the soluble enzyme yields a Hill coefficient of 1.9 (Figure 22). This indicates a process involving a positive cooperative interaction between the enzyme and the lectin.

To distinguish whether the modification of 5'-nucleotidase activity was due to general perturbations of carbohydrate moieties or specifically due to the binding of the tetravalent Con A to mannose residues, the effects of lectins specific for other monosaccharides commonly found

Figure 18. Hill plot for Con A inactivation of membrane-bound 5'-nucleotidase. Data of Figure 17 were replotted according to the method of Hill (69). V_o and v represent the velocity of the reaction in the absence and presence of lectin, respectively. All assays were at 37° C.

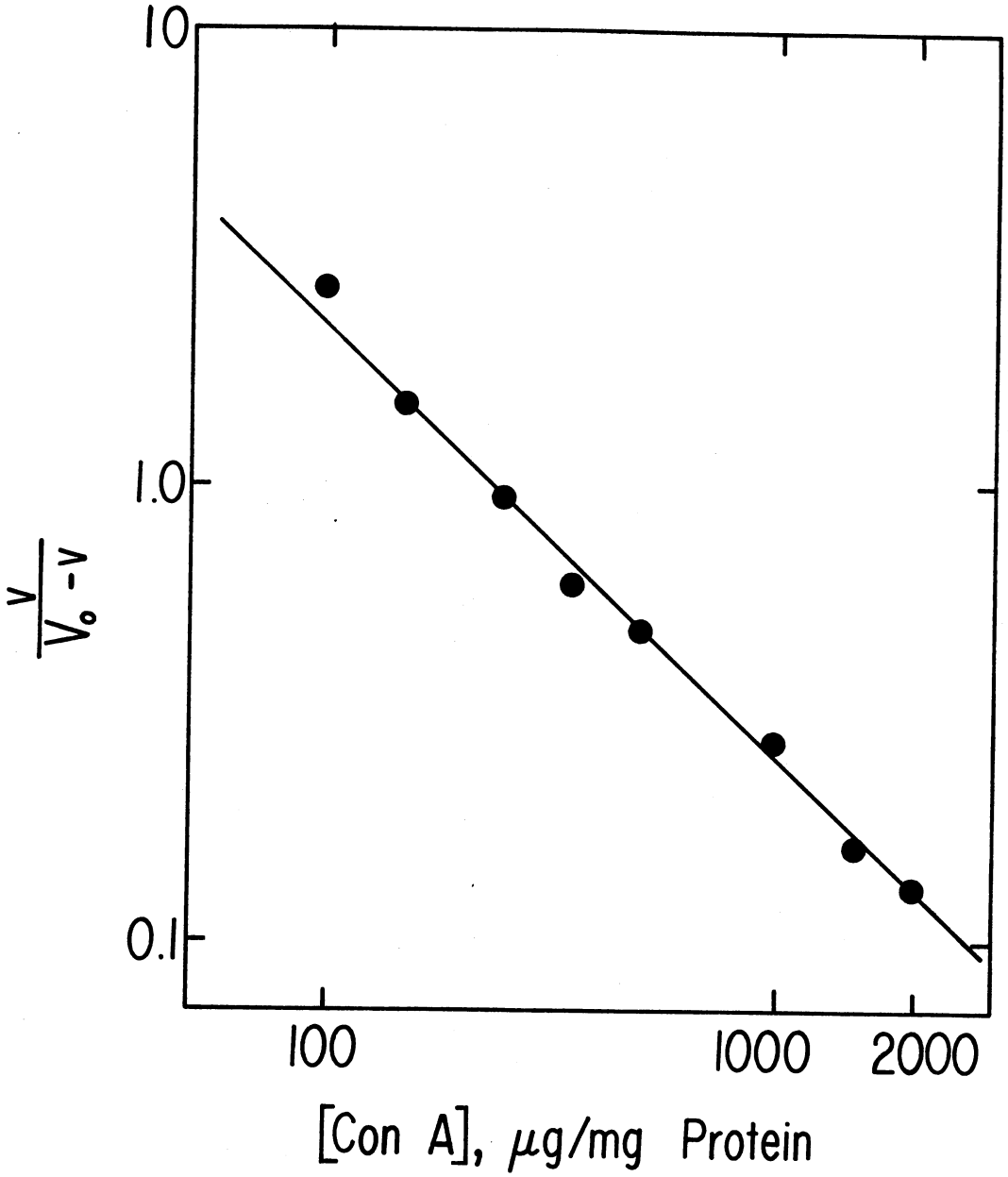

10
1.0
0.1
$\frac{v}{V_o - v}$
100
1000
2000
[Con A], μg/mg Protein

Figure 19. Lineweaver-Burke plot of velocity versus substrate concentration for the membrane-bound 5'-nucleotidase. The spectrophotometric assay was used with homogenized membrane samples. Final concentrations of reactants were 0.01 to 1.0 mM AMP, 50 mM Tris (pH 7.9), 1 mM $MgCl_2$, 2-4 units adenosine deaminase, and 0.1 mM β-glycerophosphate. After 15 min incubation at 37° C, lectin additions were made and the incubation was continued at 37° C for 15 min. Velocities were determined at 37° C in the absence or presence of 500 μg Con A/mg protein.

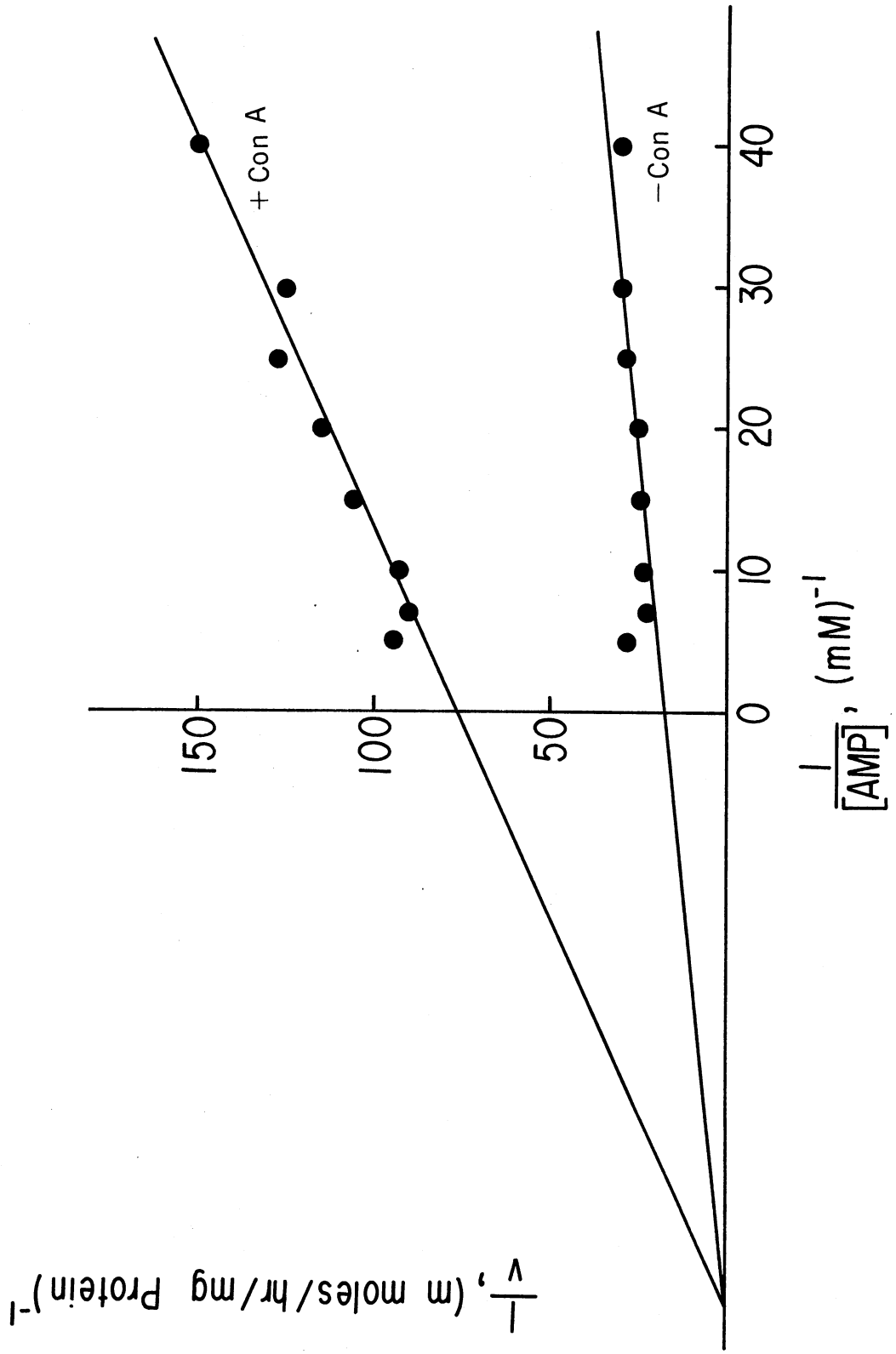

+ Con A
− Con A
150
100
50
0
10
20
30
40
$\frac{1}{v}$, (m moles/hr/mg Protein)$^{-1}$
$\frac{1}{[AMP]}$, (mM)$^{-1}$

Figure 20. Lineweaver-Burke plot of velocity versus substrate concentration for the DOC-solubilized 5'-nucleotidase. The spectrophotometric assay was used with DOC-solubilized enzyme samples. Final concentrations of reactants were 0.01 to 1.0 mM AMP, 50 mM Tris (pH 7.9), 1 mM $MgCl_2$, 2-4 units adenosine deaminase, and 0.1 mM β-glycerophosphate. After 15 min incubation at 37° C, lectin additions were made and the incubation was continued at 37° C for 15 min. Velocities were determined at 37° C in the absence or presence of 350 μg Con A/mg protein.

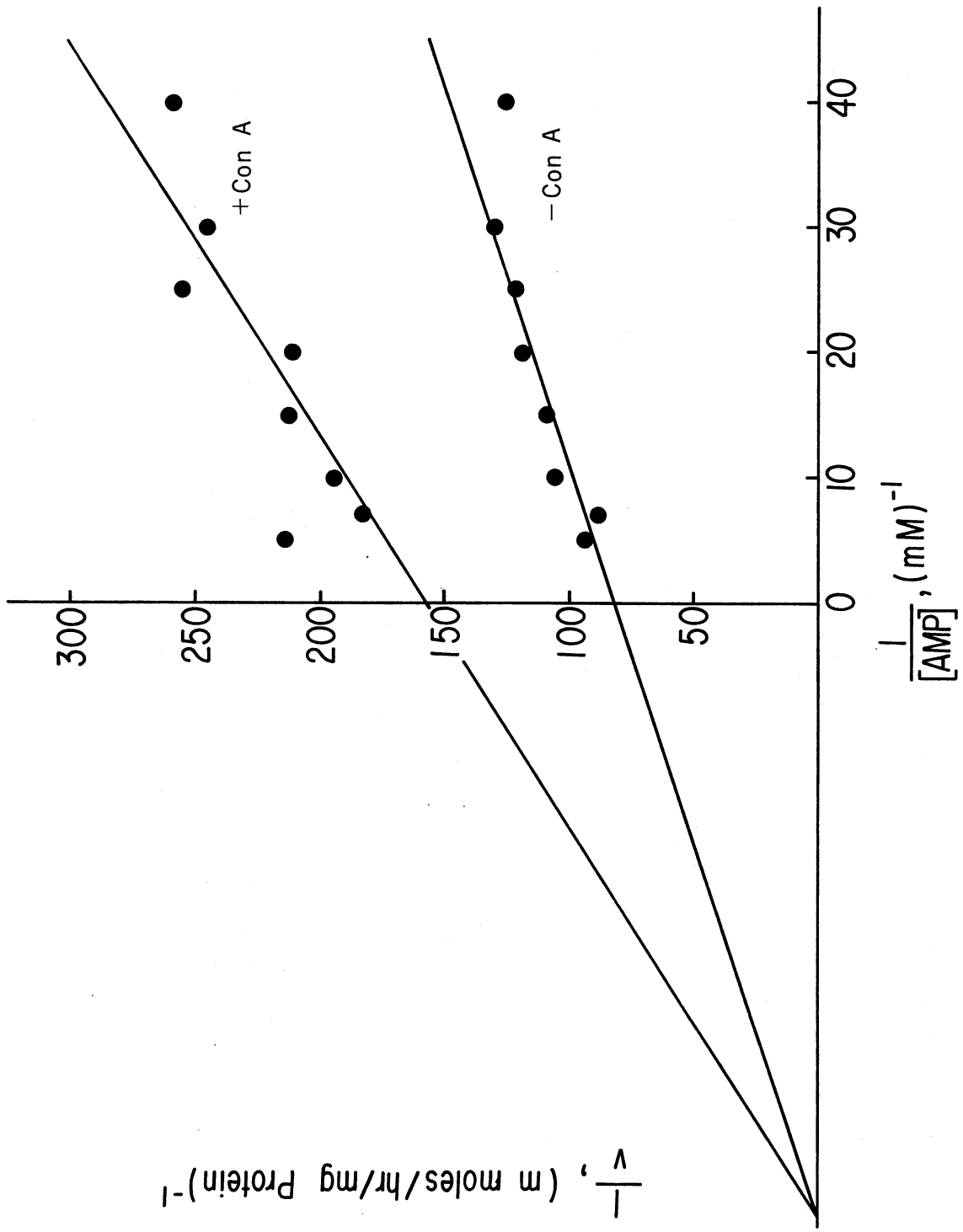

+Con A
−Con A
300
250
200
150
100
50
0
10
20
30
40
1/v, (m moles/hr/mg Protein)⁻¹
1/[AMP], (mM)⁻¹

Figure 21. Concentration dependence of Con A inhibition of DOC-solubilized 5'-nucleotidase. The spectrophotometric assay was used with DOC-solubilized enzyme supernatant. The reaction mixture contained 0.1 mM AMP, 0.1 mM β-glycerophosphate, 50 mM Tris (pH 7.0), 1 mM $MgCl_2$, and 2-4 units adenosine deaminase in a final volume of 1 ml. Preincubation before lectin addition was 15 min at 37° C followed by another 15 min incubation upon lectin addition.

