# Studies on Aspects of Glycosylation of Glycoproteins During the Acute Phase Response to Inflammation

by

Howard Arthur Kaplan

A thesis presented to
The University of Manitoba
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Winnipeg, Manitoba
November, 1983

# STUDIES ON ASPECTS OF GLYCOSYLATION OF GLYCOPROTEINS DURING THE ACUTE PHASE RESPONSE TO INFLAMMATION

by

Howard Arthur Kaplan

A thesis submitted to the Faculty of Graduate Studies of
the University of Manitoba in partial fulfillment of the requirements
of the degree of

DOCTOR OF PHILOSOPHY

© 1984

Permission has been granted to the LIBRARY OF THE UNIVERSITY OF MANITOBA to lend or sell copies of this thesis, to the NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film, and UNIVERSITY MICROFILMS to publish an abstract of this thesis.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

To Donna, Mom and my Family

ACKNOWLEDGEMENTS

I wish to express my gratitude to my supervisor, Dr. James C. Jamieson, for his valuable advice, patient guidance, continued encouragement and constant interest throughout the course of this study. I am also indebted to Dr. Harry W. Duckworth for his helpful suggestions with the work throughout this study.

I would like to thank Mike Woloski, Alex Bell, Bert Friesen and Vipin Bhayana for their assistance, encouragement and helpful discussions throughout the course of this work.

I am indebted to Wayne Buchannon, Graham Curnew, Hartley Fox, Elzbieta Gospodarek, Kay Ham, Maureen Hellman, Mike Kutryk and Chiayeng Wang for valuable technical assistance. I also wish to thank Sharon McKinley for typing this thesis.

I would like to express my sincerest appreciation to my wife, Donna, my Mother and my Family for their understanding, patience and support which made the task at hand much easier to complete.

Finally, I am indebted to The University of Manitoba and the Department of Chemistry for financial assistance.

TABLE OF CONTENTS


| | Page |
|---|---|
| ACKNOWLEDGEMENTS | iv |
| TABLE OF CONTENTS | v |
| LIST OF FIGURES | viii |
| LIST OF TABLES | xii |
| ABBREVIATIONS | xiv |
| ABSTRACT | xvi |
| INTRODUCTION | 1 |
| Inflammation | 1 |
| The Acute Inflammatory Process | 1 |
| Acute Phase Reactants | 7 |
| Glycoprotein Biosynthesis | 11 |
| Protein Synthesis | 12 |
| Structure and Synthesis of Asparagine-Linked Oligosaccharides | 16 |
| (1) Assembly | 25 |
| (2) Transfer | 30 |
| (3) Oligosaccharide processing | 31 |
| Nucleotide Sugars | 40 |
| Transport and Secretion of Glycoproteins | 45 |
| Introduction to the Present Work | 47 |
| EXPERIMENTAL | 51 |
| Materials | 51 |
| Radioactive Compounds | 51 |
| Sugars, Nucleotide Sugars and Nucleotides | 51 |
| Amino Acids and Proteins | 52 |
| Chromatographic and Electrophoretic Media | 52 |
| Chemicals | 52 |
| Methods | 53 |
| Physical and Chemical Methods | 53 |
| Determination of radioactivity | 53 |
| Gas liquid chromatography of alditol acetate derivatives of sugars | 54 |
| Polyacrylamide gel electrophoresis | 55 |
| Isoelectric focusing | 55 |
| Preparation of Dowex ion-exchangers | 56 |
| Ultrafiltration and other methods | 57 |
| Immunological Methods | 58 |
| Isolation of β-Galactosidase from Jack Bean Meal | 59 |
| Treatment of Rats for Preparation of Serum and Liver | 60 |
| Isolation of $\alpha_1$-Acid Glycoprotein from Rat Serum | 61 |

Preparation of Asialo- and Asialoagalacto-$\alpha_1$-Acid Glycoprotein . . . . . 62
Preparation of Liver Slices . . . . . 63
Subcellular Fractionation . . . . . 64
Preparation of microsome fraction and cell sap . . . 65
Preparation of rough and smooth membrane fractions . 66
Preparation of Golgi-enriched fraction . . . . . 66
Preparation of lysosome-enriched fraction . . . . . 67
Incorporation of Radioactive Sugars into Sugar-Lipid Complexes and Proteins . . . . . 68
*In vivo* studies . . . . . 68
*In vitro* studies . . . . . 68
Extraction of Sugar-Lipid Complexes from Liver . . . . 69
Labeling and Chromatographic Analysis of Oligosaccharides Derived from Oligosaccharide-Lipid Complexes . . . . . 71
Isolation and Analysis of Hepatic Nucleotide Sugars . . 72
Enzyme Assays . . . . . 75
Enzymes of nucleotide sugar metabolism . . . . . 75
Sialyl and galactosyl transferases . . . . . 78
α-Glucosidase and α-mannosidase . . . . . 82

RESULTS . . . . . 83

The Effect of Inflammation on Sialyl and Galactosyl Transferase Activities in Rat Liver and Serum . . . . . 83
Kinetic Properties of Sialyl Transferase in Liver, Medium and Serum . . . . . 97
Characterization of the Sialyl Transferase Released from Liver in Inflammation . . . . . 104
Nature of the Release of Sialyl Transferase from Liver . . . . . 112
Studies on the Effect of Inflammation of Rat Liver Nucleotide Sugar Pools . . . . . 119
Liver Nucleotide Sugar Pools . . . . . 119
UDP-N-acetylhexosamines . . . . . 119
GDP-Man, UDP-Glc, UDP-Gal and CMP-NeuAc . . . . . 120
Enzymes of Nucleotide Sugar Metabolism . . . . . 130
Studies on the Effect of Inflammation on the Formation of Lipid-Linked Sugar Intermediates Involved in the Synthesis of N-Glycosidically-Linked Glycoproteins in Rat Liver . . . . . 135
*In Vivo* Studies on the Incorporation of Radioactive Sugars into Sugar-Lipid Intermediates and Proteins from Hepatic Rough Membrane Fractions . . . . . 138
*In Vitro* Studies on the Incorporation of [$^{14}$C]Man into Sugar-Lipid Intermediates and Proteins from Hepatic Rough Membrane Fractions . . . . . 143
*In vitro* mannosylation in absence of a cell-free protein synthesizing system . . . . . 143
*In vitro* mannosylation in presence of a cell-free protein synthesizing system . . . . . 147

Table of Contents (continued) Page


Effect of cell sap on mannosylation . . . . . . . . . . 151
Analysis of Oligosaccharide-Lipid Complexes . . . . . . 158
Identification of lipid-derived oligosaccharides . . 166
Effect of Inflammation on the Activities of the Oligosaccharide Processing Glycosidases . . . . . . . . 170

DISCUSSION . . . . . . . . . . . . . . . . . . . . . . . . 179

Studies on the Effect of Inflammation on Rat Liver and Serum Sialyl and Galactosyl Transferase Activities . . . 179
Studies on the Effect of Inflammation on Nucleotide Sugar Metabolism . . . . . . . . . . . . . . . . . . . . . 193
Studies on the Effect of Inflammation on the Formation of Sugar-Lipid Intermediates and the Activities of Oligosaccharide Processing Enzymes in Rat Liver . . . . . 207

APPEXDIX I . . . . . . . . . . . . . . . . . . . . . . . . 218

REFERENCES . . . . . . . . . . . . . . . . . . . . . . . . 219

LIST OF FIGURES


Page

FIGURE 1. Schematic representation of the inflammatory process . . . . . . . . . . . . . . . . . . . . . . . . 2

FIGURE 2. Initiation and elongation reactions in eukaryotic protein synthesis . . . . . . . . . . . . . . . 13

FIGURE 3. The signal hypothesis . . . . . . . . . . . . . . . 14

FIGURE 4. Core structure of asparagine-linked oligosaccharide chains . . . . . . . . . . . . . . . . . . . 18

FIGURE 5. Structures of N-glycosidically linked oligosaccharide chains . . . . . . . . . . . . . . . . . . . . . . 19

FIGURE 6. Structure of mannosylphosphoryldolichol (Man-P-Dol) . . . . . . . . . . . . . . . . . . . . . . . . 22

FIGURE 7. Biosynthetic pathway of dolichol monophosphate in mammalian cells . . . . . . . . . . . . . . . . . . . . 23

FIGURE 8. Structure of the glucose-containing dolichol-linked oligosaccharide . . . . . . . . . . . . . . . . . . 26

FIGURE 9. The dolichol cycle showing the assembly of the dolichol-linked oligosaccharide . . . . . . . . . . . . . 27

FIGURE 10. Scheme for processing of oligosaccharide chains of N-linked glycoproteins . . . . . . . . . . . . 33

FIGURE 11. Pathways of formation of nucleotide sugars . . . 41

FIGURE 12. Procedure for extraction of sugar-lipid complexes and protein from hepatic rough membrane fractions . . . . . . . . . . . . . . . . . . . . . . . . 70

FIGURE 13. Linearity of sialyl transferase activity with time . . . . . . . . . . . . . . . . . . . . . . . . . . 84

FIGURE 14. Sialyl transferase activity as a function of amount of enzyme protein . . . . . . . . . . . . . . . . . 85

FIGURE 15. Effect of amount of acceptor protein on sialyl transferase activities . . . . . . . . . . . . . . . . . . 86

FIGURE 16. Effect of inflammation on liver sialyl and galactosyl transferase activities . . . . . . . . . . . . 87

FIGURE 17. Effect of inflammation on serum sialyl and galactosyl transferase activities . . . . . . . . . . . . 88

List of Figures (continued) Page

FIGURE 18. Effect of inflammation on sialyl and galactosyl transferase activities in liver slice medium . . . . . . 90

FIGURE 19. Effect of liver slice incubation time on release of sialyl and galactosyl transferase activities into medium . . . . . . . . . . . . . . . . . . . . . . . . 91

FIGURE 20. The effect of pH on sialyl transferase activity . 98

FIGURE 21. The effect of UMP, UDP, UTP, and CTP on liver and serum sialyl transferase activities . . . . . . . . . 102

FIGURE 22. Polyacrylamide gel electrophoresis patterns of liver slice medium and liver sialyl transferases in experiments with control and 48 h inflamed rats . . . . . 106

FIGURE 23. Sialyllactose isomers produced by liver, serum and liver slice medium sialyl transferases in experiments with control and 48 h inflamed rats . . . . . . . . 108

FIGURE 24. Sialyllactose isomers produced by human serum sialyl transferases from normal individuals and patients with neoplastic diseases . . . . . . . . . . . . . . . . . 111

FIGURE 25. Chromatogram of acid-hydrolyzed UDP-N-acetylhexosamines, for the determination of UDP-GlcNAc and UDP-GalNAc, on a Technicon Amino Acid Analyzer System model NC-2P . . . . . . . . . . . . . . . . . . . . . 121

FIGURE 26. Effect of inflammation on hepatic levels of UDP-GlcNAc, UDP-GalNAc, as determined by chromatography on the amino acid analyzer system . . . . . . . . . . . 122

FIGURE 27. Chromatogram of alditol acetate sugar derivatives . . . . . . . . . . . . . . . . . . . . . . . . 123

FIGURE 28. Effect of inflammation on hepatic levels of GDP-Man . . . . . . . . . . . . . . . . . . . . . . . . . 125

FIGURE 29. Effect of inflammation on hepatic levels of CMP-NeuAc, and UDP-Gal . . . . . . . . . . . . . . . . . 126

FIGURE 30. Effect of inflammation on hepatic levels of UDP-Glc . . . . . . . . . . . . . . . . . . . . . . . . . 127

FIGURE 31. Effect of inflammation on hepatic levels of UDP-GlcNAc, and UDP-GalNAc, as determined by gas liquid chromatographic analysis . . . . . . . . . . . . . 129

FIGURE 32. Glucosamine-6-phosphate synthase activity as a function of time and amount of enzyme protein . . . . . . 131

FIGURE 33. UDP-GlcNAc 2-epimerase activity as a function of time and amount of enzyme protein . . . . . . 132

FIGURE 34. CMP-NeuAc synthase activity as a function of time and amount of enzyme protein . . . . . . . . . . . . 133

FIGURE 35. Effect of inflammation on glucosamine-6-phosphate synthase (or, L-glutamine:D-fructose-6-phosphate aminotransferase) activity in liver . . . . . . 134