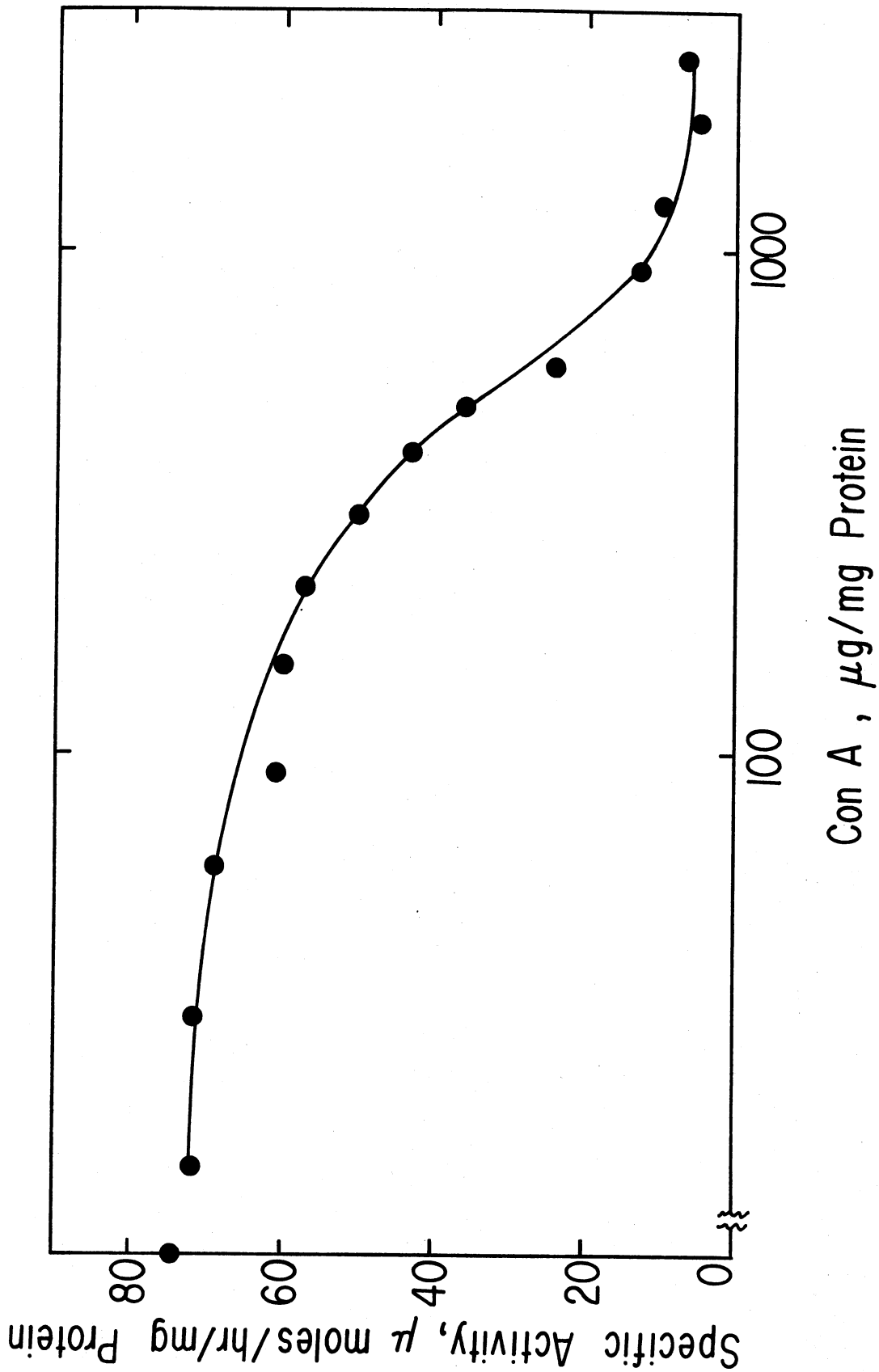
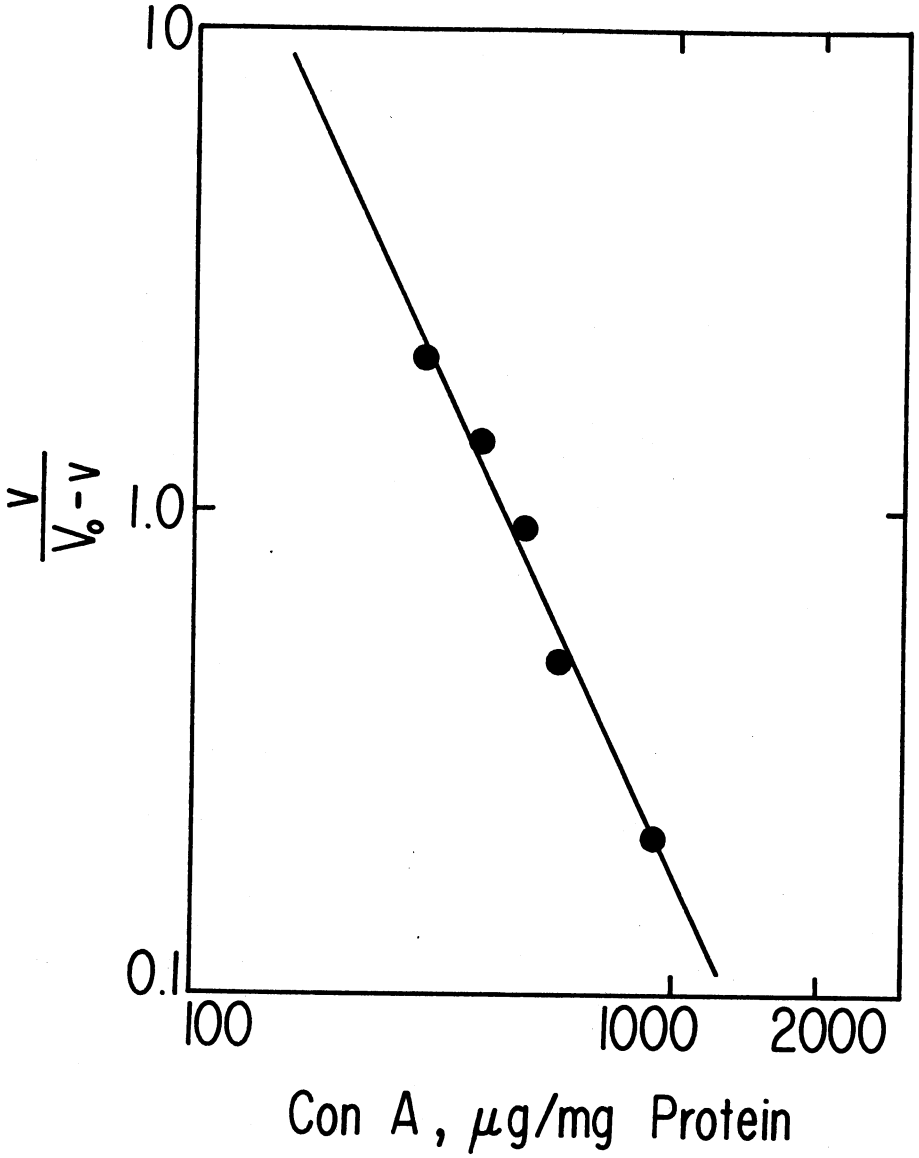

Specific Activity, μ moles/hr/mg Protein
0
20
40
60
80
100
1000
Con A , μg/mg Protein

Figure 22. Hill plot for Con A inactivation of DOC-solubilized 5'-nucleotidase. Data of Figure 21 were replotted according to the method of Hill (69). V_o and v represent the velocity of the reaction in the absence and presence of lectin, respectively. All assays were at 37° C.


10
1.0
0.1
$\frac{v}{V_o - v}$
100
1000
2000
Con A, μg/mg Protein

in glycoproteins were studied. Soy bean agglutinin showed no significant inhibition of 5'-nucleotidase activity (Figure 23). The inhibition of the enzyme by wheat germ agglutinin was only half of that observed with equivalent Con A additions (Figure 23). No positive cooperativity was detected in the wheat germ lectin-enzyme inhibition experiment.

To assess the role of the multivalency of Con A in mediating the cooperativity of enzyme inhibition, divalent lectin was prepared (57) and used in inhibition studies. The divalent Con A from two separate preparations did not inhibit deoxycholate-solubilized 5'-nucleotidase activity at concentrations as high as 1 mg divalent lectin/mg protein (Table IV). Typical inhibition was observed with native Con A controls. In contrast, membrane-bound 5'-nucleotidase activity was inhibited significantly by the same divalent Con A preparations. The amount of divalent lectin preparation required for minimum agglutination of SA-180 cells was increased 4 to 5 fold over native Con A.

Effect of Temperature on Enzyme Activity and Lectin Perturbations

Changes of activity with temperature were measured between 5 and 40° C. The hydrolysis rate of AMP at each temperature was linear over the range of enzyme concentrations used. The values obtained at 40° C were arbitrarily set as 100% for the purpose of comparison (Figure 24). The enzyme shows a large decline in activity with temperature decrease. The membrane-bound and solubilized enzymes differ markedly in temperature sensitivity. The activity of DOC-solubilized enzyme below 10° C was considerably higher (2-fold) than that of the membrane-bound enzyme. In the temperature range 10-30° C, the solubilized enzyme activity was

Figure 23. Effect of WGA and SBA on 5'-nucleotidase activity. To DOC-solubilized enzyme samples were added varied concentrations of the lectins, Con A (O—O), WGA (●—●), and SBA (▲—▲). Assays were performed as in Figure 14. Preincubation before and after addition of each lectin was for 15 min at 37° C. For each lectin the control without lectin addition was taken as 100% activity.

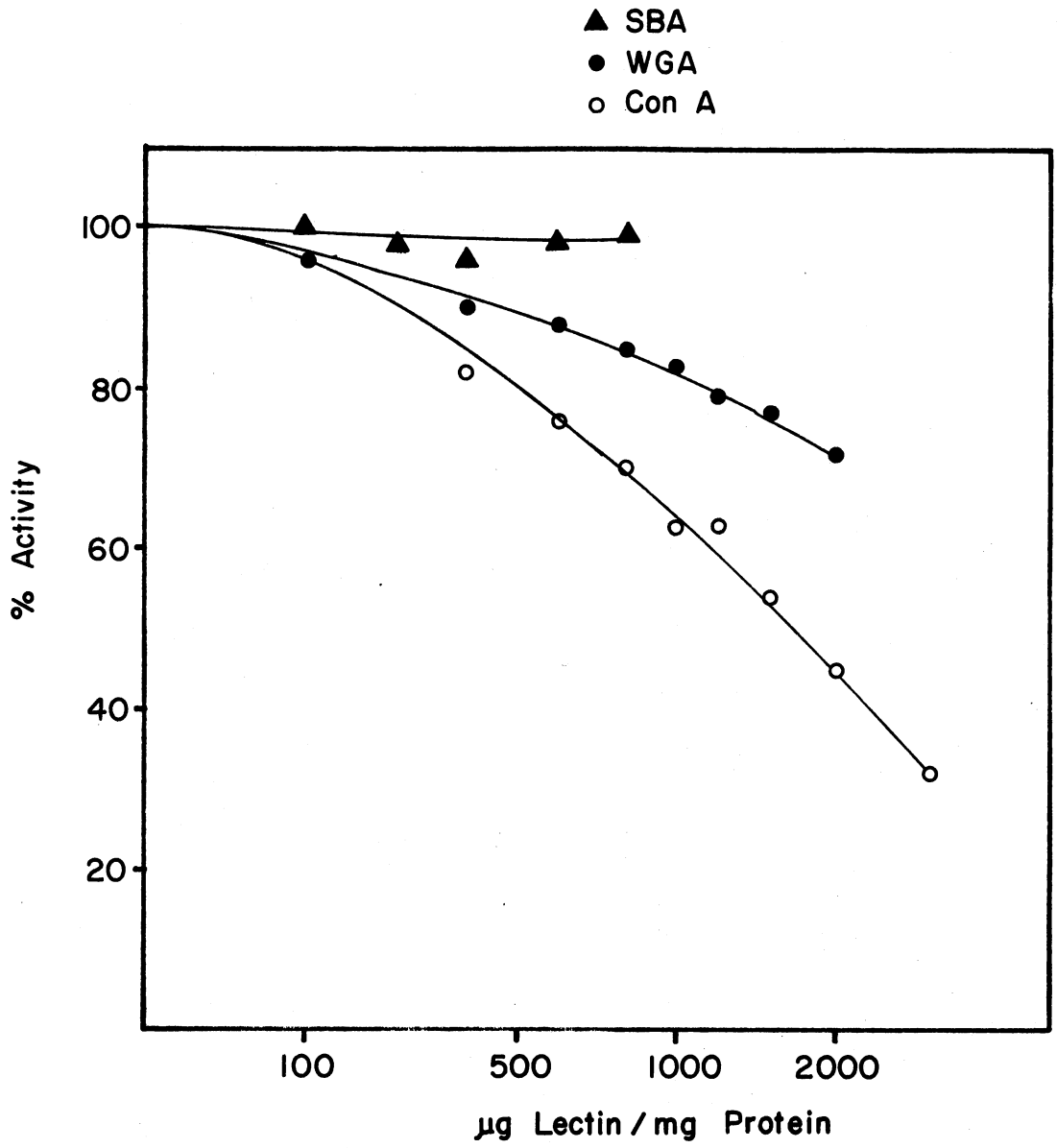
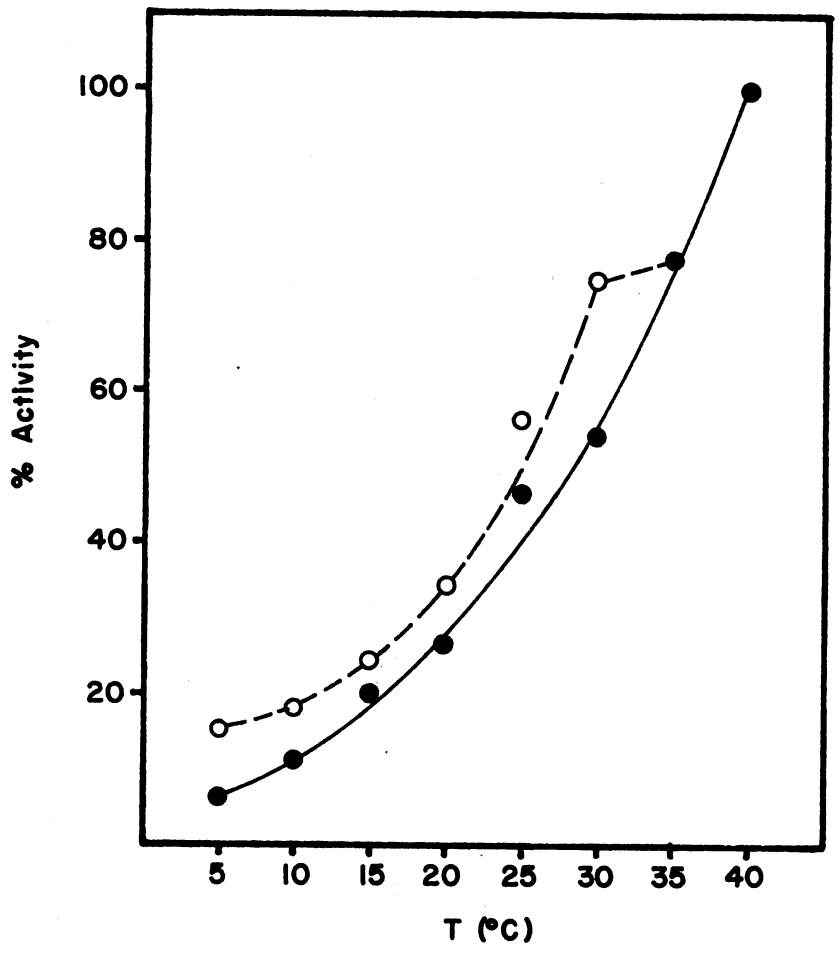

▲ SBA
● WGA
o Con A
100
80
60
40
20
% Activity
100
500
1000
2000
µg Lectin / mg Protein

TABLE IV

COMPARISON OF THE BIOLOGICAL ACTIVITIES OF CON A AND SUCCINYL-CON A

Property	Con A	Succinyl-Con A
(1) Minimum concentration for agglutination (μg/1 x 10^6 SA-180 cells)	0.75	3.5
(2) % Inhibition of DOC-solubilized 5'-nucleotidase		
(a) Lectin (300 μg/mg protein)	58	0
(b) Lectin (500 μg/mg protein)	70	0
(c) Lectin (1000 μg/mg protein)	-	0
(3) % Inhibition of membrane-bound 5'-nucleotidase Lectin (190 μg/mg protein	72	30

Figure 24. Changes of 5'-nucleotidase activity with temperature. Assays were by the spectrophotometric method with the final concentration of the reaction mixture being 0.1 mM AMP, 1 mM $MgCl_2$, 0.1 mM β-glycerophosphate, 2-4 units adenosine deaminase, 50 mM Tris (pH 7.9). The hydrolysis rate of AMP at each temperature was linear within the incubation period. The values obtained at 40° C were arbitrarily set as 100% for the purpose of comparison. ●—●, membrane-bound enzyme; 0—0, DOC-solubilized enzyme.


100
80
60
40
20
% Activity
5
10
15
20
25
30
35
40
T (°C)

consistently higher than membrane-bound enzyme activity. This effect of solubilization on enzyme activity is not noted at temperatures above 30° C.

Deoxycholate treatment releases the enzyme from the membrane and may partially remove lipid moieties associated with the enzyme. The activity-temperature curve for 5'-nucleotidase apparently changes upon deoxycholate treatment. This is consistent with the observation that in some cases the lipid type and the nature of acyl chains of lipids associated with membrane enzymes are decisive factors in determining temperature effects on activity (70, 71, 72).

The data of Figure 24 were used to construct Arrhenius plots shown in Figure 25. The apparent inflection points corresponded to temperatures of 23° C for solubilized enzyme and membrane-bound enzyme. Comparable results were found for temperature dependence of other membrane-bound enzymes (72, 73). The inflection point or break in Arrhenius plots is considered to be explicable by a conformational difference of the enzyme protein on both sides of the inflection. No apparent difference in inflection points is observed in Arrhenius plots between membrane-bound and soluble enzyme.

Temperature effects on enzyme-lectin interactions were studied by comparing Con A inhibition curves of 5'-nucleotidase activity with incubations and assays performed at 5, 20, and 37° C. Results are summarized in Table V. Using the spectrophotometric assay on DOC-solubilized enzyme, no positive cooperativity was detected at 5 or 20° C with the Hill coefficients being 1.0 and 1.05, respectively. At 37° C the same enzyme preparation had a Hill coefficient of 1.96 for Con A inhibition (Figure 26). By the phosphate assay method, membrane-bound

Figure 25. Arrhenius plots of 5'-nucleotidase activity. The values for membrane bound (●—●) and DOC-solubilized (0—0) enzyme are taken from the results of Figure 22.

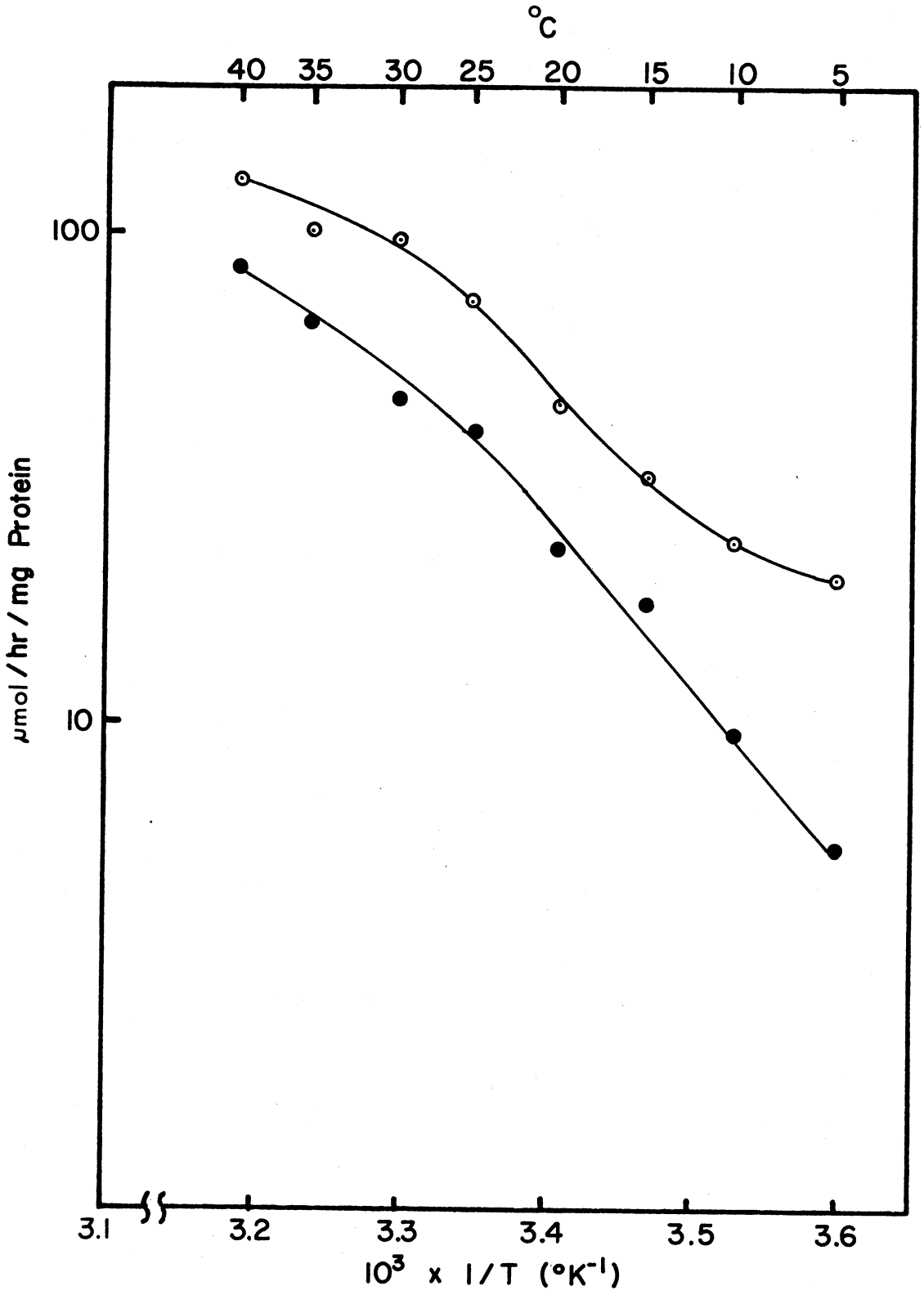
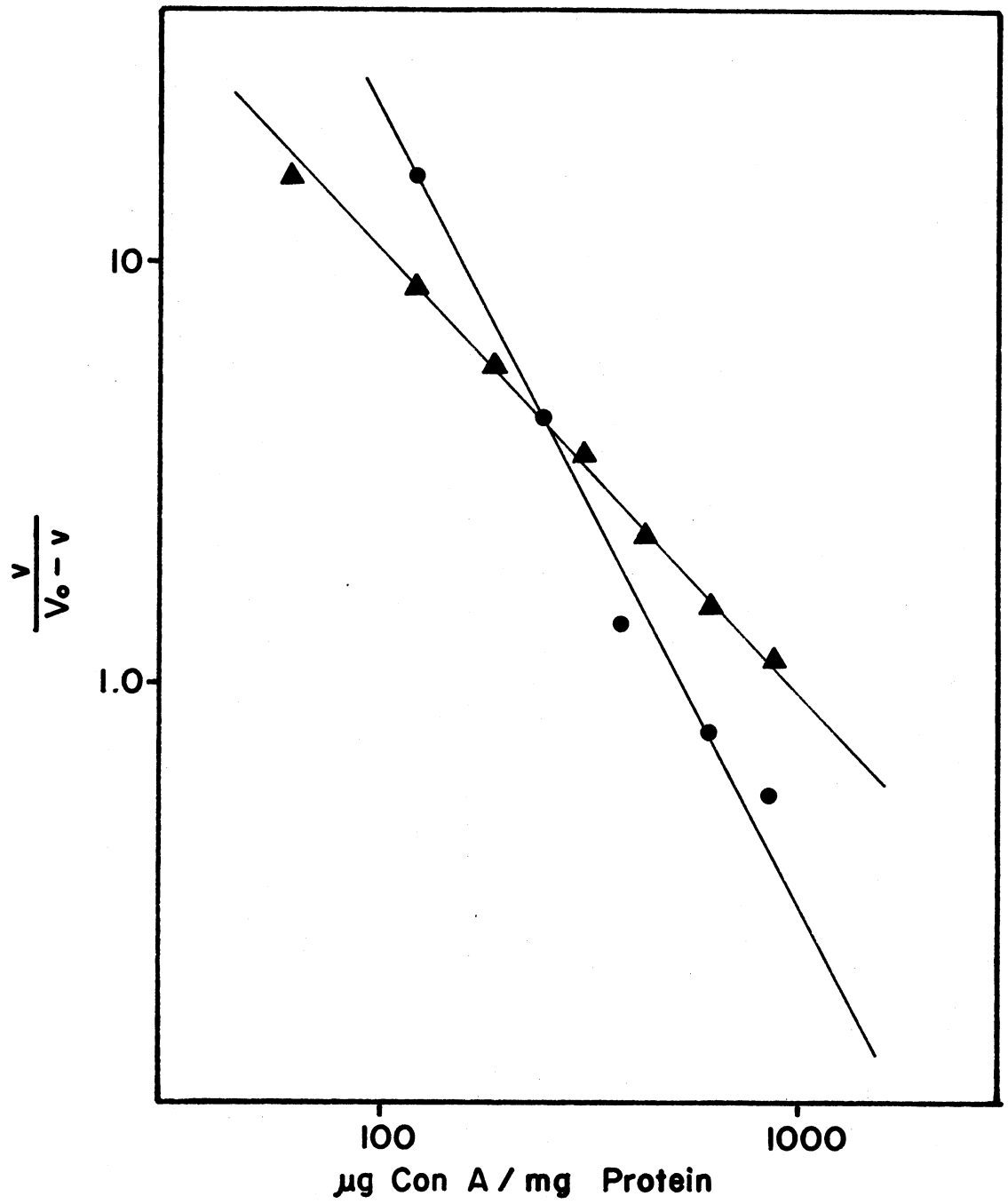