FIGURE 36. Effect of inflammation on UDP-N-acetylglucosamine 2-epimerase activity in liver . . . . . . . . . . . . . . . 136

FIGURE 37. Effect of inflammation on CMP-NeuAc synthase activity in liver . . . . . . . . . . . . . . . . . . . . . 137

FIGURE 38. In vivo incorporation of $[^3H]$GlcN and $[^{14}C]$Man into the Lipid I fraction (Man-P-Dol and $(GlcNAc)_{1-2}$-P-P-Dol) of hepatic rough membrane fractions . . . . . . . . . 139

FIGURE 39. In vivo incorporation of $[^3H]$GlcN and $[^{14}C]$Man into lipid-linked oligosaccharides (Lipid II) from hepatic rough membrane fractions . . . . . . . . . . . . 140

FIGURE 40. In vivo incorporation of $[^3H]$GlcN and $[^{14}C]$Man into proteins from hepatic rough membrane fractions . . . 141

FIGURE 41. In vitro incorporation of $[^{14}C]$Man from GDP-$[^{14}C]$Man into Dol-P from hepatic rough membrane fractions in absence of a cell-free protein synthesizing system . . . . . . . . . . . . . . . . . . . . 144

FIGURE 42. In vitro incorporation of $[^{14}C]$Man from GDP-$[^{14}C]$Man into oligosaccharide-P-P-Dol from hepatic rough membrane fractions in absence of a cell-free protein synthesizing system . . . . . . . . . . . . . . . . 145

FIGURE 43. In vitro incorporation of $[^{14}C]$Man from GDP-$[^{14}C]$Man into proteins from hepatic rough membrane fractions in absence of a cell-free protein synthesizing system . . . . . . . . . . . . . . . . . . . . 146

FIGURE 44. In vitro incorporation of $[^{14}C]$Man from GDP-$[^{14}C]$Man into Man-P-Dol from hepatic rough membrane fractions in presence of concurrent cell-free protein synthesis . . . . . . . . . . . . . . . . . . . . . . . . 148

FIGURE 45. In vitro incorporation of $[^{14}C]$Man from GDP-$[^{14}C]$Man into oligosaccharide-P-P-Dol from hepatic rough membrane fractions in presence of concurrent cell-free protein synthesis . . . . . . . . . . . . . . 149

List of Figures (continued) Page

FIGURE 46. *In vitro* incorporation of [$^{14}$C]Man from GDP-[$^{14}$C]Man into proteins from hepatic rough membrane fractions in presence of concurrent cell-free protein synthesis . . . . . . . . . . . . . . . . . . . . . . . . . 150

FIGURE 47. *In vitro* incorporation of [$^{3}$H]leucine into proteins from hepatic rough membrane fractions in presence of concurrent cell-free protein synthesis . . . 153

FIGURE 48. Chromatography of oligosaccharides derived from oligosaccharide-lipid complexes from hepatic microsome fractions . . . . . . . . . . . . . . . . . . . . . . . . . 159

FIGURE 49. Chromatography of [$^{3}$H]GlcN-labeled oligosaccharides derived from oligosaccharide-lipid complexes from liver microsome fractions . . . . . . . . 161

FIGURE 50. Chromatography of [$^{14}$C]Man-labeled oligosaccharides derived from oligosaccharide-lipid complexes from liver microsome fractions . . . . . . . . 162

FIGURE 51. Chromatography of [$^{14}$C]GlcN-labeled oligosaccharides derived from oligosaccharide-lipid complexes from microsome fractions prepared from liver slices incubated in the presence of [$^{14}$C]GlcN and puromycin . . . . . . . . . . . . . . . . . . . . . . . . . 164

FIGURE 52. Chromatography of [$^{3}$H]Man-labeled oligosaccharides derived from oligosaccharide-lipid complexes from microsome fractions prepared from liver slices incubated in the presence of [$^{3}$H]Man and puromycin . . . . . . . . . . . . . . . . . . . . . . . . . 165

FIGURE 53. The effect of pH on hepatic microsome fraction α-glucosidase activities . . . . . . . . . . . . . . . . . . 172

FIGURE 54. The effect of pH on hepatic microsome fraction α-mannosidase activities . . . . . . . . . . . . . . . . . . 173

FIGURE 55. Effect of incubation time and amount of enzyme protein on microsome fraction and lysosome α-glucosidase activities . . . . . . . . . . . . . . . . . . . . . . . 175

FIGURE 56. Effect of incubation time and amount of enzyme protein on microsome fraction and lysosome α-mannosidase activities . . . . . . . . . . . . . . . . . . . 176

LIST OF TABLES

Page

TABLE 1. Proteins of human plasma showing altered concentrations after trauma . . . . . . . . . . . . . . . . 5

TABLE 2. Physical and chemical properties of some acute phase proteins . . . . . . . . . . . . . . . . . . . . . . 8

TABLE 3. Stability of hepatic sialyl and galactosyl transferase activities in liver slice incubations . . . . 92

TABLE 4. Stability of sialyl and galactosyl transferase activities in medium in liver slice incubations . . . . . 93

TABLE 5. Effect of endogenous, heat stable, low molecular weight factors on liver, medium and serum sialyl transferase activities . . . . . . . . . . . . . . . . . 96

TABLE 6. Apparent $K_m$ values of sialyl transferase . . . . . 100

TABLE 7. Effect of nucleotides on liver, medium and serum sialyl transferase activities . . . . . . . . . . . . . . 103

TABLE 8. Human serum sialyl transferase activities in normal individuals and metastatic cancer patients . . . . 110

TABLE 9. Effect of colchicine on release of sialyl transferase into slice medium . . . . . . . . . . . . . . 114

TABLE 10. Effect of puromycin, cycloheximide and tunicamycin on liver slice and medium sialyl transferase activities . . . . . . . . . . . . . . . . . . . . . . . 116

TABLE 11. Effect of puromycin and tunicamycin on [$^3$H]-glucosamine incorporation into total liver and medium proteins . . . . . . . . . . . . . . . . . . . . . . . 117

TABLE 12. Effect of trypsin and pronase B on sialyl transferase activity in a liver slice system . . . . . . 118

TABLE 13. Effect of experimental liver cell saps on the incorporation of [$^{14}$C]mannose and [$^3$H]leucine into sugar-lipid intermediates and proteins from control liver rough membrane fractions . . . . . . . . . . . . . . . . . . . 154

TABLE 14. Effect of experimental cell saps on the incorporation of [$^{14}$C]mannose and [$^3$H]leucine into sugar-lipid intermediates and proteins from 12 h experimental rough membrane fractions . . . . . . . . . . . . . . . . 155

List of Tables (continued) Page

TABLE 15. Effect of a high molecular weight fraction of liver cell sap on the incorporation of [$^{14}$C]mannose and [$^{3}$H]leucine into sugar-lipid complexes and proteins from control liver rough membrane fractions . . . . . . . 157

TABLE 16. Identification of chromatographed lipid-derived [$^{3}$H]glucosamine-labeled oligosaccharides from control and 12 h experimental liver slices . . . . . . . . . . . 167

TABLE 17. Identification of chromatographed [$^{14}$C]-glucosamine-labeled lipid-derived oligosaccharides from control and 12 h experimental liver slices incubated with puromycin . . . . . . . . . . . . . . . . . . . . . . 168

TABLE 18. Identification of chromatographed [$^{3}$H]mannose-labeled lipid-derived oligosaccharides from control and 12 h experimental liver slices incubated with puromycin . . . . . . . . . . . . . . . . . . . . . . . . 169

TABLE 19. Effect of inflammation on α-glucosidase activities in liver microsome subfractions and lysosomes . . . . . . . . . . . . . . . . . . . . . . . . 177

TABLE 20. Effect of inflammation on α-mannosidase activities in liver microsome subfractions lysosomes . . . . . . . . . . . . . . . . . . . . . . . . 178

ABBREVIATIONS

| | |
|---|---|
| A | Adenosine |
| aa | Amino acid |
| aa-tRNA | Aminoacyl-transfer ribonucleic acid |
| ADP | Adenosine 5'-diphosphate |
| AMP | Adenosine 5'-monophosphate |
| ATP | Adenosine 5'-triphosphate |
| Asn | Asparagine |
| Ci | Curie |
| CDP | Cytidine 5'-diphosphate |
| CHO | Chinese hamster ovary |
| CM- | Carboxymethyl- |
| CMP | Cytidine 5'-monophosphate |
| CTP | Cytidine 5'-triphosphate |
| DEAE- | Diethylaminoethyl- |
| DNA | Deoxyribonucleic acid |
| Dol-P | Dolichol monophosphate |
| Dol-P-P | Dolichol pyrophosphate |
| dpm | Disintegrations per minute |
| EDTA | Ethylenediaminetetracetate |
| eIf | Initiation factor |
| FA | Fatty acid |
| FACoA | Fatty acyl coenzyme A |
| Fru-6-P | Fructose-6-phosphate |
| Fuc | Fucose |
| G | Guanosine |
| Gal | Galactose |

| | |
|---|---|
| GalN | Galactosamine |
| GalNAc | N-Acetylgalactosamine |
| GDP | Guanosine 5'-diphosphate |
| Glc | Glucose |
| GlcN | Glucosamine |
| GlcNAc | N-Acetylglucosamine |
| GMP | Guanosine 5'-monophosphate |
| GTP | Guanosine 5'-triphosphate |
| kd | Kilodalton |
| $K_m$ | Michaelis constant |
| mA | Milliampere |
| met-$tRNA_f$ | N-formylmethionyl-transfer ribonucleic acid |
| mRNA | Messenger ribonucleic acid |
| NeuAc | N-Acetylneuraminic acid |
| P | Phosphate |
| RNA | Ribonucleic acid |
| rpm | Revolutions per minute |
| rRNA | Ribosomal ribonucleic acid |
| Ser | Serine |
| SRP | Signal recognition particle |
| Thr | Threonine |
| U | Uridine |
| UDP | Uridine 5'-diphosphate |
| UMP | Uridine 5'-monophosphate |
| UTP | Uridine 5'-triphosphate |
| UV | Ultraviolet |
| VSV | Vesicular stomatitis virus |

ABSTRACT

The acute phase response to inflammation is accompanied by elevated hepatic biosynthesis of serum glycoproteins, termed acute phase reactants. This thesis examines the effect of inflammation on aspects of glycosylation of acute phase glycoproteins. Rat hepatic and serum sialyl and galactosyl transferase activities increased, reaching a maximum at 48 h after inflammation. Kinetic evidence from liver slice experiments coupled with linkage specificity studies indicated that elevated serum Galβ1→4GlcNAcα2→6 sialyl transferase activity was due to increased and preferential release of the enzyme from liver indicating that the enzyme is an acute phase reactant. The origin of serum galactosyl transferase is not known. Alterations in nucleotide sugar pools occurred as early as 4 h after inflammation. Glucosamine-6-phosphate synthase and UDP-GlcNAc 2-epimerase activities increased two-fold at 24 h and 8 h, respectively, after inflammation; CMP-NeuAc synthase did not show large changes after inflammation. *In vivo* and *in vitro* studies indicated an increase in glycosylation of dolichol phosphate derivatives and proteins from experimental rough membrane fractions; cell sap from experimental livers stimulated cell-free glycosylation of oligosaccharide-lipids and proteins. Only minor differences were observed in gel fitration elution profiles of lipid-derived oligosaccharides from control and experimental livers. The activities of the oligosaccharide processing α-glucosidases and α-mannosidases did not change significantly in inflammation. The results indicate that elevated glycoprotein biosynthesis in acute

inflammation is accompanied by alterations in several biochemical processes associated with glycoprotein glycosylation reactions.