°C
40 35 30 25 20 15 10 5
100
10
μmol / hr / mg Protein
3.1
3.2
3.3
3.4
3.5
3.6
10³ x 1/T (°K⁻¹)

TABLE V

EFFECT OF TEMPERATURE ON HILL COEFFICIENTS OF 5'-NUCLEOTIDASE

Enzyme Preparation	Assay Method	Temperature	Hill Coefficient
Membrane-bound enzyme in homogenized membrane particles	Spectrophotometric	37°	1.0
	Phosphate	37°	1.26
	Phosphate	20°	1.14
	Phosphate	5°	1.26
DOC-Solubilized enzyme in 100,000 x g supernatant	Spectrophotometric	37°	1.96
	Spectrophotometric	20°	1.05
	Spectrophotometric	5°	1.0
	Phosphate	37°	1.8
	Phosphate	20°	1.12

Figure 26. Alteration of Hill coefficient for 5'-nucleotidase-Con A interaction by temperature. DOC-solubilized enzyme was prepared by the routine described in Experimental Procedure. The spectrophotometric assay was used with the medium containing 0.1 mM AMP, 0.1 mM $MgCl_2$, 0.1 mM β-glycerophosphate, 3 units adenosine deaminase, and 50 mM Tris (pH 7.9) in a volume of 3 ml. Samples were preincubated 15 min at the designated temperature and the incubation was continued for 15 min after lectin addition. Incubation temperatures were 37° C (●—●) and 20° C (▲—▲).


10
1.0
$\frac{v}{V_o - v}$
100
1000
µg Con A / mg Protein

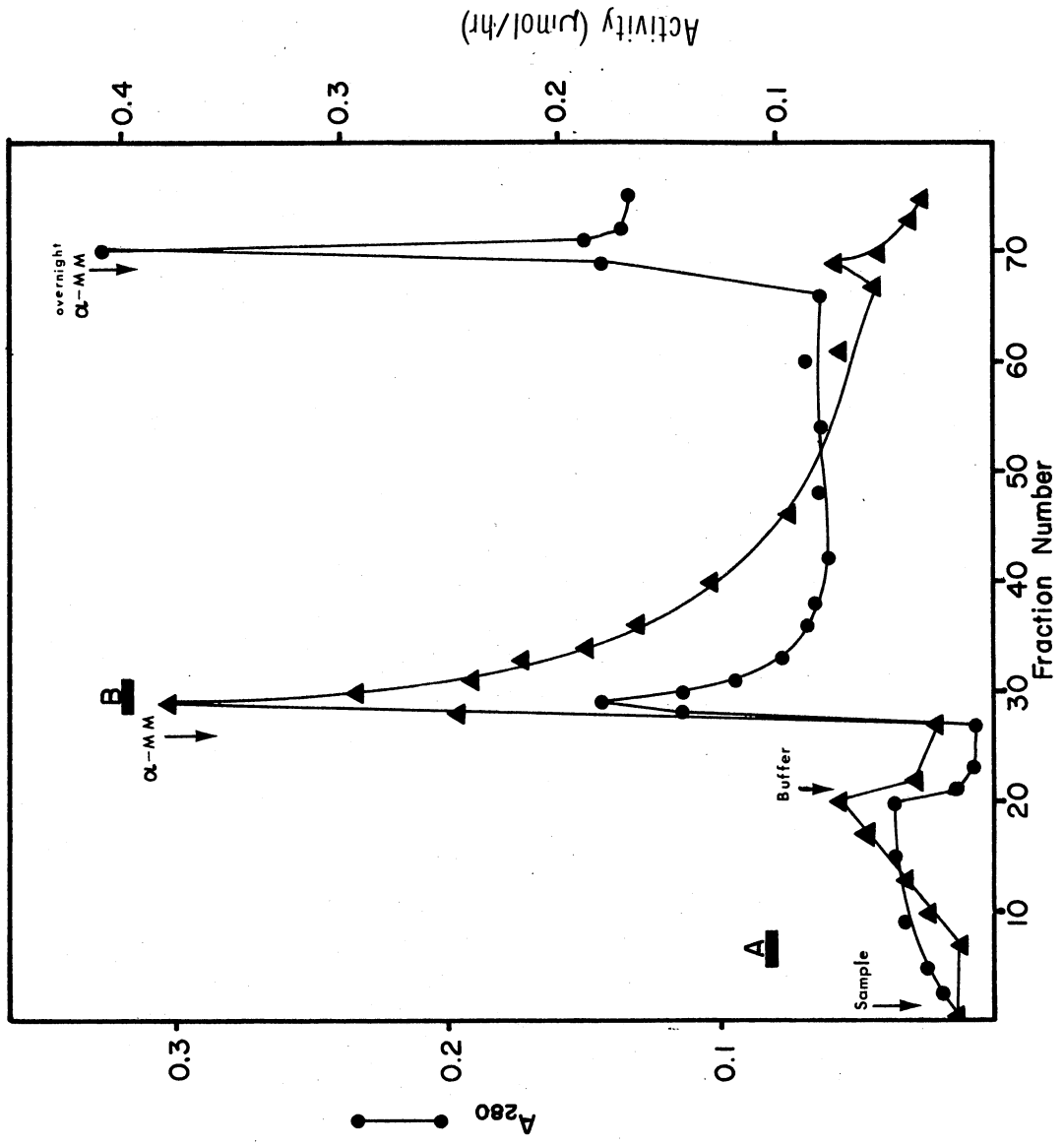
enzyme had a Hill coefficient of about 1.2 for all three temperatures, whereas DOC-solubilized enzyme had Hill coefficients of 1.8 at 37° C and 1.12 at 20° C.

Affinity Chromatography of 5'-Nucleotidase on Con A-Agarose

The substantial interaction of Con A and 5'-nucleotidase plus the reversibility of the process by α-methylmannoside provided a property that might be exploited for affinity chromatography.

Figure 27 shows a typical elution profile obtained when enzyme solution (795 μmol/hr in 6.67 mg protein) was applied to a column of Con A-Agarose. When the solution was washed through with DOC-Tris buffer (pH 7.9), 99.4 per cent of the enzyme was retained. The remainder of the activity and 75 per cent of the protein passed through with the wash. When the Con A-Agarose columns were eluted with 0.1 M α-methylmannoside in the DOC-Tris buffer, the recovery of adsorbed enzyme activity was quite variable. Elution with 0.1 M α-methylmannoside removed 38 per cent of the enzyme from the column. The recovery of 5'-nucleotidase was 302 μmol/hr units contained in 1.45 mg of protein (Figure 27). To promote the release of bound enzyme activity from the column, the Con A-Agarose column after its initial elution was allowed to stand 12 hours at room temperature in the presence of the 0.1 M α-methylmannoside-DOC-Tris elution medium. No further release of enzyme activity was noted (Figure 27). The A_{280} peak obtained after overnight elution in 0.1 M α-methylmannoside consisted mostly of Con A. Similar elution results were obtained when columns were eluted with α-methylmannoside at 4° C. α-methylglucoside gave similar elution results when

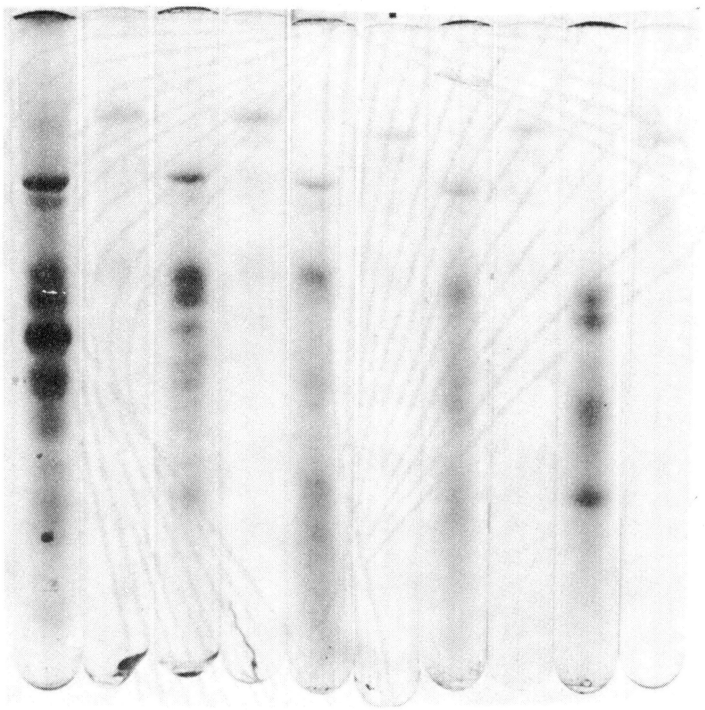
Figure 27. Affinity chromatography of 5'-nucleotidase. Samples of DOC-solubilized membrane were bound to and eluted from columns of Con A-Agarose as described in Experimental Procedure. Absorbance at 280 nm (●—●) and 5'-nucleotidase activity (▲—▲) were monitored. Fractions denoted by black bars were pooled and characterized by SDS gel electrophoresis. Elution from the column was with 0.1 M α-methylmannoside denoted by arrows.


Activity (μmol/hr)
0.4
0.3
0.2
0.1
overnight α-MM
B
α-MM
Buffer
A
Sample
A_{280}
0.3
0.2
0.1
10
20
30
40
50
60
70
Fraction Number

it was included in the elution buffer. Protein yields were difficult to quantitate from the columns since the addition of α-methylmannoside not only elutes bound glycoproteins but also releases considerable amounts of Con A. This is not uncommon for such columns (75). The low recovery of bound enzyme upon elution and the introduction of Con A contamination presents a significant limitation for including this method in a purification scheme. The incomplete elution has been observed by others who have used Con A for affinity chromatography of glycoproteins (75).

The two fractions separated by affinity chromatography had protein and glycoprotein profiles which differed in the distribution of some major MFGM components (Figure 28). The major membrane protein components, proteins II and V, were not retained on the column and appear only in the electrophoretic profiles of the sample application fractions (fraction A). GP-4 and component IV interacted strongly with the Con A column and appear only in fractions obtained after α-methylmannoside elution. The retention of protein IV which stains heavily with Coomassie blue yet has no apparent PAS polypeptide counterpart suggests that in the DOC-Tris supernatant employed in this experiment this polypeptide may be associated with a glycoprotein component or alternatively the protein IV may have a carbohydrate percentage which permits it to bind to the lectin yet does not interfere with Coomassie blue staining. Protein III and GP-2, 3, and 5 appear in SDS gels of both column fractions. This suggests a weaker interaction between these glycoproteins and Con A and is probably related to differences in the carbohydrate moieties of the glycoproteins and the more firmly bound GP-4. Another interpretation of these results could be that the PAS bands of GP-2, 3, and 5 each represent a mixture of proteins belonging to a particular

Figure 28. Acrylamide gel electrophoresis of Con A-Agarose chromatography fractions. Pooled fractions designated in Figure 27 were dialyzed, lyophilized, and prepared for SDS gel electrophoresis. Letters at the bottom represent fractions from the column. Samples are arranged (left to right) with alternating gels stained for protein and carbohydrate. M indicates membrane control. S indicates solubilized-enzyme sample.


M
S
A
A
B

molecular weight class. This is not the case at least for GP-2 since the glycoprotein has been purified by SDS gel filtration on the basis of molecular weight and shown to give only one PAS band on gels varying from 5 to 12.5% acrylamide.

It has been suggested that protein III and GP-5 are the same molecular species due to the similarity of their SDS gel electrophoresis mobility. These two polypeptides show identical distribution patterns in Con A-Agarose chromatography which supports this suggestion.

The fact that all protein bands applied to the column are accounted for in the eluate or wash suggests that there is no non-specific irreversible binding taking place.

It is possible that a more discrete fractionation of glycoproteins might be obtained by eluting columns with various concentrations of different sugars. Also it seems likely that glycoproteins could be fractionated by using lectins with different specificities.

Purification of 5'-Nucleotidase

The 5'-nucleotidase isolation scheme of Evans and Gurd (40) was modified for use with the enzyme solubilized from MFGM. A shallow sucrose gradient (5-20%) in 0.25% DOC-Tris medium was used to separate the enzyme activity from the bulk of the solubilized proteins (Figure 29). This step resulted in a 74-fold purification of the enzyme, which was associated with 6.8% of the total protein applied (Table VI). The distribution of protein and glycoprotein across the gradient was examined by SDS gel electrophoresis (Figure 30). The 5'-nucleotidase peak contained GP-1, 2, 3, 4, and 5. A sample from the outer edge of the rotor showed bands of GP-1, 2, 4, 5, and 6 as well as proteins II

Figure 29. Sucrose density gradient centrifugation of solubilized 5'-nucleotidase. Concentrated DOC-solubilized enzyme derived from 50 mg of MFGM was applied to a sucrose-detergent gradient and centrifuged at 200,000 x g for 9.2 hr. Details of gradient construction and collection are described in Experimental Procedure. The profile represents (left to right) a 5 to 20% sucrose-detergent gradient. Absorbance at 280 nm (——) and 5'-nucleotidase activity (---) were monitored. 5'-Nucleotidase was measured by the release of inorganic phosphate. Results are expressed as absorbance at 740 nm per 15 min incubation at 37° C of 50 µl of each fraction in 1.0 mM AMP, 50 mM Tris (pH 7.9).

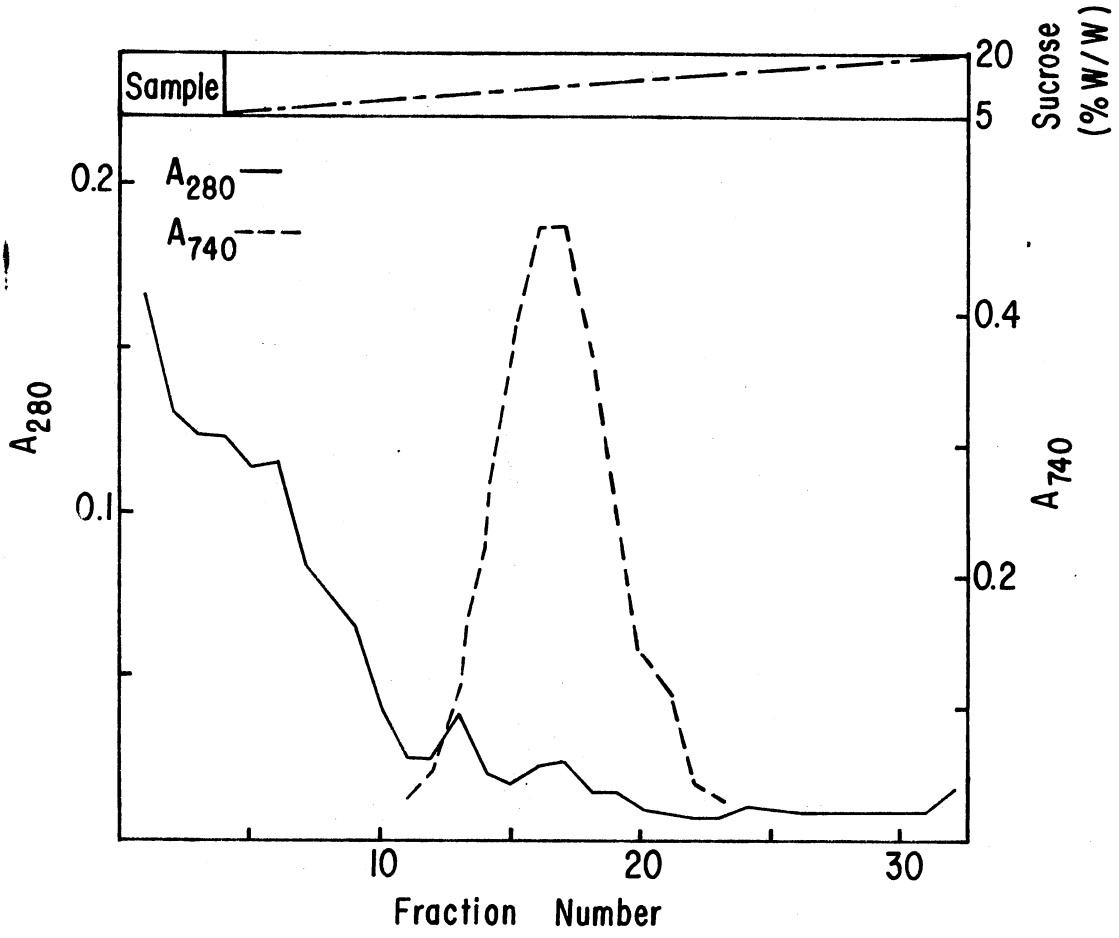
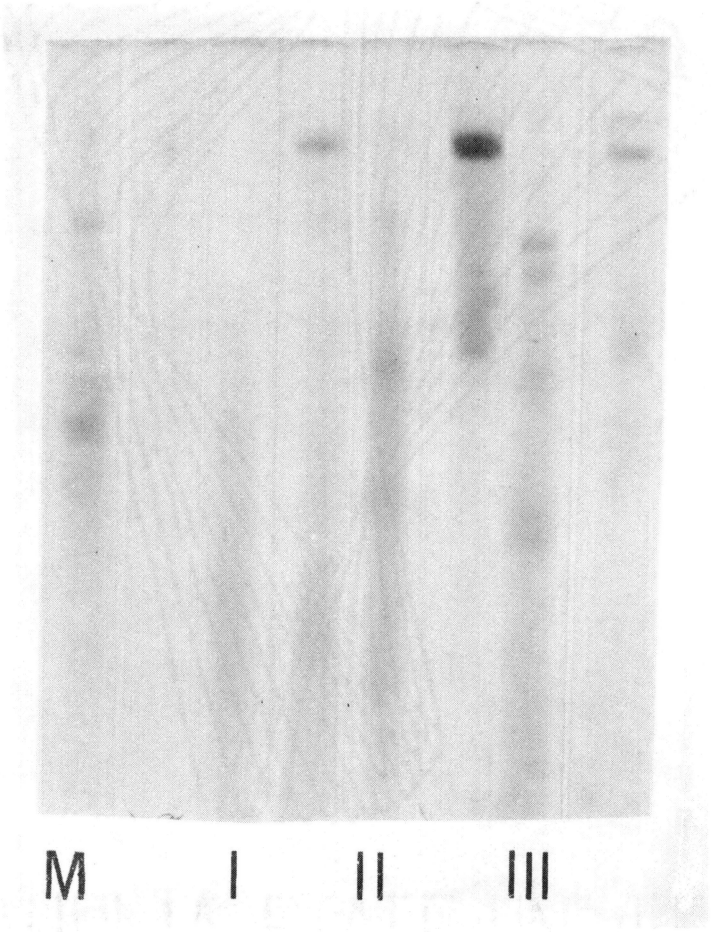

Sample
20
5
Sucrose (% W/W)
0.2
A280
A740
0.4
0.2
0.1
A280
A740
10
20
30
Fraction Number

TABLE VI

PURIFICATION OF 5'-NUCLEOTIDASE FROM MFGM

Fraction	Protein Recovered (mg)	Enzyme Activity Recovered (units)	Specific Activity (µmol of Substrate liberated/hr per mg of protein)	Yield (%)	Purification Ratio
Raw Cream	871	6332	7.27		
Washed Cream	841	7358	8.75	116	1.21
MFGM	49	3077	62.8	48.6	8.64
DOC-Tris Supernatant	11.8	872	73.9	13.8	10.2
Enzyme Peak from Sucrose-DOC Gradient	0.805	434	539	6.85	74.1
Sepharose 4B Enzyme Peak	0.520	442	850	6.98	117.0
Sephadex G-200 Enzyme Peak	0.0496	173	3500	2.73	481

Figure 30. SDS gel electrophoresis of fractions from a sucrose gradient containing detergent. Gradients were prepared and run as described in Experimental Procedure. A 5 ml sucrose gradient (5-20% (w/w)) was divided into three fractions, Fraction I (tubes 1-12); Fraction II (13-20); and Fraction III (21-31). The samples were prepared for electrophoresis as described in Figure 15. Fraction I represents the portion of the gradient from the inner edge of the rotor. Fraction II represents the enzyme peak from the central portion of the gradient. Fraction III contains the portion of the gradient near the outer edge of the rotor.


M
I
II
III

and IV.

Fractions containing enzyme activity were pooled, concentrated and chromatographed on Sepharose 4B in deoxycholate-Tris buffer (Figure 31). Excess detergent eluted at the void volume and was devoid of enzyme activity. The 5'-nucleotidase activity eluted at a molecular weight of approximately 80,000. Two major A_{280} peaks were retained in the column but were not coincident with the enzyme activity. The pooled enzyme activity peak from the Sepharose 4B column was concentrated and applied to Sephadex G-100, G-150, or G-200 columns in the same medium. All enzyme activity and protein eluted near the void volume of Sephadex G-100 and G-150. Figure 32 shows the elution profile of a concentrated enzyme activity peak applied to a G-200 column. The first two A_{280} peaks represent excess detergent which is not retained significantly by the column. The third A_{280} peak has a broad trailing shoulder which is coincident with the enzyme activity peak. The enzyme activity eluted at an apparent molecular weight of 94,000. The SDS gel electrophoresis profile of tubes across the peaks indicated the presence of two major glycoprotein components, GP-4 and 5 (Figure 33). The SDS gel electrophoresis profile suggests that GP-4 is the predominant polypeptide in Fraction 2 while GP-5 is apparently the predominant polypeptide in Fractions 3 and 4 from the G-200 column. Gel filtration resulted in a 480-fold purification of the enzyme, which was associated with 0.42% of the solubilized protein applied to the sucrose gradient. The purification ratio, yield, and protein recovery for each of the purification steps are summarized in Table VI.

Samples from the peak tube of enzyme activity were run on polyacrylamide gels in DOC-Tris buffer to correlate enzyme activity with

Figure 31. Sepharose 4B gel filtration of enzyme peaks from sucrose gradients. The enzyme peaks from sucrose gradients were pooled, concentrated, and applied to a 1.5 x 80 cm Sepharose 4B column in 0.25% DOC, 50 mM Tris (pH 7.9). Fractions of 3.1 ml were collected and monitored for absorbance at 280 nm (——), 5'-nucleotidase activity (---), and bromophenol blue (-·-·-). 5'-Nucleotidase was assayed by the phosphate method which measures the release of inorganic phosphate. Results are expressed as 740 nm absorbance per 15 min incubation at 37° C of 50 µl of each fraction in 1.0 mM AMP, 50 mM Tris (pH 7.0). Marker proteins were: A, Blue Dextran; B, β-galactosidase; C, bovine serum albumin; D, α-chymotrypsinogen; E, cytochrome c; F, bromophenol blue.