# INTRODUCTION

# INFLAMMATION

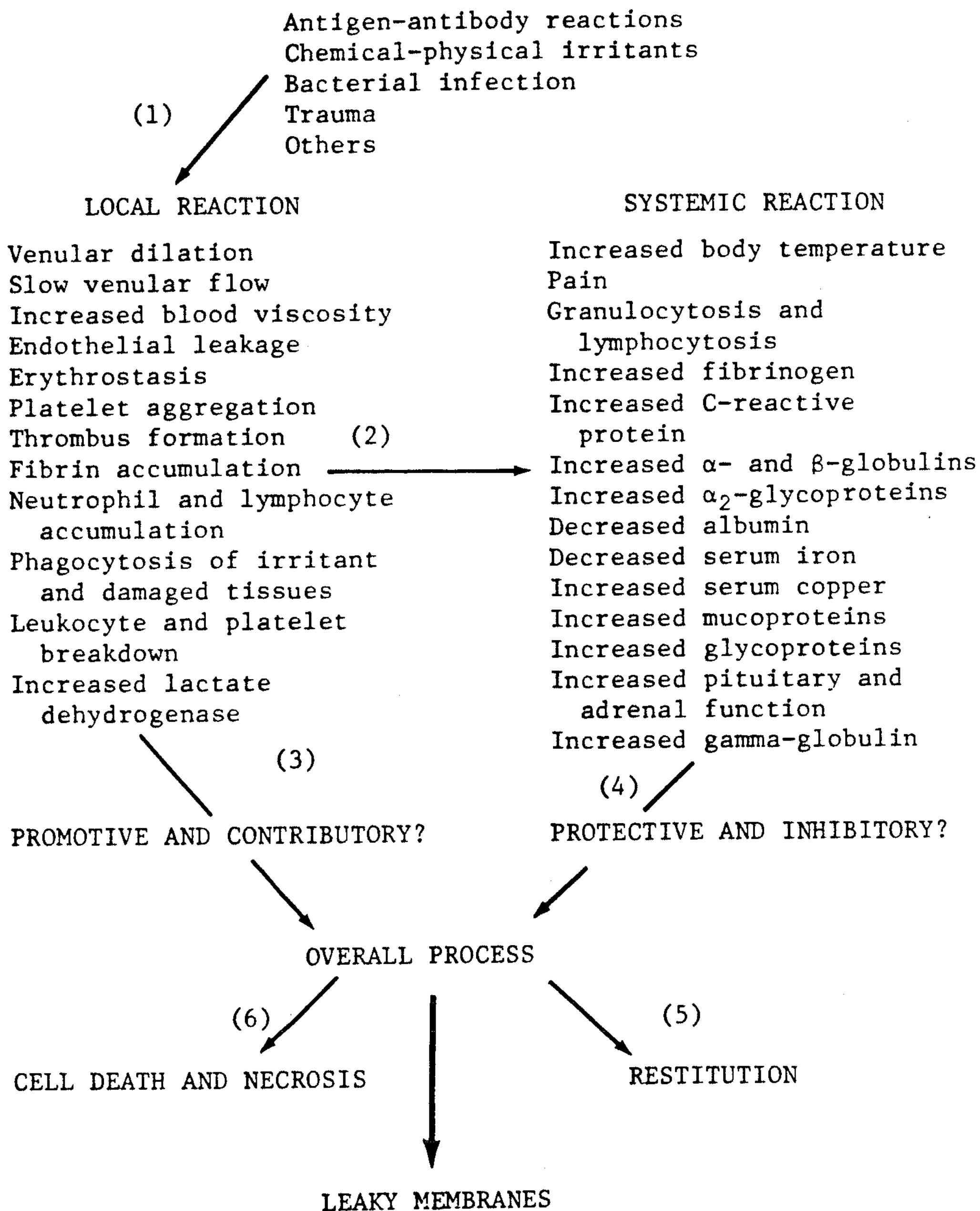
## The Acute Inflammatory Process

In mammals, inflammation may be caused by a variety of injurious stimuli including bacterial infections, neoplastic diseases, rheumatoid arthritis, chemical inflammatory agents, and physical trauma (Winzler, 1965; Gordon, 1970; Koj, 1974). The occurrence of tissue injury or infection, which represent a threat to the integrity of the organism, requires readjustments in the usual metabolic and physiologic equilibria. The local reaction of tissue to such injury or infection is termed acute inflammation; the large number of systemic and metabolic changes which occur are termed the acute phase response (Kushner, 1982). The events of the inflammatory process are appropriately summarized by the scheme proposed by Glenn *et al*. (1968) and shown in Fig. 1. Glenn *et al*. (1968) suggest that the systemic reaction is induced by the local reaction.

The first events in the local response to inflammation are believed to be venular dilation and alterations in blood flow (Kulka, 1964). This is followed by platelet aggregation, the formation of thrombi, and then the migration of neutrophils, monocytes, and lymphocytes into the area of tissue damage (Gorog and Kovacs, 1969; Ross, 1972). This process is thought to be accompanied by the release of lysosomal enzymes at the site of inflammation (Houck *et al*., 1968; Woessner, 1979). It has been suggested that the lysosomal enzymes which are released activate cutaneous collagenase which degrades collagen in the structural components of damaged tissue into soluble breakdown products which then drain away from site of inflammation (Weissman and Uhr, 1968).

As shown in Fig. 1, the systemic or acute phase response is characterized by a variety of metabolic, humoral and physiologic

Fig. 1. Schematic representation of the inflammatory process. The numbers in parentheses describe the order in which the events are believed to occur. Taken from Glenn *et al*. (1968).


The "Inflammatory Process"
DAMAGING AGENT(S)
Antigen-antibody reactions
Chemical-physical irritants
Bacterial infection
Trauma
Others
(1)
LOCAL REACTION
Venular dilation
Slow venular flow
Increased blood viscosity
Endothelial leakage
Erythrostasis
Platelet aggregation
Thrombus formation
Fibrin accumulation
Neutrophil and lymphocyte accumulation
Phagocytosis of irritant and damaged tissues
Leukocyte and platelet breakdown
Increased lactate dehydrogenase
(2)
SYSTEMIC REACTION
Increased body temperature
Pain
Granulocytosis and lymphocytosis
Increased fibrinogen
Increased C-reactive protein
Increased α- and β-globulins
Increased $\alpha_2$-glycoproteins
Decreased albumin
Decreased serum iron
Increased serum copper
Increased mucoproteins
Increased glycoproteins
Increased pituitary and adrenal function
Increased gamma-globulin
(3)
(4)
PROMOTIVE AND CONTRIBUTORY?
PROTECTIVE AND INHIBITORY?
OVERALL PROCESS
(6)
(5)
CELL DEATH AND NECROSIS
RESTITUTION
LEAKY MEMBRANES

alterations. One of the earliest alterations recognized was fever (Atkins and Bodel, 1979). Elevated body temperature results from altered hypothalamic control of thermoregulation induced by endogenous pyrogen; endogenous pyrogen may be released from phagocytes at the site of inflammation, or chemotactic factors produced by invading bacteria or released from injured tissue (Dinarello and Wolff, 1978; Bernheim et al., 1979). Fever, which forms a part of the acute phase response to many types of stimuli, functions to provide sub-optimum growth conditions for invasive microorganisms (Beisel, 1980; Kushner, 1982). Elevated temperatures also induce a hypermetabolic state resulting in increased utilization of nutrients to meet the elevated energy requirements of body cells. The stimulation of cellular metabolic rates results in changes in available energy sources including: increased gluconeogenesis and glycogenolysis; elevated rates of amino acid degradation with accelerated ureogenesis and ammoniogenesis, increased catabolism of somatic proteins, and reduced ketogenesis and hypertriglyceridemia (Beisel, 1975, 1980; Shutler et al., 1977; Langstaff et al., 1980; Kushner, 1982). These changes result in significant alterations in the utilization of available protein, carbohydrate and lipid for synthesis of macromolecules.

During the acute phase response elevated serum levels of a number of hormones, including insulin, glucagon, adrenocorticotropic hormone (ACTH), cortisol, catecholamines, growth hormone, thyroxine, thyroid stimulating hormone, vasopressin and aldosterone, have been described (Beisel, 1977; Egdahl et al., 1977; Langstaff et al., 1980). The extent to which these hormones may participate in bringing about other elements of the acute phase response has yet to be delineated.

The serum concentrations of the cations, zinc, iron, and copper, are also altered during the acute phase response. Copper levels are increased, apparently as a result of elevated rates of secretion of the copper-binding protein, ceruloplasmin, from the liver (Poulik and Weiss, 1975). Plasma zinc and iron levels decrease. This is due to increased sequestration of these cations by the liver which contains elevated levels of the zinc-binding protein, metallothionein (Sobocinski and Canterbury, 1982) and the iron binding-protein, ferritin (Pekarek et al., 1972; Weinberg, 1978). There is evidence that glucocorticoids, glucagon and ACTH, acting through different mechanisms, may also contribute to the zinc-binding capability of the liver (Falchuk, 1977; Etzel and Cousins, 1981).

Perhaps one of the more important changes occurring during the acute phase response is in the levels of some plasma proteins. Table 1 lists changes induced by trauma for many important serum proteins. Those plasma proteins which increase following stress are referred to as acute phase reactants (Koj, 1974); proteins that decrease in concentration following stress, such as albumin, have been described as "negative acute phase reactants" (Kushner, 1982). At least two common features have been ascribed to the acute phase reactants; almost all are glycoproteins and all are synthesized by the liver (Darcy, 1965; Balegno and Neuhaus, 1970; Jamieson and Ashton, 1973a; Koj, 1974; Kushner and Feldmann, 1978; Benson and Kleiner, 1980; Selinger et al., 1980). Hormonal-like factors, originating at the site of tissue injury, are believed to be transported by blood (John and Miller, 1969) to the liver where they stimulate increased synthesis of acute phase reactants (Koj, 1974; Beisel, 1975). Work by Woloski et al. (1983a) in this

Table 1. Proteins of human plasma showing altered concentrations after trauma.[a]

| Protein | | % of preoperative values |
|---|---|---|
| Increased: | fibrinogen | >200 |
| | haptoglobin | 206 |
| | orosomucoid[b] | >200 |
| | C-reactive protein | >200 |
| | $\alpha_1$-antitrypsin | >200 |
| | slow $\alpha$-globulin | 173 |
| | complement | 122 |
| | ceruloplasmin | 124 |
| Decreased: | albumin | 80 |
| | $\beta$-lipoprotein | 77 |
| | transferrin | 78 |
| | thyroxine-binding globulin | 69 |

[a]The information in the table was taken from Gordon (1970). The data were obtained from patients who had undergone minor surgery 8 h or more before the second blood sample was collected.

[b]Orosomucoid is $\alpha_1$-acid glycoprotein which is the major component of the seromucoid fraction of serum or plasma. The seromucoid fraction consists of those proteins soluble in 0.6 M perchloric acid, but precipitated by 5% phosphotungstic acid.

laboratory and by others (Edington et al., 1972; Kampschmidt et al., 1973; Beisel, 1975, 1980; Bornstein, 1982; Fuller and Ritchie, 1982; Kampschmidt et al., 1982; McAdam et al., 1982) has suggested that leukocytes may be the source of factors, called cytokines, which exert a direct effect on the liver to stimulate acute phase protein synthesis. Cytokines are generally small molecular weight proteins (Oppenheim et al., 1982; Simon and Willoughby, 1982) which are known to be released from a variety of white blood cells that are known to accumulate at the site of tissue injury or infection. In the case of the ongoing studies in our laboratory, Woloski (1983) has shown that monocyte-derived factor(s) are able to stimulate the reactions characteristic of the acute phase response in liver including elevated glycoprotein biosynthesis. The mechanism by which cytokines stimulate hepatic synthesis of acute phase reactants is not known. Although cytokines can stimulate the changes characteristic of the acute phase response, the response of the acute phase proteins appears to be lower than that found following trauma (Woloski, 1983; Woloski et al., 1983a). It has been suggested that hormones, such as cortisol, may be involved in conjunction with cytokines to stimulate the liver to synthesize elevated levels of acute phase reactants. Cortisol has been suspected of being involved in stimulating the acute phase response of glycoproteins to inflammation in the past (Barnabei and Sereni, 1964; Stackhouse et al., 1968), but attempts to duplicate the acute phase response with cortisol alone have usually resulted in a general increase in the synthesis of liver synthesized proteins, rather than specific increases in the acute phase reactants. For example, John and Miller (1969), using cortisol in an in vitro liver perfusion system,

successfully elevated synthesis of haptoglobin, fibrinogen and an $\alpha_1$-globulin, but synthesis of albumin, which is a "negative acute phase reactant," was also increased. Also, the inclusion of growth hormone and insulin with cortisol enhanced the stimulation of protein synthesis observed for cortisol alone, however, no stimulation was observed by these hormones without cortisol. Thus, although hormones may be involved in the stimulation of elevated hepatic protein synthesis, current ideas suggest that cytokines are required for full expression of elevated synthesis of acute phase reactants. Clearly, the acute inflammatory process represents a coordinated system to limit, modulate and/or otherwise direct host responses during periods of intense inflammation and tissue destruction.

## Acute Phase Reactants

As previously mentioned, although the acute phase reactants are a diverse group of proteins, almost all of them are glycoproteins (Table 2). The carbohydrate content of these acute phase glycoproteins is quite variable. For example, fibrinogen contains only 4% carbohydrate, whereas $\alpha_1$-acid glycoprotein contains about 40% carbohydrate (Table 2). The oligosaccharide chains of the acute phase reactants are usually of the N-linked complex type (Jamieson, 1983) however, alterations in the carbohydrate moieties of some acute phase reactants following an inflammatory challenge have been observed (Koj *et al.*, 1982). It is interesting to note that while rat C-reactive protein is a glycoprotein, the human and rabbit counterparts are not glycoproteins (Table 2).

The biological activity of some of the acute phase reactants is well defined. Haptoglobin binds free hemoglobin thus removing hemoglobin

Table 2. Physical and chemical properties of some acute phase proteins.

| Protein | Molecular Weight (kd) Native | Molecular Weight (kd) Subunit | pI | Percent Carbohydrate | Reference |
|---|---|---|---|---|---|
| Rat $\alpha_1$-acid glycoprotein | 43 | 43 | 2.95 | 34 | Jamieson *et al*. (1972a) |
| Human $\alpha_1$-acid glycoprotein | 40 | 40 | 2.7 | 42 | Schmid (1975) |
| Rat $\alpha_1$-major acute phase protein | 86 | 86 | 4.6 | 20 | Urban *et al*. (1979) |
| Rat $\alpha_2$-macroglobulin | 700-800 | 190 | 4.1-4.6 | 10-16 | Jamieson *et al*. (1972a) Hudig and Sell (1979) |
| Rat C-reactive protein | 100 | 20 | 3.8 | 11 | Baltz *et al*. (1982) |
| Human C-reactive protein | 120 | 20 | 7.9 | 0 | Baltz *et al*. (1982) |
| Rabbit C-reactive protein | 120 | 20 | - | 0 | Baltz *et al*. (1982) |
| Rat fibrinogen | 340 | 61,58,51 | - | 4 | Nickerson and Fuller (1981a) |
| Human fibrinogen | 340 | - | 5.5 | 4 | Doolittle (1975) |
| Rat Haptoglobin | 90 | 35,9.5 | 4.2 | 20 | Haugen *et al*., 1981 |

which could support bacterial growth (Eaton *et al.*, 1982). Fibrinogen is involved in localizing infections through clot formation (Beisel, 1980). Ceruloplasmin transports copper and is thought to protect cells from damage by superoxide anion radicals generated at the site of tissue damage (Goldstein *et al.*, 1982). $\alpha_2$-Macroglobulin, $\alpha_1$-antitrypsin and $\alpha_1$-antichymotrypsin are protease inhibitors (Laurell and Jeppsson, 1975). Some acute phase reactants have been shown to be able to inhibit certain lymphocyte responses *in vitro*; these include C-reactive protein (Mortensen *et al.*, 1975; Mortensen and Gewurz, 1976), $\alpha_1$-acid glycoprotein (Chiu *et al.*, 1977; Bennet and Schmid, 1980), and $\alpha$-fetoprotein (Murgita and Tomasi, 1975; Yachnin, 1975). However, the significance of these *in vitro* inhibitory activities on lymphocyte function *in vivo* is still uncertain. C-reactive protein has a particularly wide spectrum of activity *in vitro* as it can react with platelets (Fiedel *et al.*, 1982) and also activate complement by the classical pathway (Kaplan and Volankis, 1974; Volankis, 1982). The serum complement system represents a major host defence mechanism against invading pathogens. It is a highly organized and tightly regulated system which is activated in a sequential "cascade-like" fashion; fourteen distinct proteins participate in sequence activation and at least six other proteins are responsible for regulation and control of the system (Stroud *et al.*, 1979; Reid and Porter, 1981). In the classical pathway, the initiation of complement activation in blood is caused mainly by the formation of antibody-antigen aggregates or by antibody bound to cellular or particulate antigens (Reid and Porter, 1981). In the case of C-reactive protein, the initiation of activation of the complement system involves the binding of C-reactive protein to pneumonococcal

C-polysaccharide (Kaplan and Volankis, 1974). Thus, from the above information, it is suggested that acute phase reactants play important roles in the nonspecific immune responses of the host.

As mentioned previously, the liver is the main, or only, site of synthesis of acute phase reactants. In every study performed thus far, increased plasma concentrations of these acute phase proteins have been found to be accompanied by increased hepatic synthesis (Koj, 1974; Kushner, 1982; Jamieson et al., 1983). During the acute phase response, increased synthesis of acute phase reactants has been found to occur concomitantly with alterations in ultrastructural elements and chemical constituents of the liver. One of the major changes in the liver is a proliferation of membranes of the Golgi complex (Earp, 1975; Lombart et al., 1980). This alteration is of particular importance, for as we shall see, the Golgi complex plays a major role in glycoprotein biosynthesis. Dilation of the rough endoplasmic reticulum, increased amounts of smooth endoplasmic reticulum, and increased synthesis of hepatocyte plasma membranes have been reported (Turchen et al., 1977; Little, 1981). There is also increased formation and volume of microtubules (Maurice et al., 1980; Feldmann, 1982), and increased synthesis of cytoplasmic actin (Morrow et al., 1981). There is increased synthesis of RNA, particularly ribosomal RNA (Thompson and Wannemacher, 1973), alterations in free amino acid levels (Woloski et al., 1983b), and increased synthesis of cholesterol and other lipids by the endoplasmic reticulum (Canonico et al., 1977). These alterations are consistent with increased synthesis, transport and secretion of acute phase reactants.

Clearly, the liver is an important organ during the acute phase

response. Not only is it the site of synthesis of the acute phase reactants, but it also responds in a variety of diverse ways which can be equated with an elevation in glycoprotein biosynthetic machinery. It is for this reason that this thesis is mainly concerned with the involvement of liver in the acute phase response to inflammation with particular emphasis on the synthesis of glycoproteins.

## GLYCOPROTEIN BIOSYNTHESIS

Glycoproteins are a class of proteins containing covalently linked carbohydrate. Glycoproteins are widely distributed in nature, occurring not only in vertebrate and invertebrate animals, but also in plants, fungi, bacteria and viruses (Spiro, 1973). In animals, glycoproteins occur as an integral part of cell membranes and structural tissues, and are also free in various body fluids and secretions. Virtually all plasma proteins are glycoproteins, with the notable exception of albumin, the plasma protein which is present in the highest concentration in blood (Peters *et al.*, 1971; Spiro, 1973). With the noteworthy exception of the immunoglobulins, most plasma glycoproteins are synthesized by the liver (Spiro, 1973).

Therefore, it is important for a consideration of the contents of this thesis that the current ideas on hepatic glycoprotein biosynthesis should be outlined. This has been divided into a consideration of the details of the synthesis of the polypeptide and carbohydrate moieties of serum glycoproteins.

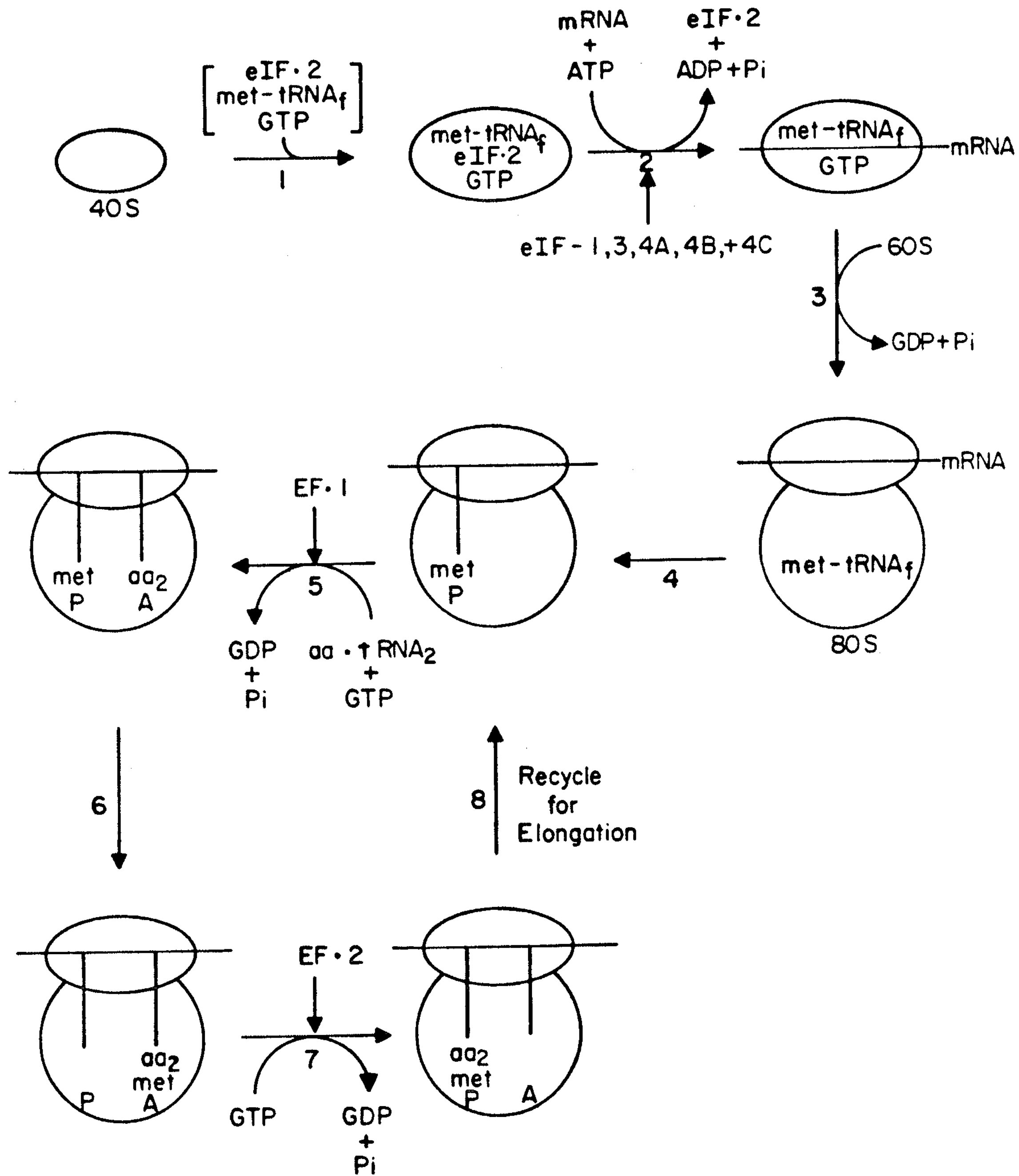
## Protein Synthesis

Since the elucidation of the genetic code, considerable insight has been obtained into the process of protein synthesis (Greenberg, 1975; Weissbach and Ochoa, 1976; Revel, 1977; Shafritz, 1977; Revel and Groner, 1978). In eukaryotic cells, the genetic information is encoded in the DNA, located mainly in the nucleus, while protein synthesis is mainly a cytoplasmic event. Genetic information is transferred from DNA by transcription to precursor RNA molecules which are subsequently modified to produce messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). It is believed that the modification of the precursor RNA forms is a nuclear event and that the modified forms pass into the cytoplasm where they are utilized for protein synthesis. Prevailing concepts on initiation and elongation of polypeptide chains in eukaryotic cells are outlined in Fig. 2. There is considerable evidence that the polypeptide moieties of plasma proteins, such as the acute phase reactants, are translated on membrane-bound polyribosomes and translocated through the rough endoplasmic reticulum membrane (Schachter, 1974a,b; Morrison and Lodish, 1975; Jamieson, 1977; Schachter, 1978; Schachter, 1981; Walter and Blobel, 1981a,b; Walter *et al.*, 1981; Sabatini *et al.*, 1982). A mechanistic model to describe this translocation event has been presented by Blobel and Dobberstein (1975a,b) and is referred to as the "signal hypothesis." According to this hypothesis, which is outlined in Fig. 3, polypeptide synthesis is initiated on free polyribosomes. The mRNA for the protein contains a unique sequence which codes for an N-terminal signal peptide consisting of 15-30 amino acids. The appearance of the signal peptide triggers the attachment of the ribosome to the membrane. This attachment

Fig. 2. Initiation and elongation reactions in eukaryotic protein synthesis (Weissbach and Ochoa, 1976; Revel and Groner, 1978).