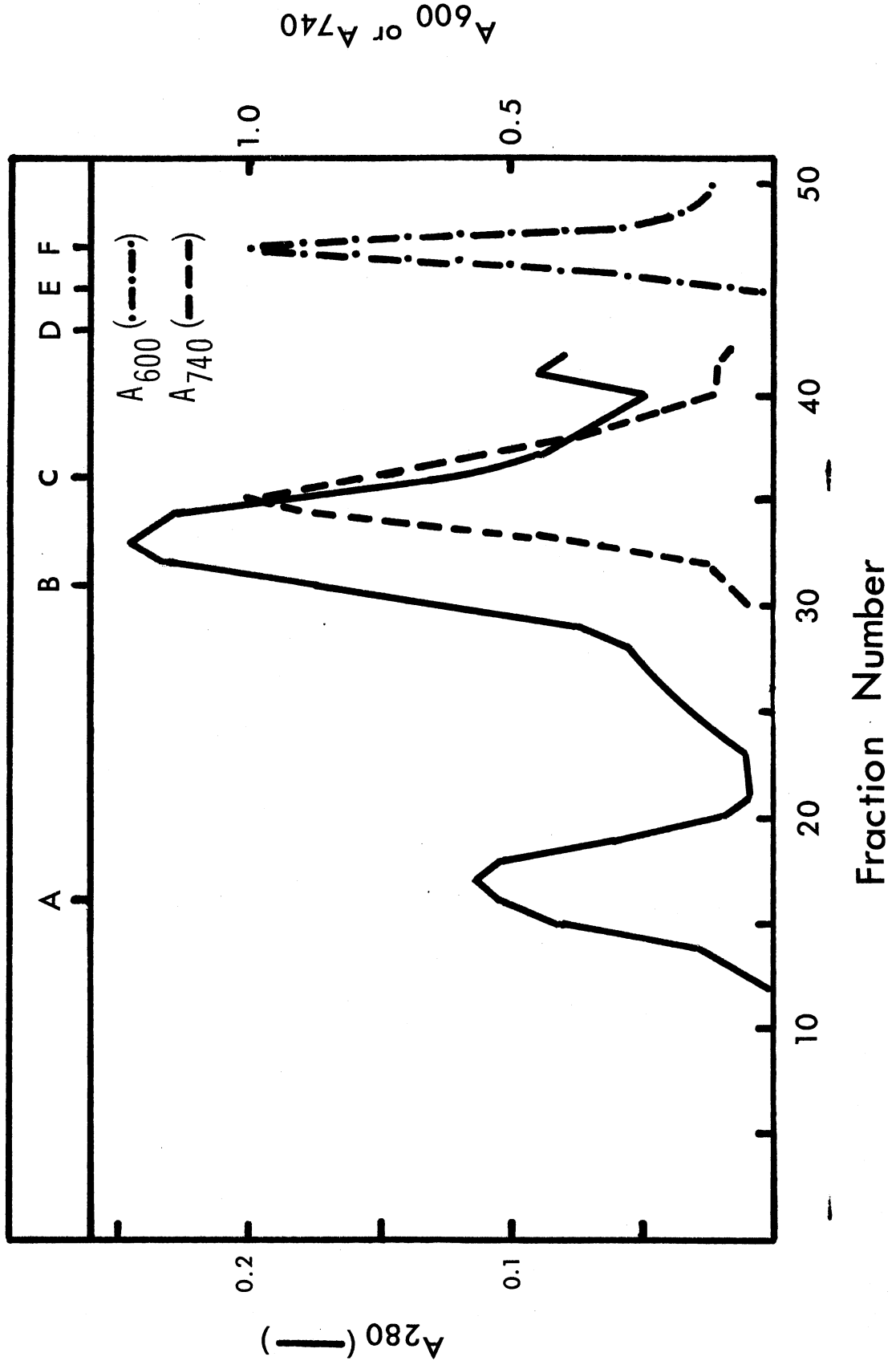

A600 or A740
1.0
0.5
A
B
C
D
E
F
A600 (·—·—·)
A740 (— — —)
0.2
0.1
A280 (——)
10
20
30
40
50
Fraction Number

Figure 32. Gel filtration of partially purified 5'-nucleotidase on Sephadex G-200. The 5'-nucleotidase peak from Sepharose 4B columns was concentrated and applied to Sephadex G-200 in 0.251 DOC- 50 mM Tris (pH 7.9). Absorbance at 280 nm (——) and 5'-nucleotidase activity (---) were monitored. Enzyme activity was monitored as absorbance at 740 nm as described in Figure 31. Marker proteins were: A, Blue Dextran; B, Bovine serum albumin-dimer; C, Bovine serum albumin-monomer. Enzyme activity was monitored as absorbance at 740 nm as described in Figure 31.

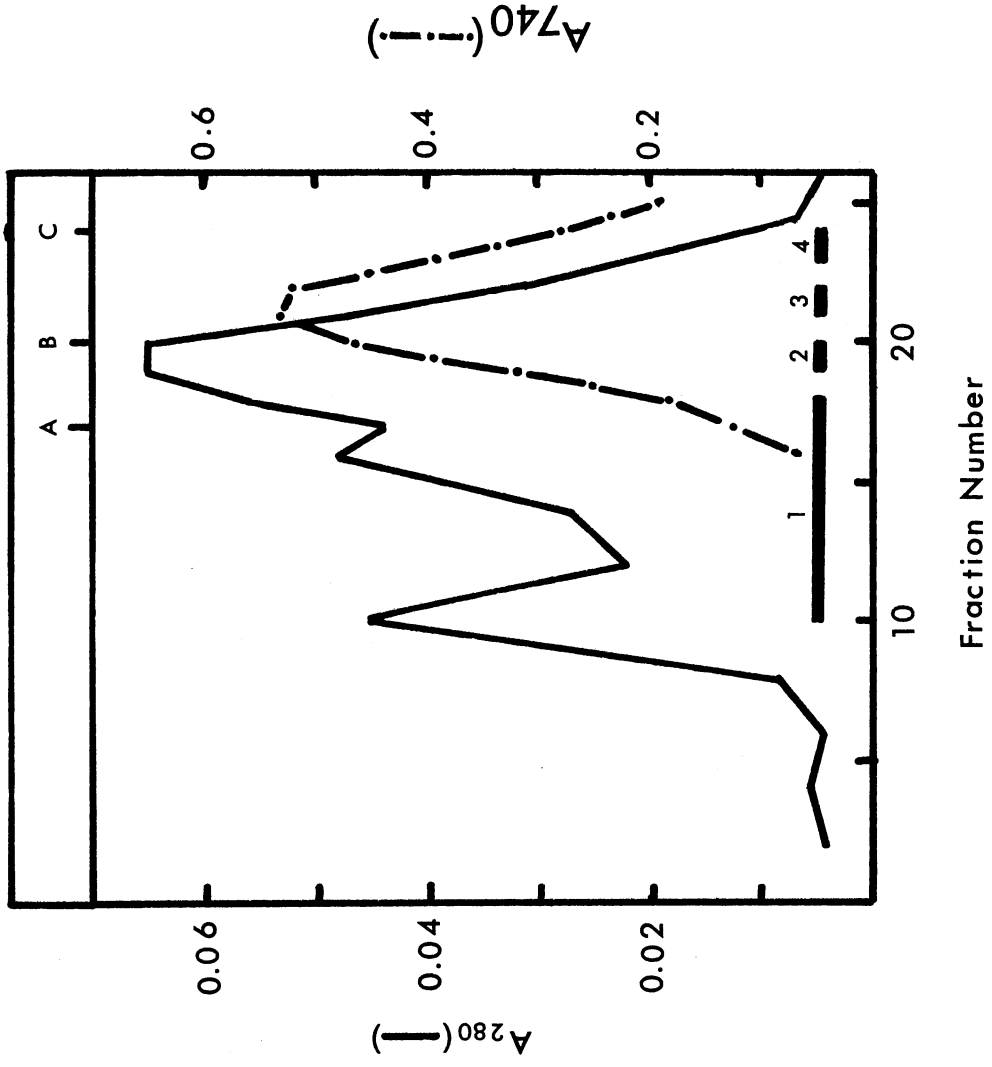
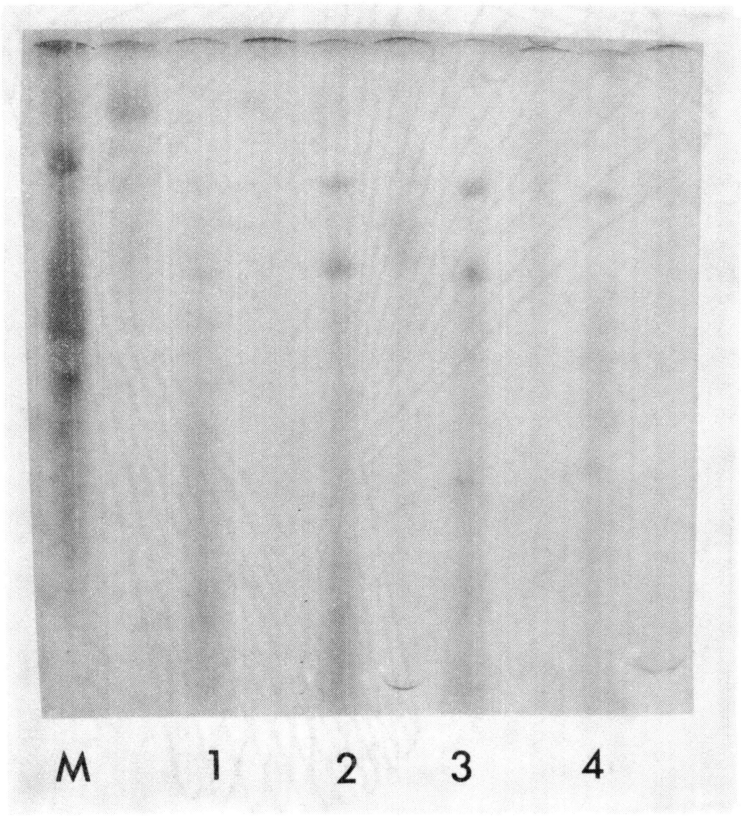
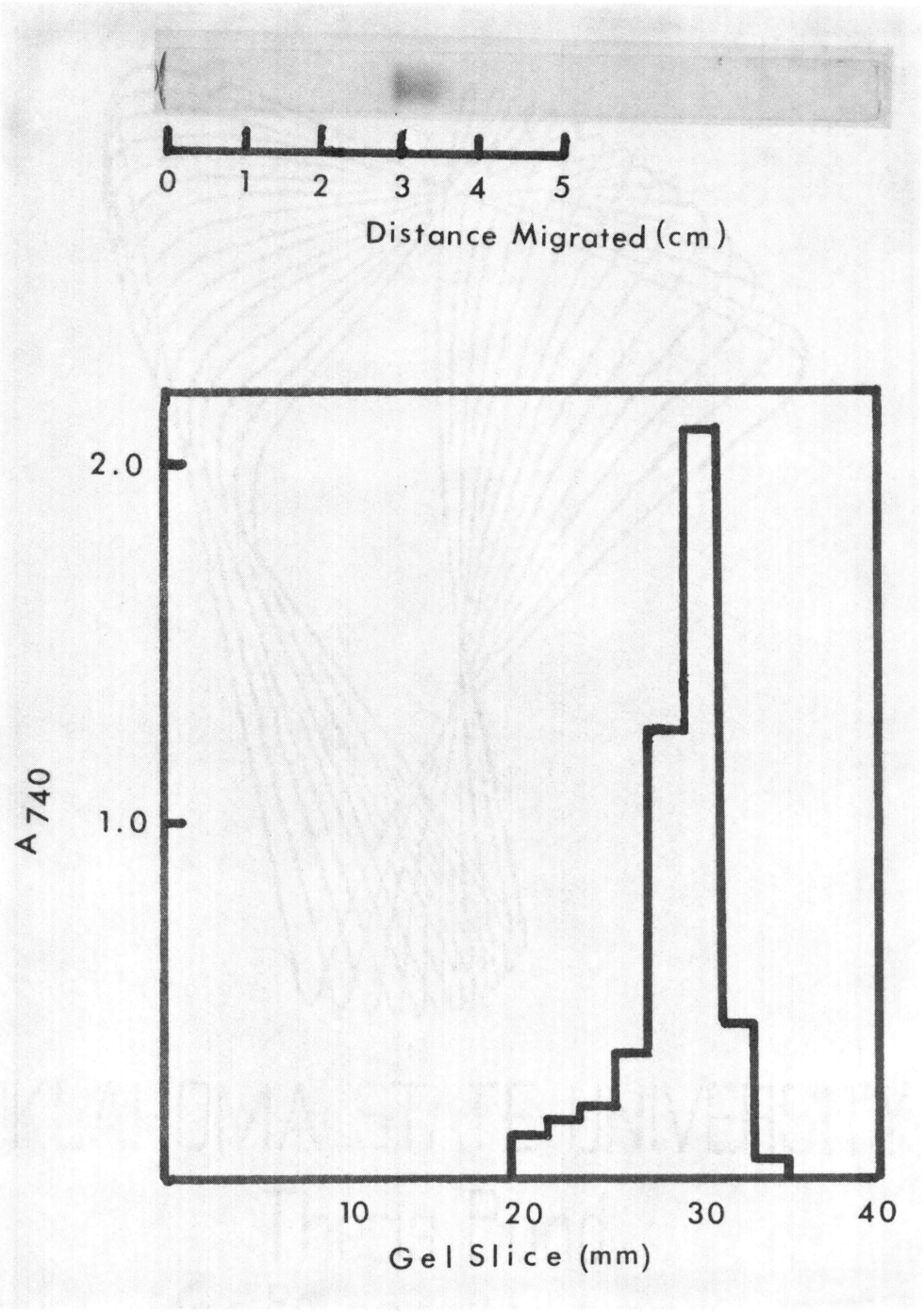

A740 (-·-·-)
0.6
0.4
0.2
A
B
C
1
2
3
4
10
20
Fraction Number
0.06
0.04
0.02
A280 (——)

Figure 33. SDS gel electrophoresis of 5'-nucleotidase fractions from a G-200 column. Fractions indicated in Figure 32 were prepared for electrophoresis. M indicates membrane control. Samples are arranged (left to right) with alternating gels stained for protein and carbohydrate.


M
1
2
3
4

stained protein bands (Figure 34). A major band staining for both protein and glycoprotein corresponded to a single enzyme activity peak. There was a minor diffuse protein contaminant band which had no enzymic activity.