Initiation involves the formation of a complex involving mRNA, a 40S ribosome subunit, formylmethionine-tRNA (met-$tRNA_f$), and initiation factors (eIF) with energy for the reactions supplied by GTP and ATP (reactions 1 and 2). A 60S ribosome subunit interacts with the 40S initiation complex to form an 80S complex (reaction 3).

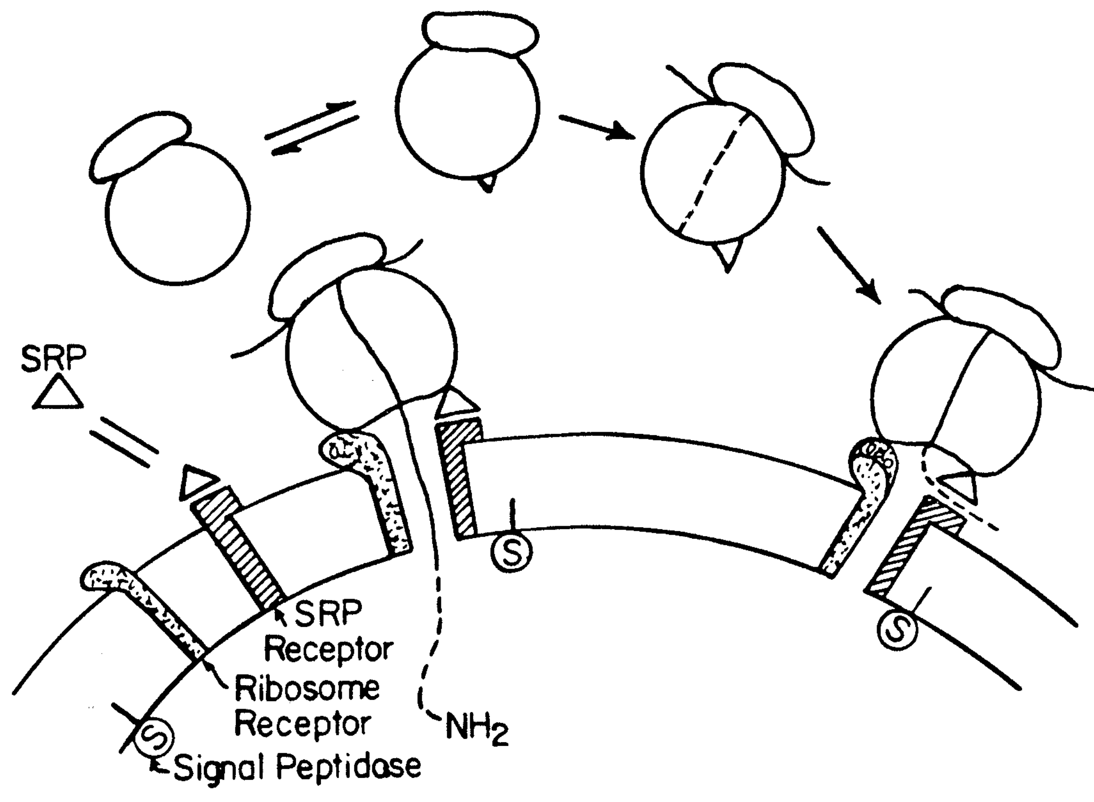
Elongation begins with the met-$tRNA_f$ occupying the peptidyl site (P) in the 60S subunit in such a manner that the tRNA anticodon pairs with the AUG initiation codon on mRNA (reaction 4). Elongation proceeds with the incoming aminoacyl–tRNA occupying the aminoacyl site (A) on the 60S subunit, followed by the transfer reaction (reactions 5 and 6) in which the methionine forms a peptide bond with incoming aminoacyl-tRNA. The next phase of elongation is the translocation of the peptide into the peptide binding site (reaction 7). Elongation continues by repeated cycling of reactions 5-8. Polypeptide chain termination then occurs when the mRNA termination codons (UGA, UAA or UAG arrive in position to be read by the incoming aminoacyl–tRNA.

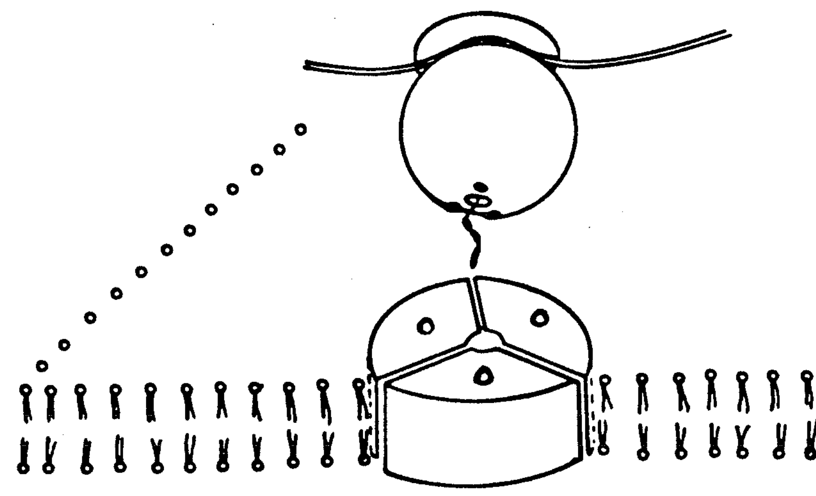


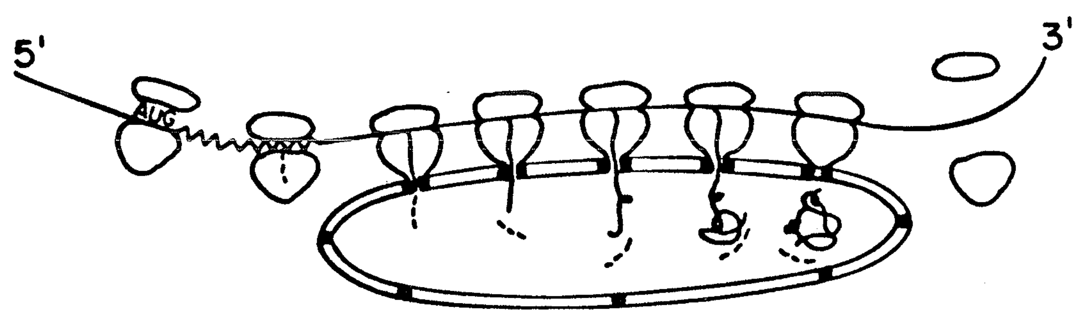
40S
eIF·2
met-tRNA$_f$
GTP
1
met-tRNA$_f$
eIF·2
GTP
mRNA
+
ATP
eIF·2
+
ADP+Pi
2
eIF-1,3,4A,4B,+4C
met-tRNA$_f$
GTP
mRNA
3
60S
GDP+Pi
mRNA
met-tRNA$_f$
80S
4
met
P
EF·1
5
GDP
+
Pi
aa·tRNA$_2$
+
GTP
met
P
aa$_2$
A
6
8
Recycle
for
Elongation
aa$_2$
met
P
A
EF·2
7
GTP
GDP
+
Pi
aa$_2$
met
P
A

Fig. 3. The signal hypothesis as proposed by Blobel and Dobberstein (1975a,b).

According to the scheme, polypeptide synthesis is initiated on free ribosomes. The mRNA for a protein that is synthesized on membrane-bound polyribosomes contains a nucleotide sequence which codes for a unique peptide sequence referred to as the signal peptide (illustrated by ----). Attachment to the membrane is mediated by a signal recognition particle (SRP) which binds to the ribosome and to an SRP receptor on the membrane. (The SRP consists of six different polypeptides plus a 7S RNA particle (Walter and Blobel, 1982)); the SRP also has an affinity for the signal peptide and if it binds this peptide before binding to the SRP receptor, translocation of the peptide across the membrane is blocked (these steps are illustrated in diagram A). Successful association of ribosome and SRP with membrane results in aggregation of membrane proteins causing a tunnel to be formed through which the nascent polypeptide can pass (diagram B). The signal peptide is rapidly removed by signal peptidases as the nascent polypeptide is elongated on the ribosome (diagram C).


A
SRP
SRP
Receptor
Ribosome
Receptor
S
Signal Peptidase
$NH_2$


B


5'
3'
AUG
C

is mediated by the "signal recognition particle" (SRP) which has a receptor on the endoplasmic reticulum membrane (Walter and Blobel, 1982) (see Fig. 3). Once the ribosome-SRP-SRP receptor complex is formed, a channel is created in the membrane which allows passage of the nascent polypeptide either into the lumen, or into the membrane of the endoplasmic reticulum. The signal peptide, usually referred to as the prepeptide, is believed to be removed shortly after transfer by a signal peptidase located on the luminal face of the endoplasmic reticulum membrane. All secretory proteins examined to date have been found to contain N-terminal signal peptides (Sabatini et al., 1982). Although the amino acid compositions of prepeptides are variable, all that have been found are rich in hydrophobic amino acids, a feature which presumably aids in the translocation of the polypeptide across the highly hydrophobic membrane (Lingappa et al., 1977; Strauss et al., 1977; Palmiter et al., 1978; Cohn and MacGregor, 1981).

Some proteins synthesized on bound polyribosomes, such as albumin (Redman and Cherian, 1972; Palade, 1975; Peters, 1977; Kreil, 1981), also have an additional sequence of amino acids situated between the signal peptide and the N-terminus of the native protein; this sequence is rich in basic amino acids and is referred to as the propeptide (Quinn, 1975; Peters, 1977; Cohn and MacGregor, 1981). The function of the polypeptide has not yet been fully determined. It has been suggested that it may facilitate the formation of correct tertiary structure (Steiner et al., 1974), permit anchoring of the protein to the negatively charged membrane through the formation of salt bridges (Schreiber et al., 1976; Schreiber and Urban, 1978), regulate the secretion of proteins from cells (Judah and Quinn, 1976; Schreiber et al..

1976), or to inactivate normally highly active enzymes such as trypsin and chymotrypsin (Kassell and Kay, 1973) during the secretory process.

Compartmentalization of proteins in different intracellular structures during secretion has received some recent attention (Shore and Tata, 1977). Location within the lumen of the endoplasmic reticulum presumably serves to properly channel proteins through the secretory apparatus. Certain proteins are also required inside membrane vesicles like the endoplasmic reticulum, Golgi complex and mitochondria. The signal hypothesis of Blobel and Dobberstein (1975a,b) describes a mechanism for polypeptide insertion into these vesicles. Localization of proteins in proximity to membrane-bound enzymes can serve to properly orient nascent polypeptides for modification by proteolytic cleavage, disulfide bridge formation, hydroxylation, phosphorylation, iodination, lipidation and glycosylation. Since glycosylation is the major modification of proteins destined to become glycoproteins, a description of the structure and biosynthesis of the carbohydrate moieties of glycoproteins is important for an understanding of the work described in this thesis.

Structure and Synthesis of Asparagine-Linked Oligosaccharides.

Glycosylation is one of the major modifications of proteins in liver after polypeptide synthesis. Since most acute phase reactants are glycoproteins, a discussion of the structure and synthesis of the oligosaccharide moieties of glycoproteins is of particular relevance to the studies presented in this thesis.

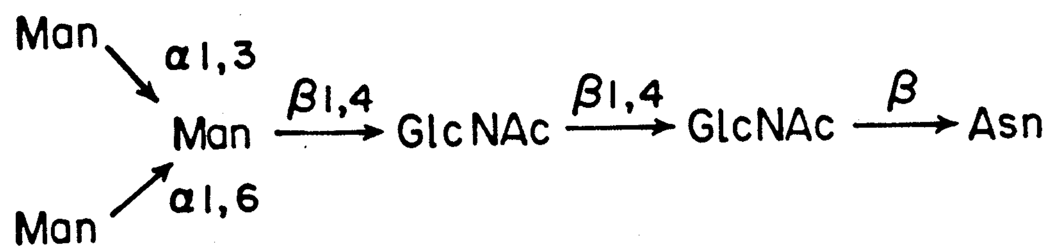
With a few exceptions, such as fetuin (Nilsson *et al*., 1979), the carbohydrate moieties of serum glycoproteins are linked to polypeptide *via* a covalent glycosylamine bond involving the amide group of asparagine

and the C-1 hydroxyl of an N-acetylglucosamine residue in the carbohydrate chain. The other type of linkage, which is much less common in serum glycoproteins, is the O-glycosidic linkage normally found between a N-acetylgalactosamine residue and serine or threonine in mucus glycoproteins (Zinn et al., 1977), and a galactose residue and hydroxylysine in collagen (Marshall, 1974; Zinn et al., 1977). The glycosylamine type of linkage was first characterized by Marshall and Neuberger (1964) and has since been found widely distributed in nature (Kornfeld and Kornfeld, 1980).