Figure 34. Distribution of protein staining and enzymic activity in a polyacrylamide gel in DOC-Tris medium. Samples (0.1-0.2 ml) from the peak tube of enzyme activity from the G-200 column were added to 50 µl of 40% sucrose containing Bromophenol blue (5 mg/ml). Electrophoresis was carried out on 5% polyacrylamide gels in 30 mM Tris, 30 mM glycine, 0.25% DOC (pH 7.4). Companion gels were stained for protein and sliced into 2 mm sections for enzyme assay. To locate enzyme activity each 2 mm section was incubated in 50 mM Tris (pH 7.9), 0.1 mM AMP, 1 mM $MgCl_2$ with a final volume of 0.4 ml. Incubation time was 1 hr. Color development was by the procedure of Lazarus (59) for measurement of inorganic phosphate. Results are expressed as 740 nm absorbance per 1 hr incubation at 37° C.


0 1 2 3 4 5
Distance Migrated (cm)
2.0
1.0
A 740
10 20 30 40
Gel Slice (mm)

CHAPTER IV

DISCUSSION

The glycoproteins of MFGM were shown to be selectively extracted from washed cream or isolated membranes without contamination with other membrane proteins by a chloroform/methanol procedure. After SDS solubilization of the glycoprotein extract, repetitive chromatography on Sephadex G-200 permitted the isolation of the major membrane glycoprotein, GP-2. The purified glycoprotein behaves anomalously on SDS gels where the molecular weight of the single glycoprotein band ranges from 185,000 daltons on 5% gels to 70,000 daltons on 12.5% gels. By gel filtration in Sepharose 4B in 1% SDS, the calculated molecular weight of GP-2 was 105,000 daltons. The discrepancy between the results of these methods may derive from a lack of binding of SDS by the carbohydrate portion of the glycoprotein molecule making the glycoprotein retarded more than the standard proteins used to calibrate both columns and polyacrylamide gels.

In comparison with the major glycoprotein from beef erythrocytes membranes which was characterized by Capaldi (76), GP-2 is lower in molecular weight by gel electrophoresis in SDS (185,000 versus 285,000) and by SDS column chromatography (105,000 versus 150,000). Although both methods introduce discrepancies, the GP-2 results are consistently lower by both methods. This is in accord with the apparent lower carbohydrate content of GP-2 (50%) compared to the erythrocyte glyco-

protein (68) carbohydrate content. However, the ratio of hexosamine: hexose:sialic acid for the total glycoproteins is quite similar between the two.

The amino acid compositions of the glycoproteins differ significantly, but both contain large amounts of aspartic acid, glutamic acid, serine, and threonine. The glycopeptide bonds of glycoproteins from mammalian sources appear to be primarily of two types. Glycosylamine linkages involve C1 of glucosamine and the amide nitrogen of asparagine. O-glycosidic linkages involve the C1 of galactose, N-acetylgalactosamine, mannose, or xylose to hydroxyl groups of serine, threonine, or sometimes hydroxylysine. Sialic acid and fucose are found as terminal molecules or on the nonreducing end in the carbohydrate chain of glycoproteins (77). Hence the serine and threonine content of GP-2 is commensurate with its high carbohydrate content.

Harrison (68) used Pronase to cleave sialoglycopeptides from the surface of intact MFGM. Five fractions were obtained upon DEAE-Sephadex chromatography. The molar percentage values for two of these sialoglycopeptides are very similar to the carbohydrate molar percentages determined for GP-2. Fucose is present in the carbohydrate of GP-2 as well as these two sialoglycopeptides. The other three sialoglycopeptides lack fucose. The threonine/serine ratios for these sialoglycopeptides and GP-2 are similar also. This suggests that the carbohydrate moiety of GP-2 may be externally disposed on the surface of the intact MFGM.

Previously, two reports have appeared of partial purification and partial characterization of MFGM glycoprotein species. Jackson (29) partially purified a mucoprotein of 123,000 daltons. Following papain digestion of the glycoprotein mixture, cleaved glycopeptides were

isolated from Sephadex gels and found to contain 32% hexose, 22.4% hexosamine, and 13.6% sialic acid. These values are two-fold greater for hexose and similar for hexosamine and sialic acid values observed for GP-2. Swope et al. (30) used differential centrifugation and 1 M KCl extraction to obtain glycoproteins from MFGM. Two fractions were obtained from a P-30 column. One peak contained 7.8% hexose and had a molecular weight of 300,000 which suggested the formation of associated species in the buffer system used. Total carbohydrate content of the glycoprotein fraction was estimated at 15-19%. The amino acid composition was reported but differs significantly from the analysis of GP-2. The glycoprotein isolated by Swope had a similar glutamic acid content but higher aspartic acid and lower serine content than GP-2. Due to the nature of the methods employed for characterization, it is not clear which glycoprotein components these partially characterized species correspond to.

The SDS-hydroxylapatite procedure described is a promising technique for glycoprotein isolation schemes. The technique permits the fractionation of SDS solubilized membrane glycoproteins as a class free of Coomassie blue staining protein components. This technique may be profitably utilized in procedures where glycoprotein species are desired for immunological or enzymatic characterization if non-denaturing detergents such as deoxycholate or Triton X-100 are substituted for SDS.

The 5'-nucleotidase activity from MFGM is associated with a glycoprotein component of the membrane. The enzyme has a broad neutral pH optimum, and as isolated in this study shows no significant Mg^{+2} requirement. Most of the enzyme activity could be selectively solubilized from membranes or washed cream by low concentrations of deoxycholate.

The enzyme was very stable in the detergent during a series of purification steps at room temperature.

Sucrose density centrifugation resolved several glycoprotein components from the bulk of deoxycholate solubilized membrane protein (Figure 29). The 5'-nucleotidase was isolated to near homogeneity by gel filtration. The molecular weight determined for the enzyme on Sephadex G-200 was 94,000, while on Sepharose 4B a molecular weight of 80,000 was obtained. These discrepancies probably arise from differences in the composition and nature of the beads of the two chromatographic media. These molecular weights differ from those obtained for the enzyme obtained from sheep brain (78) and mouse liver plasma membrane (40) where a molecular weight of 140,000 was reported. The liver plasma membrane enzyme was considered a dimer composed of identical glycoprotein subunits of molecular weight 75,000.

With the purest fractions we have obtained, SDS gel electrophoresis on 5 per cent gels shows one major band corresponding to GP-5 with a molecular weight of approximately 92,000. From these fractions the major band is coincident with all of the enzyme activity on DOC-Tris gel electrophoresis using 5 per cent gels. A minor contaminant appears to correspond to GP-4 with a molecular weight of approximately 145,000 on 5 per cent SDS gels and approximately 160,000 on Sephadex G-200 in the presence of DOC. The possibility that 5'-nucleotidase is composed of two non-identical subunits with only one subunit retaining activity has not been excluded.

For rat liver plasma membrane 5'-nucleotidase, Widnell and Unkeless (38) found a major enzyme peak eluted near the void volume of a 6 per cent agarose column and a minor enzyme peak was retained on the

column. With longer preparative procedures, the proportion of the retained peak increased and was ascribed to degradation of the major peak. Huang and Keenan (37) used the same procedure to extract 5'-nucleotidase from MFGM. They likewise obtained two enzyme peaks, however, the major enzyme peak was retained well into the column and a minor peak eluted near the void volume. After comparing the enzyme fractions by K_m, substrate specificity, and metal requirements, they concluded that the two fractions represented two separate 5'-nucleotidases. In the chromatographic profiles obtained in this study only one peak of the 5'-nucleotidase is present.

The specific activity of the 5'-nucleotidase increased during purification but there was a loss in total enzymic activity. This may result from replacement of lipid bound to the enzyme with detergent. The altered environment created by lipid-detergent exchange may be less favorable to the enzyme activity with extended time.

A further purification step involving hydroxylapatite chromatography of DOC-solubilized 5'-nucleotidase preparations may permit resolution of the partially purified enzyme. Another alternative final step might utilize a Sepharose 4B-α-methylene adenosine monophosphate conjugated affinity column to purify the enzyme.

DOC-treatment probably removes or perturbs the lipid association of the membrane-bound 5'-nucleotidase. At low temperature (5-10° C) and intermediate temperatures (15-25° C), the change of membrane-bound activity with temperature is considerably less than for solubilized enzyme. At higher temperatures (above 30° C) both preparations show identical temperature effects on change in activity. A contribution of lowered fatty acid fluidity to membrane-bound enzyme behavior would

be consistent with these results. The solubilized enzyme would be at least partially released from such constraints and activated by the environment provided by bound detergent. A decrease in the specific activity of 5'-nucleotidase has been noted in brain homogenates and liver plasma membranes from rats maintained on essential fatty acid deficient diets (74).

Several membrane associated enzymes including sarcoplasmic reticulum ATPase and hepatic microsomal UDP-glucuronyl transferase are inactivated by removal of phospholipids (40). The partially purified enzyme preparations of Huang and Keenan (37) and Widnell and Unkeless (38) contained large amounts of phospholipid (primarily sphingomyelin) associated with the enzyme. However phospholipase C or butanol-ether extractions did not affect 5'-nucleotidase activity of rat liver plasma membrane suggesting that it is not a phospholipid dependent enzyme (40). The isolation procedure of Evans and Gurd (40) permitted the isolation of active enzyme without detectable amounts of phospholipid. They attributed previous reports of phospholipid dependence for enzyme activity to the complexity of the double detergent system used for solubilization.

When the 5'-nucleotidase is solubilized, the Arrhenius plot becomes more complex for the solubilized enzyme than for the membrane-bound enzyme. However, the similarity of the location of breaks or inflections in the Arrhenius plots for solubilized and membrane-bound enzyme suggest that the bound detergent is apparently able to replace the membrane hydrophobic environment by one equally favorable to the enzyme in which the detergent is acting as a lipid substitute.

For membrane enzyme changes in temperature characteristics and loss

of discontinuity in Arrhenius plots have been interpreted to mean that the temperature induced change in activation energy denoted by the break or inflection was associated with a phase change in the lipid component of membranes whereas similar breakpoints in both membrane-bound and solubilized enzyme systems are interpreted as indicating temperature-dependent conformational changes in the enzyme (72, 79). The inflection points for 5'-nucleotidase occur within similar temperature ranges as the inflections for Arrhenius plots of other membrane-bound enzymes (73). Total ($Na^+ + K^+ + Mg^{++}$) ATPase showed a discontinuity at 26° C for both essential fatty acid deficient and control plasma membranes from rat liver (74).

The 5'-nucleotidase activity of MFGM was further characterized by perturbations with lectins. The Con A inhibition of the enzyme in both bound and soluble forms is essentially reversible by incubation in the presence of 50 mM α-methylmannoside. Both bound and solubilized enzyme are inhibited in an apparently noncompetitive manner with essentially no change in apparent K_m and a substantial reduction in V_{max}. This suggests that similar inhibitory mechanisms are operating in both preparations. These observations are consistent with a direct interaction of Con A with the enzyme molecule at an oligosaccharide site to produce a conformational change which alters enzyme activity. A very similar inhibitory effect was demonstrated for intact cultured C6 glioma cells (50) and 13762 MAT-A rat mammary tumor intact cells and isolated membranes (46).

At 37° C, the cooperativity of Con A response for membrane-bound and solubilized enzymes differ in MFGM. One possible explanation for the observed positive cooperativity in lectin-solubilized enzyme

interaction could be the classical explanation for allosteric behavior involving cooperative interactions between subunits of a multisubunit nucleotidase. An alternative explanation might invoke the multivalent nature of Con A as the source of cooperativity by way of Con A induced associations of the nucleotidase molecules. In the latter case cooperativity would be dependent not only on the enzyme structure but also on the lectin association, the enzyme association with the membrane, and/or the mobility of the enzyme. Solubilization apparently removes the constraints imposed by the membrane and permits free enzyme association.

The Con A inhibition of soluble enzyme also differs in concentration required for half-maximal inhibition which is 2-fold higher for the solubilized preparation than for the isolated membranes. Similar results were noted with solubilized enzyme preparations from rat mammary gland and 13762 rat mammary tumor. In contrast, Riordan (49) observed that solubilized enzyme from rat liver plasma membrane was more susceptible to Con A inhibition with smaller Con A additions being required for half-maximal inhibition. Several experiments are consistent with the cooperative effect being derived from the tetravalent nature of Con A. Of three lectins tested, WGA (divalent) and Con A (tetravalent) did inhibit the solubilized enzyme while SBA did not. However, only the tetravalent Con A inhibited the solubilized enzyme in a cooperative manner. Con A undergoes a tetramer to dimer transition below 25° C (80). At lower temperatures (5 and 20° C), the cooperative effect was abolished although substantial inhibition was still observed for the solubilized enzyme-Con A interaction. Divalent Con A did not inhibit the enzyme at divalent lectin concentrations that were 10-fold greater than native lectin concentrations which produced 50 per cent inhibition.

Presumably higher concentrations of divalent lectin are required to produce inhibition. Fraser (81) reported that a 9-fold increase in divalent Con A was necessary to induce agglutination in mouse spleen cells when compared to native Con A agglutination results. In this study divalent-Con A preparations produced agglutination of SA-180 cells at divalent lectin concentrations 4 to 5 fold greater than native Con A. The divalent Con A inhibited membrane-bound 5'-nucleotidase. This demonstrates that the saccharide binding capacity of the divalent lectin was maintained. The data suggests that the cooperative alteration of 5'-nucleotidase activity is not due to mere general perturbations of carbohydrate moieties but rather is specifically related to the binding of tetravalent Con A to the enzyme.

Bornens et al. (82) described the cooperative binding of Con A to rat thymocytes plasma membranes. When succinyl-concanavalin A was used or when cells were pre-fixed with gluteraldehyde, there was no cooperative lectin binding. They interpreted their results as showing that a cooperative membrane modification was brought about by the binding of the tetravalent lectin. Schmidt-Ullrich and Wallach (83) suggest that the cooperative binding process which they observe for the binding of Con A to rat thymocyte membranes is derived from polymerization of a specific 55,000 dalton glycoprotein receptor during lectin binding. Specific crosslinking of this one receptor abolishes the cooperative binding of Con A. Their data supports the concept of membrane enzyme activity being modified by association of enzyme molecules induced by the tetravalent lectin.

In addition to the tetravalence of Con A, the enzyme association with the membrane and perhaps its mobility are equally important in the

expression of the cooperative effect. Until the function of 5'-nucleotidase is known and the molecule's association with the membrane is determined, it is not possible to connect any of the cellular effects of Con A with the lectin's ability to affect the enzyme. However, the valence of the lectin and receptor mobility are probably important factors in the expression of the mechanism of action of the lectin at the cell surface.

CHAPTER V

SUMMARY

The major glycoprotein from the bovine milk fat globule membrane was solubilized by selective extraction of washed cream with chloroform/methanol/water. The glycoprotein was purified to homogeneity by repetitive chromatography of the dodecyl sulfate-solubilized extract on Sephadex G-200 in 1% dodecyl sulfate. The purified glycoprotein gave one band upon SDS gel electrophoresis at gel concentrations from 5 to 12.5%, and the apparent molecular weight of the glycoprotein varied from approximately 185,000 to 70,000, with gel percentage. The amino acid composition of the glycoprotein was characterized by a relatively high content of serine, threonine, and glutamic acid residues. The carbohydrate portion of the molecule may represent as much as 50 per cent by weight and consisted of hexosamine (20% of the total glycoprotein), sialic acid (13%), and hexose (16%). The mole percentages of total carbohydrate for individual monosaccharides were galactose (15.9), mannose (11.1), fucose (5.84), N-acetylgalactosamine (14.0), N-acetylglucosamine (22.3), and sialic acid (30.5).

The glycoprotein enzyme 5'-nucleotidase was selectively extracted from isolated milk fat globule membranes with low concentrations of deoxycholate, which left most of the membrane protein unsolubilized. The enzyme was purified by sucrose gradient centrifugation and by gel filtration on Sepharose 4B and Sephadex G-200 columns equilibrated with

detergent. One peak of activity was obtained from Sephadex G-200 chromatography. Two glycoprotein bands were obtained on SDS gel electrophoresis and deoxycholate-Tris gel electrophoresis. The enzyme activity was coincident with the major glycoprotein band, while the second band apparently represented a minor contaminant. The molecular weights calculated for the enzyme activity by gel filtration in deoxycholate and by SDS gel electrophoresis was approximately 94,000 and 92,000, respectively. The enzyme is apparently solubilized and isolated as a glycoprotein monomer.

The nature of the cooperative effect of Concanavalin A (Con A) on 5'-nucleotidase was studied in milk fat globule membrane and for solubilized enzyme. Both membrane-bound and solubilized enzyme have an apparent K_m of about 15-25 μM and are inhibited by Con A in an apparently noncompetitive process. The inhibition is reversed by α-methylmannoside. Membrane bound enzyme is substantially inhibited by the lectin, but there is no cooperativity for the Con A-enzyme interaction at 5, 20, or 37° C. In contrast, the Con A-solubilized enzyme interaction shows significant cooperativity (Hill coefficient 1.9) at 37° C but the cooperativity is absent at 20° and 5° C although substantial inhibition is still observed. The latter observation suggested that the dimer-tetramer equilibrium of the Con A molecule may play a role in eliciting the cooperative effect. A divalent-dimer Con A sample was prepared and did not inhibit the solubilized enzyme although it would agglutinate SA-180 tumor cells and would inhibit membrane-bound 5'-nucleotidase. The data suggest that the tetravalence of the lectin is necessary for the cooperative effect to occur. Furthermore, the mode of interaction of enzyme with the

membrane seems to affect the expression of the cooperative effect for enzyme-lectin interaction.

A SELECTED BIBLIOGRAPHY

(1) Singer, S. J. (1974) Ann. Rev. Biochem., 43, 805.

(2) Nicolson, G. L. (1974) Internatl. Rev. Cytol., 39, 89.

(3) Patton, S., and Keenan, T. W. (1975) Biochim. Biophys. Acta, 415, 273.

(4) Briley, M. S., and Eisenthal, R. (1975) Biochem. J., 147, 417.

(5) Stein, O., and Stein, Y. (1967) J. Cell Biol., 34, 251.

(6) Wooding, F. B. P. (1971) J. Cell Sci., 9, 805.

(7) Bargmann, W., and Knoop, A. (1959) Z. Zellforsch., 49, 344.

(8) Patton, S. (1974) FEBS Lett., 48, 85.

(9) Hood, L. F., and Patton, S. (1973) J. Dairy Sci., 56, 858.

(10) Keenan, T. W., Morre, D. J., and Huang, C. M. (1972) FEBS Lett. 24, 204.

(11) Martel-Pradal, M. B., and Got, R. (1972) FEBS Lett., 21, 220.

(12) Keenan, T. W., Huang, C. M., and Morre, D. J. (1972) J. Dairy Sci., 55, 51.

(13) Kobylka, D., and Carraway, K. L. (1972) Biochim. Biophys. Acta, 288, 282.

(14) Keenan, T. W., Morre, D. J., Olson, D. E., Yunghans, W. N., and Patton, S. (1970) J. Cell Biol., 44, 80.

(15) Wooding, F. B. P. (1971) J. Ultrastructure Res., 37, 388.

(16) Henson, A. F., Holdsworth, G., and Chandan, R. C. (1971) J. Dairy Sci., 54, 1752.

(17) Anderson, M., Cheeseman, G. C., and Knight, D. J. (1972) J. Dairy Res., 39, 409.

(18) Kobylka, D., and Carraway, K. L. (1973) Biochim. Biophys. Acta, 307, 133.

(19) Mather, I. H., and Keenan, T. W. (1975) J. Memb. Biol., 21, 65.

(20) Keenan, T. W., Franke, W. E., and Kartenbeck, J. (1974) FEBS Lett. 44, 274.

(21) Baumrucker, C. R., and Keenan, T. W. (1973) J. Dairy Sci., 56, 1092.

(22) Wooding, F. B. P. (1973) J. Cell Sci., 13, 221.

(23) Anderson, M., Cawston, T., and Cheeseman, G. C. (1974) Biochem. J., 139, 653.

(24) Kitchen, B. J. (1974) Biochim. Biophys. Acta, 356, 257.

(25) Anderson, M., Brookes, B. E., Andrews, A. T., and Alichanidis, E. (1974) J. Dairy Sci., 57, 1448.

(26) Anderson, M., Brookes, B. E., Andrews, A. T., and Alichonidia, E. (1975) J. Dairy Res., 42, 401.

(27) Huang, C. M., Keenan, T. W., and Morre, D. J. (1972) J. Dairy Sci., 55, 662.

(28) Allen, J. C., and Humphries, C. (1975) in Isoelectric Focusing, J. P. Arbuthnott and J. A. Beeley, eds., Butterworths, London, p. 347.

(29) Coulson, E. J., and Jackson, R. H. (1962) Arch. Biochem. Biophys. 97, 378.

(30) Swope, F. C., Rhee, K. C., and Brunner, J. R. (1968) Milchwissenschaft 23, 744.

(31) Anderson, M., and Cawston, T. E. (1975) J. Dairy Res., 42, 459.

(32) Avis, P. G., Bergel, F., and Bray, R. C. (1955) J. Chem. Soc., 1100.

(33) Zittle, C. A., and Dellamonica, E. S. (1952) Arch. Biochem. Biophys., 35, 321.

(34) Zittle, C. A., Dellamonica, E. S., Custer, J. H., and Rudd, R. R. (1956) J. Dairy Sci., 39, 528.

(35) Mather, I. H., and Keenan, T. W. (1974) FEBS Lett., 44, 79.

(36) Patton, S., and Trams, E. G. (1971) FEBS Lett., 14, 230.

(37) Huang, C. M., and Keenan, T. W. (1972) Biochim. Biophys. Acta, 274, 246.

(38) Widnell, C. C., and Unkeless, J. C. (1968) Proc. Nat. Acad. Sci., 61, 1050.

Edelman, G. M. (1963) Proc. Nat. Acad. Sci., 70, 1012.

(58) Morre, D. J. (1971) Methods in Enzymol., 22, 130.

(59) Lazarus, L. H., and Chou, S. C. (1972) Anal. Biochem., 45, 557.

(60) Ipata, P. L. (1967) Anal. Biochem., 20, 30.

(61) Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem., 193, 265.

(62) Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956) Anal. Chem., 28, 350.

(63) Warren, L. (1959) J. Biol. Chem., 234, 1971.

(64) Spackman, D. H., Stein, W. H., and Moore, S. (1958) Anal. Chem., 30, 119.

(65) Glick, M. C. (1974) in Methods in Membrane Biology, E. D. Korn, ed., 2, 157.

(66) Hudson, B. G., Wegener, L. J., Wingate, J. M., and Carraway, K. L. (1975) Comp. Biochem. Physiol., 51B, 127.

(67) Reinhold, V. N. (1972) Methods in Enzymol., 25, 244.

(68) Harrison, R., Higginbotham, J. D., and Newman, R. (1975) Biochim. Biophys. Acta, 389, 449.

(69) Hill, A. V. (1910) J. Physiol. (London), 40, 190.

(70) Morero, R. D., Bloj, B., Farias, R. N., Trucco, R. E. (1972) Biochim. Biophys. Acta, 282, 157.

(71) Galo, M. G., Bloj, B., and Farias, R. N. (1975) J. Biol. Chem., 250, 6204.

(72) Farias, R. N., Bloj, B., Morero, R. D., Sineriz, F., and Trucco, R. E. (1975) Biochim. Biophys. Acta, 415, 231.

(73) Tanaka, R., and Teruga, A. (1973) Biochim. Biophys. Acta, 473, 584.

(74) Brivio-Haugland, R. P., Louis, S. L., Musch, K., Waldeck, N., and Williams, M. A. (1976) Biochim. Biophys. Acta, 433, 150.

(75) Gottlieb, C., Baenziger, J., and Kornfeld, S. (1975) J. Biol. Chem., 250, 3303.

(76) Capaldi, R. A. (1953) Biochim. Biophys. Acta, 311, 386.

(77) Spiro, R. G. (1973) Adv. Prot. Chem., 27, 350.

(78) Ipata, P. L. (1968) Biochemistry, 7, 507.

(79) Sineriz, F., Farias, R. N., Trucco, R. E. (1975) FEBS Lett., 32, 30.

(80) Huet, M. (1975) Eur. J. Biochem., 59, 627.

(81) Fraser, A. R., Hemperly, J. J., Wang, J. L., and Edelman, G. M. (1965) Proc. Nat. Acad. Sci., 73, 790.

(82) Bornens, M., Karsenti, E., Avrameus, S. (1976) Eur. J. Biochem. 65, 61.

(83) Schmidt-Ullrich, R., and Wallach, D. F. H. (1976) Biochem. Biophys. Res. Commun., 69, 1011.

VITA 2

Lloyd Dale Snow

Candidate for the Degree of

Doctor of Philosophy

Thesis: ISOLATION AND CHARACTERIZATION OF GLYCOPROTEINS FROM THE MILK FAT GLOBULE MEMBRANE

Major Field: Biochemistry

Biographical:

Personal Data: Born near Lake City, Arkansas, December 3, 1947, the son of Mr. and Mrs. William Lloyd Snow.

Education: Graduated from Lake City Public High School, Lake City, Arkansas in 1965; received the Bachelor of Science degree in Zoology from Arkansas State University in May, 1969; received the Masters of Science degree in Biology, Arkansas State University in August, 1972; completed requirements for the Doctor of Philosophy degree in December, 1976.

Professional Experience: Technician, Biology Department, Arkansas State University, 1966-1969; Biology Teacher, Bay-Brown Public High School, Bay, Arkansas, 1969-1971; Assistant, Biology Department, Arkansas State University, 1971-1972; Research Assistant, 1972-1976, Biochemistry Department, Oklahoma State University.

Honorary Societies: Phi Eta Sigma, Beta Beta Beta, Phi Lambda Upsilon, and Phi Kappa Phi.