Glycoproteins that contain the glycosylamine type of linkage involving asparagine are usually referred to as asparagine-linked or N-linked glycoproteins and they have been found to fall into two categories referred to as the simple or high-mannose type and the complex type (Kornfeld and Kornfeld, 1976, 1980; Montreuil, 1980; Jamieson, 1983). Both categories of oligosaccharide chains contain the common core structure shown in Fig. 4. However, the high-mannose type contains only two sugars, mannose (Man) and N-acetylglucosamine (GlcNAc) (Fig. 5), whereas the complex type contains in addition, galactose (Gal) and N-acetylneuraminic acid (NeuAc) (Fig. 5). Complex type chains can also contain the deoxy sugar fucose (Fuc).

Prior to 1970, the most widely accepted theory for glycoprotein biosynthesis assumed that the assembly of the oligosaccharide chains occurred by the stepwise addition of sugars from nucleotide sugars to the growing oligosaccharide chain. The sugar additions were believed to be specified by the glycosyl transferases as the polypeptide chain was synthesized and channelled from the lumen of the rough endoplasmic reticulum to the smooth endoplasmic reticulum and Golgi apparatus before

Fig. 4. Core structure of asparagine-linked oligosaccharide chains. Taken from Jamieson (1983).




Core Structure

Fig. 5. Structures of N-glycosidically linked oligosaccharide chains. The high-mannose structure shown is found in bovine thyroglobulin (Ito *et al*. (1977), but a variety of this type of structure with varying amounts of mannose and varying degrees of branching has been characterized. The complex type of oligosaccharide chains are of the bi-, tri- and tetra-antennary type. The bi- and tri-annetennary structures have been reported to be present in rat serum $\alpha_1$-acid glycoprotein; the tetra-antennary has been reported in human serum $\alpha_1$-acid glycoprotein (Yoshima *et al*., 1981). Complex type chains can also contain the deoxy sugar fucose which is usually linked either $\alpha 1 \rightarrow 3$ to GlcNAc residues in the terminal triplet regions or $\alpha 1 \rightarrow 6$ to the GlcNAc involved in the glycosylamine bond with asparagine (Yoshima *et al*., 1981). Taken from Kornfeld and Kornfeld (1980). Abbreviations: Asn, asparagine; GlcNAc, N-acetylglucosamine; Man, mannose; Gal, galactose; NeuAc, N-acetylneuraminic acid.

Man $\xrightarrow{\alpha 1,2}$ Man $\xrightarrow{\alpha 1,2}$ Man $\searrow^{\alpha 1,3}$
Man $\xrightarrow{\beta 1,4}$ GlcNAc $\xrightarrow{\beta 1,4}$ GlcNAc $\xrightarrow{\beta}$ Asn
Man $\xrightarrow{\alpha 1,2}$ Man $\xrightarrow{\alpha 1,3}$ Man $\nearrow_{\alpha 1,6}$
Man $\xrightarrow{\alpha 1,2}$ Man $\nearrow_{\alpha 1,6}$

High mannose

NeuAc $\xrightarrow{\alpha 2,3}$ Gal $\xrightarrow{\beta 1,4}$ GlcNAc $\searrow^{\beta 1,4}$
Man $\searrow^{\alpha 1,3}$
NeuAc $\xrightarrow{\alpha 2,6}$ Gal $\xrightarrow{\beta 1,4}$ GlcNAc $\nearrow_{\beta 1,2}$
Man $\xrightarrow{\beta 1,4}$ GlcNAc $\xrightarrow{\beta 1,4}$ GlcNAc $\xrightarrow{\beta}$ Asn
NeuAc $\xrightarrow{\alpha 2,6}$ Gal $\xrightarrow{\beta 1,4}$ GlcNAc $\searrow^{\beta 1,2}$
Man $\nearrow_{\alpha 1,6}$
NeuAc $\xrightarrow{\alpha 2,3}$ Gal $\xrightarrow{\beta 1,4}$ GlcNAc $\nearrow_{\beta 1,6}$

tetraantennary

NeuAc $\xrightarrow{\alpha 2,3}$ Gal $\xrightarrow{\beta 1,4}$ GlcNAc $\searrow^{\beta 1,4}$
NeuAc $\xrightarrow{\alpha 2,6}$ Gal $\xrightarrow{\beta 1,4}$ GlcNAc $\xrightarrow{\beta 1,2}$ Man $\searrow^{\alpha 1,3}$
Man $\xrightarrow{\beta 1,4}$ GlcNAc $\xrightarrow{\beta 1,4}$ GlcNAc $\xrightarrow{\beta}$ Asn
NeuAc $\xrightarrow{\alpha 2,6}$ Gal $\xrightarrow{\beta 1,4}$ GlcNAc $\xrightarrow{\beta 1,2}$ Man $\nearrow_{\alpha 1,6}$

triantennary

NeuAc $\xrightarrow{\alpha 2,6}$ Gal $\xrightarrow{\beta 1,4}$ GlcNAc $\xrightarrow{\beta 1,2}$ Man $\searrow^{\alpha 1,3}$
Man $\xrightarrow{\beta 1,4}$ GlcNAc $\xrightarrow{\beta 1,4}$ GlcNAc $\xrightarrow{\beta}$ Asn
NeuAc $\xrightarrow{\alpha 2,6}$ Gal $\xrightarrow{\beta 1,4}$ GlcNAc $\xrightarrow{\beta 1,2}$ Man $\nearrow_{\alpha 1,6}$

biantennary

Complex

being secreted. This concept is still accepted as the mechanism for the addition of the terminal triplet sugars of complex oligosaccharide chains, but not for the synthesis of the core region of these chains.

Recent evidence suggests that the high-mannose and complex chains have a common biosynthetic origin which involves the initial formation of an oligosaccharide-lipid complex (Parodi and Leloir, 1979). The oligosaccharide portion, which is formed in what is now referred to as the dolichol cycle, contains three glucose (Glc), nine Man and two GlcNAc residues. The oligosaccharide moiety of this complex is transferred to protein and then undergoes subsequent modification by a process collectively referred to as oligosaccharide processing. Details of the dolichol cycle and the oligosaccharide processing reactions have been the subject of extensive study in recent years and are now fairly well understood.

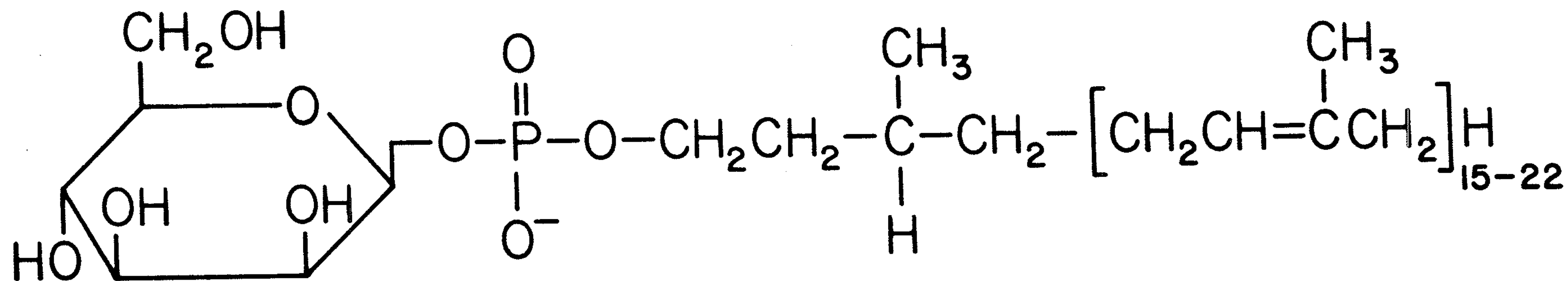
The involvement of lipids in the preassembly of various saccharides in the synthesis of complex glycans in bacteria had been known to exist for some time (Struck and Lennarz, 1980). In 1970, Behrens and Leloir (1970) provided the first firm evidence for the existence of a similar mechanism in eukaryotes. Work by Leloir's group (Parodi and Leloir, 1979) and Lennarz's group (Waechter and Lennarz, 1976; Struck and Lennarz, 1980) showed that the hydrophobic moiety of the intermediate complex is from a family of polyisoprenol alcohols, known as the dolichols. In animal tissues these compounds are usually composed of 16-23 isoprene units with 2 internal _trans_-olefinic bonds -- the remainder of the internal olefinic bonds are _cis_-oriented -- and with no unsaturation in the α-isoprene unit (Hemming, 1974).

There are two possible sources of dolichol in liver, the diet and

*de novo* synthesis (Keenan *et al*., 1977; Keller *et al*., 1982); it has been determined that *de novo* synthesis accounts for 98% of new dolichol in the liver (Keller *et al*., 1982; Adair and Keller, 1982). The major forms of dolichol found in mammalian tissues are the free polyisoprenol alcohols and fatty acyl ester derivatives (Butterworth and Hemming, 1968; Rupar and Carroll, 1978). The acylated derivatives, commonly referred to as dolichol esters, have been shown to be synthesized by a transesterification reaction in which phosphatidylcholine can act as the acyl donor (Keenan and Kruczek, 1976). The enzyme responsible for the transesterification reaction, dolichol fatty acyl ester synthetase (Keenan and Kruczek, 1976), as well as the enzyme catalyzing the reverse reaction, dolichol fatty acyl ester esterase (Scher and Waechter, 1981) have been demonstrated *in vitro* in mammalian cells. It is the phosphorylated derivatives, dolichol monophosphate and dolichol pyrophosphate, which serve as carriers of saccharide residues in the assembly of N-glycosidically linked oligosaccharides of glycoproteins (see Fig. 6) (Hemming, 1974; Waechter and Lennarz, 1976; Parodi and Leloir, 1979; Struck and Lennarz, 1980; Hubbard and Ivatt, 1981).

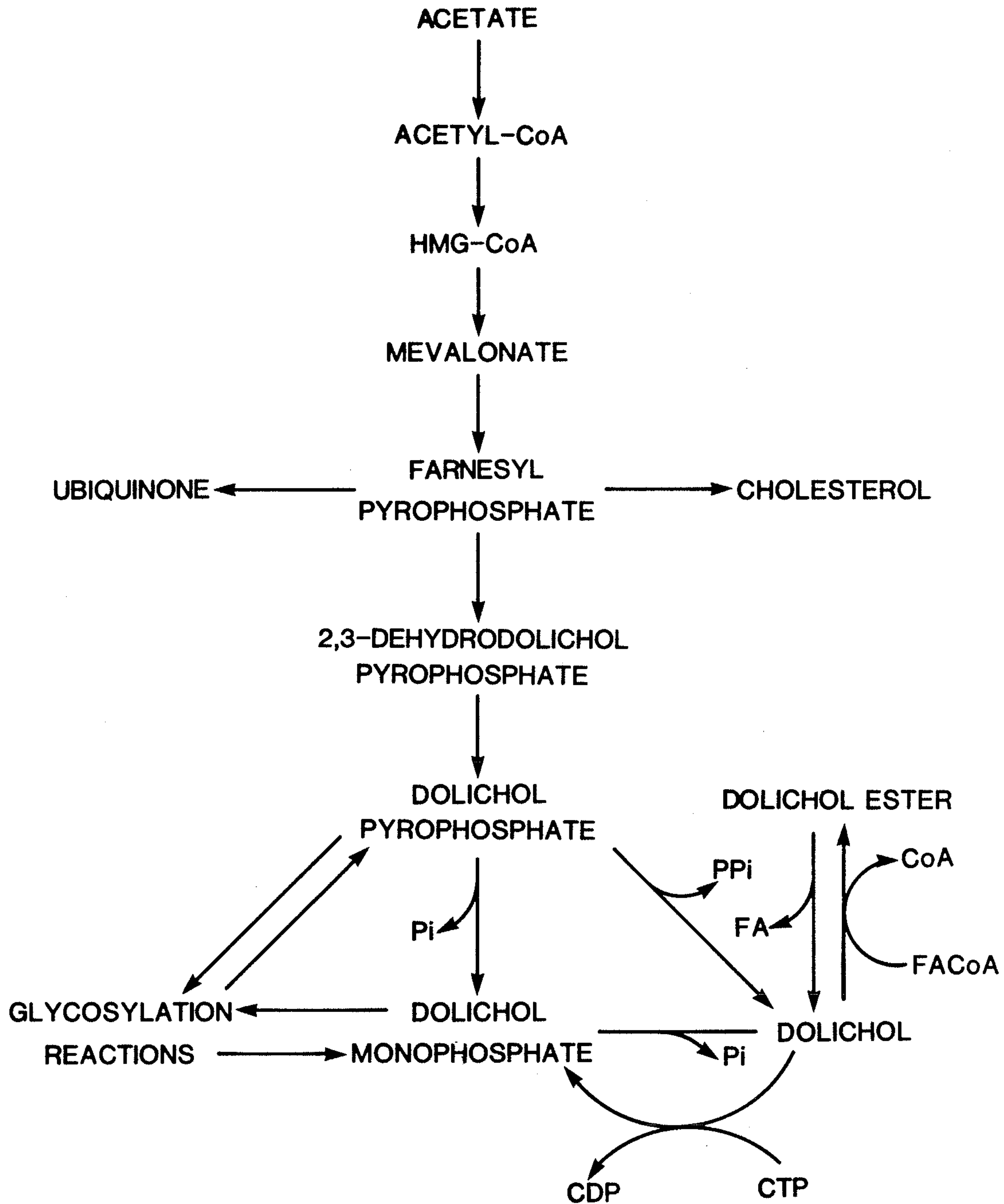
The pathway for the *de novo* synthesis of dolichol monophosphate is shown in Fig. 7 where it can be seen that dolichol monophosphate, cholesterol and ubiquinone share a common biosynthetic pathway from acetate to farnesyl pyrophosphate, at which point the pathways diverge. The isoprene chain is lengthened by the addition of 13-20 *cis*-isoprene units to farnesyl pyrophosphate in a reaction catalyzed by a long chain prenyltransferase, followed by saturation of the α-isoprene unit (Chojnacki and Dallner, 1983). Using rat liver subcellular fractions, Wong and Lennarz (1982) have established the microsome

Fig. 6. Structure of mannosylphosphoryldolichol (Man-P-Dol). The group of compounds referred to as dolichols consist of a linear chain of isoprene units in which the α-isoprene unit is saturated. See text for further details.




Man–P–Dol

Fig. 7. Biosynthetic pathway of dolichol monophosphate in mammalian cells. Adapted from Hemming (1977) and Burton _et al_. (1979). See text for further details. Abbreviations: CoA, Coenzyme A; HMG-CoA, hydroxymethylglutaryl-CoA; $P_i$, monophosphate; $PP_i$, pyrophosphate; FA, fatty acid.


ACETATE
ACETYL-CoA
HMG-CoA
MEVALONATE
UBIQUINONE
FARNESYL PYROPHOSPHATE
CHOLESTEROL
2,3-DEHYDRODOLICHOL PYROPHOSPHATE
DOLICHOL PYROPHOSPHATE
DOLICHOL ESTER
PPi
CoA
Pi
FA
FACoA
GLYCOSYLATION REACTIONS
DOLICHOL MONOPHOSPHATE
DOLICHOL
Pi
CDP
CTP

fraction as the primary site of synthesis of dolichol from farnesyl pyrophosphate. As shown in Fig. 7, there are two possible sources of dolichol monophosphate; de novo synthesis from acetate and phosphorylation of dolichol. The enzyme responsible for catalyzing the formation of dolichol monophosphate from dolichol is the CTP-dependent dolichol phosphokinase (Allen et al., 1978; Burton et al., 1979, Rip and Carroll, 1980); the enzyme catalyzing the reverse reaction is dolichol phosphate phosphatase (Adrian and Keenan, 1979; Wedgewood and Strominger, 1980; Rip et al., 1981). Both of these enzyme activities have been found in liver microsome fractions (Rupar et al., 1982). The results from the above studies suggest that the microsome fraction of liver is the main site for synthesis of both dolichol and dolichol monophosphate. Hemming's group (Richards and Hemming, 1972) showed that while other lipids could also function as acceptors in liver, dolichol monophosphate was the most efficient. Recently, retinyl phosphate has been shown to act as an acceptor of mannose in liver, but its role in the synthesis of glycoproteins is not clear (Wolf et al., 1979; Quill and Wolf, 1981).

As mentioned previously, the involvement of dolichol in oligosaccharide-lipid formation is now fairly well understood and it is clear that the reactions involving dolichol-linked sugar complexes are obligatory for N-linked glycoprotein biosynthesis. The synthesis of the oligosaccharide chains of N-linked glycoproteins via dolichol-linked oligosaccharide intermediates can be divided into three distinct steps: (1) assembly of oligosaccharide core regions utilizing phosphorylated dolichol sugar complexes; (2) transfer of the oligosaccharide from lipid carrier to polypeptide; and (3) modification of the oligosaccharide by processing, followed by synthesis of the terminal triplet structures

(Elbein, 1979; Struck and Lennarz, 1980; Hubbard and Ivatt, 1981).

(1) Assembly

In 1972, Parodi et al. (1972) reported that a glucose-containing lipid-linked oligosaccharide containing about 20 sugar units could be synthesized in liver and the oligosaccharide transferred to protein in cell-free preparations from rat liver. Since then, studies involving liver and other tissues have led to the identification of an oligosaccharide-pyrophosphate-dolichol complex (oligosaccharide-P-P-Dol), which represents the end product of a series of reactions prior to the transfer of the oligosaccharide to protein (Struck and Lennarz, 1980; Hubbard and Ivatt, 1981). The structure of this oligosaccharide-lipid complex is shown in Fig. 8, and the series of reactions involved in its formation, termed the dolichol cycle, is shown in Fig. 9. Although evidence supporting the dolichol cycle has come from sources other than liver, there is evidence that all the reactions shown in Fig. 9 are operative in the liver (Parodi and Leloir, 1979; Friesen and Jamieson, 1980; Struck and Lennarz, 1980; Sharon and Lis, 1981; Jamieson, 1983). As shown in Fig. 9, the initial step in the assembly of the glucose-containing oligosaccharide or G-oligosaccharide, as it is sometimes called, involves the formation of N-acetylglucosaminylpyrophosphoryl-dolichol (GlcNAc-P-P-Dol) in which GlcNAc-1-P is transferred from UDP-GlcNAc to dolichol monophosphate (Dol-P) (reaction 1). A second GlcNAc is then added to this complex, the donor again being UDP-GlcNAc (reaction 2). The addition of the first five Man residues to this complex is believed to proceed directly from GDP-Man (reaction 3). Elongation of $Man_5GlcNAc_2$-P-P-Dol occurs by the subsequent transfer of four Man residues from Man-P-Dol (reaction 4). The final reaction in

Fig. 8. Structure of the glucose-containing dolichol-linked oligosaccharide. The structure is based on that proposed by Robbin's group (Liu _et al_., 1979) and Kornfeld's group (Kornfeld and Kornfeld, 1980).

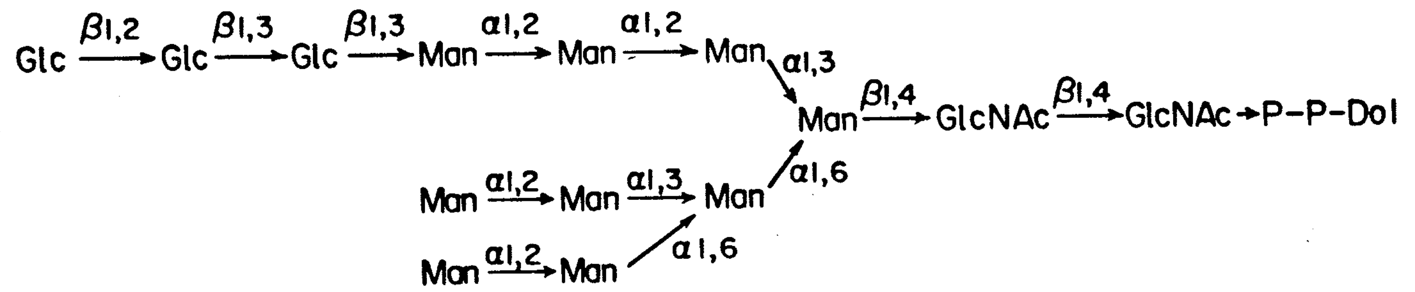
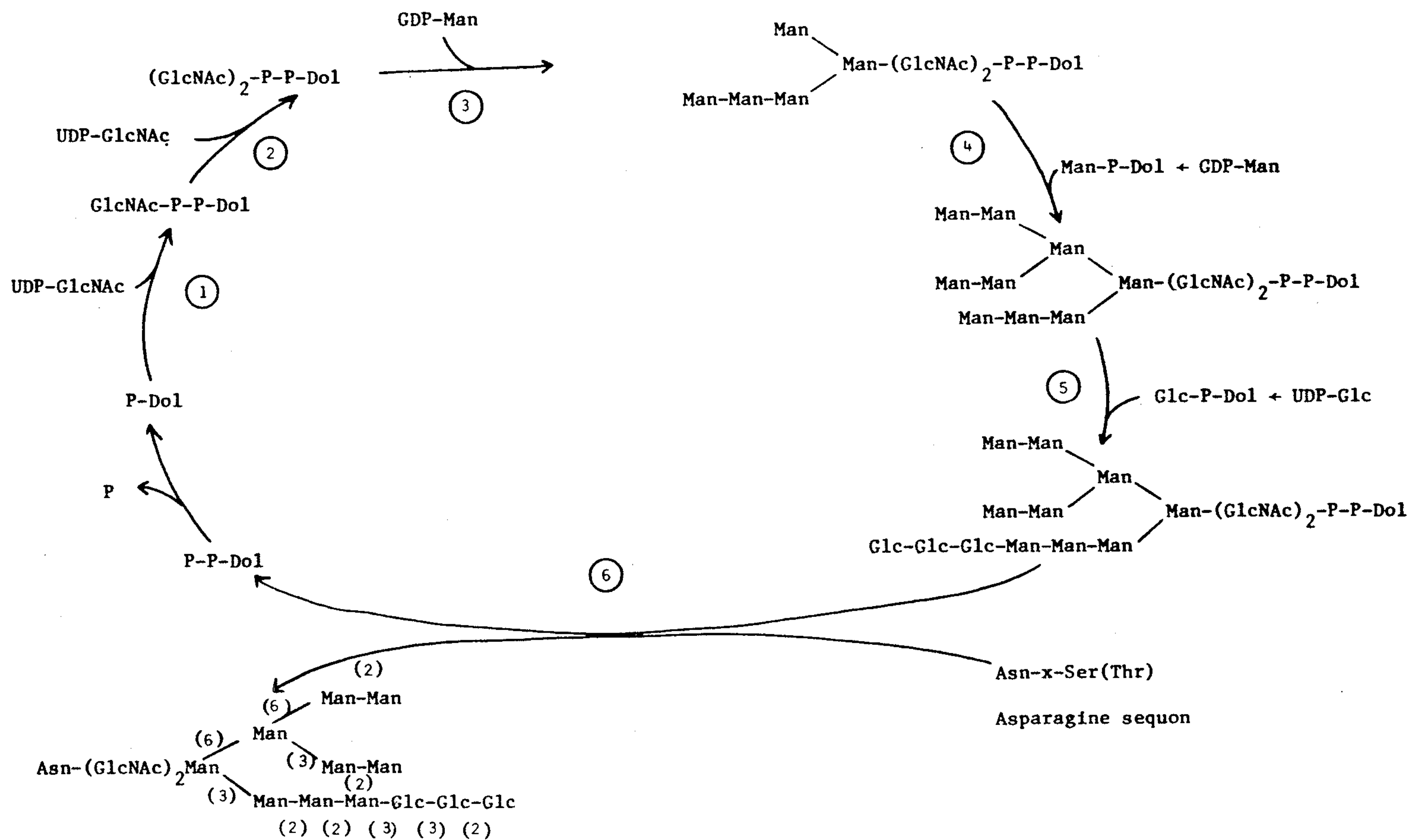

Glc
β1,2
Glc
β1,3
Glc
β1,3
Man
α1,2
Man
α1,2
Man
α1,3
Man
β1,4
GlcNAc
β1,4
GlcNAc→P–P–Dol
Man
α1,2
Man
α1,3
Man
α1,6
Man
α1,2
Man
α1,6

Fig. 9. The dolichol cycle showing the assembly of the dolichol-linked oligosaccharide. The circled numbers represent the main steps in the dolichol cycle; see text for further details. The numbers in parentheses indicate the nature of the linkages (see Fig. 8). Taken from Jamieson (1983).


GDP-Man
(GlcNAc)$_2$-P-P-Dol
3
Man
Man-(GlcNAc)$_2$-P-P-Dol
Man-Man-Man
UDP-GlcNAc
2
GlcNAc-P-P-Dol
4
Man-P-Dol + GDP-Man
Man-Man
Man
Man-Man
Man-(GlcNAc)$_2$-P-P-Dol
Man-Man-Man
UDP-GlcNAc
1
P-Dol
5
Glc-P-Dol + UDP-Glc
Man-Man
Man
Man-Man
Man-(GlcNAc)$_2$-P-P-Dol
Glc-Glc-Glc-Man-Man-Man
P
P-P-Dol
6
(2)
(6)
Man-Man
(6)
Man
(3)
Man-Man
(2)
Asn-(GlcNAc)$_2$Man
(3)
Man-Man-Man-Glc-Glc-Glc
(2) (2) (3) (3) (2)
Asn-x-Ser(Thr)
Asparagine sequon

the synthesis of the lipid-linked oligosaccharide is the addition of three Glc residues from Glc-P-Dol (reaction 6). It has not yet been established whether the Glc residues are added before or after transfer of the ninth Man residue to the lipid-linked $Man_8GlcNAc_2$ complex. Indeed, all of the transfer reactions stated above have not been fully elucidated; this will require the isolation and characterization of the various intermediate oligosaccharide complexes coupled with a study of the glycosyl transferases responsible for the reactions.

Several studies have been performed to determine if the enzymes involved in the assembly of lipid-linked oligosaccharide complexes in the dolichol cycle are localized on the cytoplasmic or luminal surface of the rough endoplasmic reticulum. The significance of these studies resides in the fact that while protein-linked carbohydrate is primarily cisternal (Rothman and Lenard, 1977; Rodriquez-Boulan et al., 1978; Hanover and Lennarz, 1980), nucleotide sugars are located in the cytoplasm and the lipid-linked oligosaccharide cannot transverse the membrane unaided (Hanover and Lennarz, 1979; McCloskey and Troy, 1980). Thus, we have to explain how the oligosaccharide-lipid complex traverses the membrane so that the oligosaccharide can be transferred to the protein on the luminal side. Protease treatment of rat liver microsome fraction vesicles demonstrated that enzymes involved in the synthesis of Man-P-Dol and Glc-P-Dol, from GDP-Man and UDP-Glc, respectively, and those transferring GlcNAc and Glc to the oligosaccharide-lipid are located at the cytoplasmic surface of the vesicles (Snider et al., 1980); the possibility that they are transmembrane enzymes, with their catalytic transfer capabilities at the luminal surface, could not be excluded. A study by Nilsson et al.

(1978), using a similar experimental approach, concluded that part of the Man-transferring activity of rat liver rough endoplasmic reticulum is cytoplasmic. However, Hanover and Lennarz (1979, 1982) using galactosyl transferase as a membrane-impermeable probe to determine the orientation of $GlcNAc_2$-P-P-Dol in hen oviduct microsome fraction vesicles, observed the formation of Gal-$GlcNAc_2$-P-P-Dol only after disruption of the membranes with detergent. This implies a luminal location for the lipid-linked saccharide. Limited proteolysis of the hen oviduct microsome fraction vesicles indicated a cytoplasmic accessibility of the enzymes involved in saccharide-lipid synthesis (Hanover and Lennarz, 1982), as was observed by Snider _et al_. (1980) using rat liver microsome fraction vesicles in experiments described above. Hanover and Lennarz (1982) also demonstrated the impermeability of GDP-Man and UDP-GlcNAc to microsome fraction membranes. Due to the luminal orientation of saccharide-lipid products and the cytoplasmic location of nucleotide sugars, it was concluded that the enzymes involved in the synthesis of the lipid-linked oligosaccharides span the membrane of the rough endoplasmic reticulum, with portions of the polypeptide chains of the enzymes exposed on both cytoplasmic and luminal faces. Thus, Hanover and Lennarz (1982) suggest that a transmembrane multienzyme complex directs the assembly of the lipid-linked oligosaccharide through the ordered addition of monosaccharides from both nucleotide sugars and lipid intermediates. In this model, the polyisoprenol lipid moiety is believed to be involved in anchoring the growing oligosaccharide to the membrane-bound multienzyme complex.

(2) Transfer

It is now well established that N-glycosylation of proteins involves the *en bloc* transfer of the glucose-containing high-mannose oligosaccharides from the lipid carrier to an asparagine residue of a nascent polypeptide chain (Waechter and Lennarz, 1976; Struck and Lennarz, 1980; Hubbard and Ivatt, 1981). A number of lines of evidence indicate that the glycosylation event occurs within the lumen of the rough endoplasmic reticulum (Rothman and Lodish, 1975; Bergman and Kuehl, 1977; Hanover and Lennarz, 1979). Early studies of the primary structure of glycoproteins suggested that the asparagine to which the oligosaccharide chain became attached must be part of the sequence -Asn-X-Ser(Thr)-, now referred to as the "asparagine sequon" (Marshall, 1974). The amino acid -X- can be any of the 20 naturally occurring amino acids except proline (Ronin *et al.*, 1979) and aspartic acid (Marshall, 1974) which prevent glycosylation. Pless and Lennarz (1977) have suggested that an additional requirement for the glycosylation of asparagine is the unfolding of the polypeptide structure in the region of the sequon.

Conflicting evidence exists concerning the mechanism of glycosylation of nascent polypeptide chains. As mentioned previously, the polypeptide moiety of serum glycoproteins is synthesized on membrane-bound polyribosomes and translocated across the rough endoplasmic reticulum membrane. Studies in liver indicate that glucosamine and mannose incorporation into nascent polypeptide chains of $\alpha_1$-acid glycoprotein and B$\beta$ chains of fibrinogen is mainly a post-ribosomal event (Jamieson, 1977; Woloski and Jamieson, 1980; Nickerson and Fuller 1981a). However, studies in other cell types on the glycosylation of immunoglobulins (Bergman and Kuehl, 1977, 1978), lactalbumin (Lingappa *et al.*,

1978), ovalbumin (Kiely et al., 1976; Glabe et al., 1980; Hanover and Lennarz, 1980) and viral proteins (Rothman and Lodish, 1977; Sefton, 1977) have suggested that glycosylation occurs during polypeptide elongation. Based on these conflicting results, there would appear to be two mechanisms for initial glycosylation of glycoproteins. One would involve glycosylation during elongation of the polypeptide and the other would involve glycosylation after release of the polypeptide from the ribosome; a combination of both mechanisms could also exist. The particular mechanism employed may depend on the cell type, the accessibility of the asparagine sequon, and the location of the oligosaccharide-lipid complexes in the rough endoplasmic reticulum membrane.

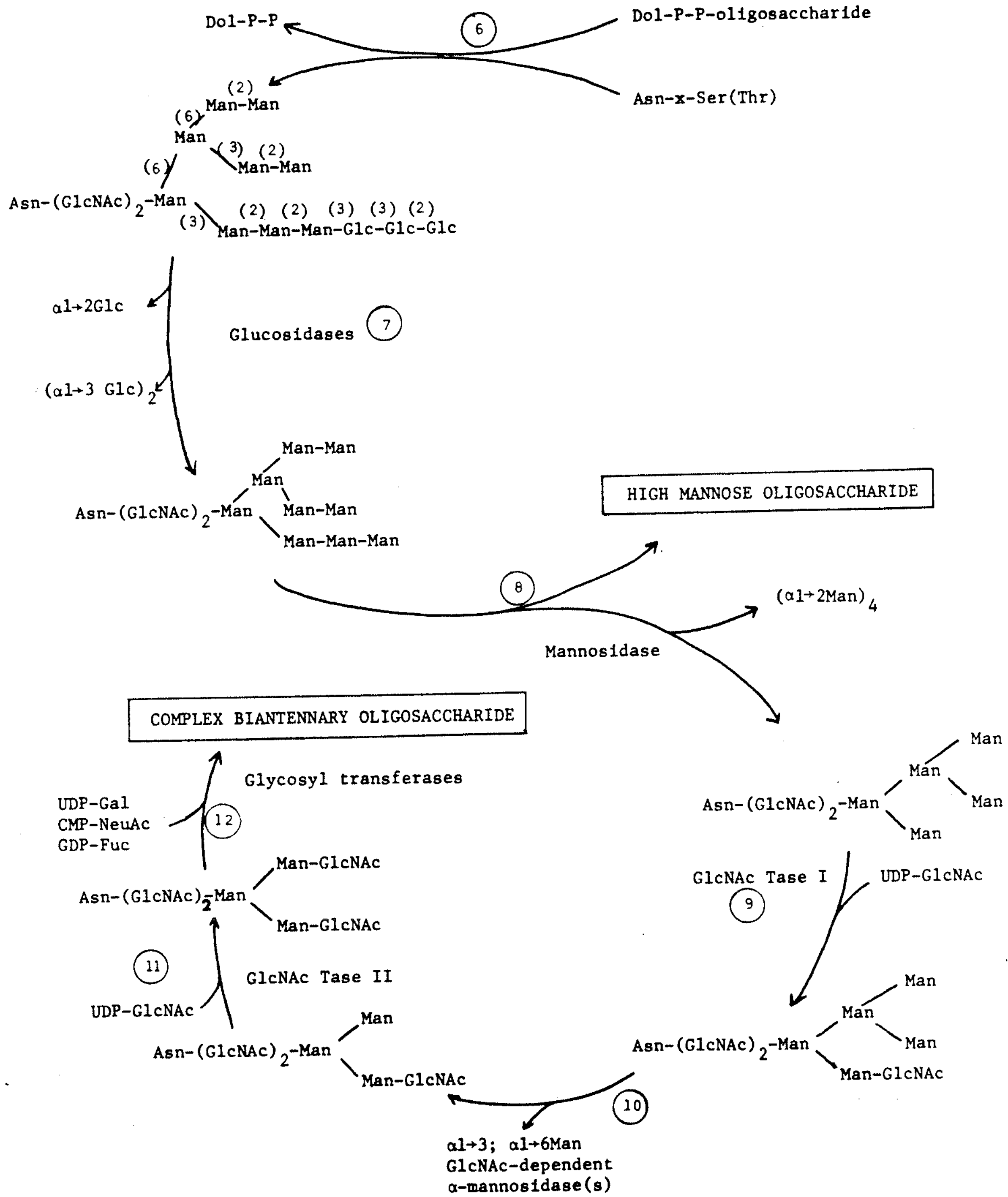
The presence of glucose in the lipid-linked oligosaccharide was surprising since this sugar is not present in the complex-type oligosaccharides of serum glycoproteins (for structures see Fig. 5). Turco et al. (1977) showed that glucose-containing oligosaccharides were transferred from lipid carriers to acceptor proteins at about a six-fold higher initial rate than oligosaccharides without glucose. Thus, it was suggested that glucose is required for the en bloc transfer of the oligosaccharide from lipid to protein; this idea was also supported in studies by Spiro et al. (1979). It appears that the glucose residues on the terminal positions of the oligosaccharide-lipid complexes (for structure see Fig. 8) may serve as a recognition factor for the oligosaccharide-transferring enzyme.

(3) Oligosaccharide processing

After transfer to acceptor proteins, the initially homogeneous population of precursor oligosaccharides begins to undergo a series of modifications that will eventually produce the diverse range of N-linked

carbohydrate chains of mature glycoproteins. The removal of all of the glucose and all but three of the mannose residues is referred to as oligosaccharide processing. Most of the information on oligosaccharide processing has come from studies on virus proteins, immunoglobulins, or cells in tissue culture (Kornfeld *et al*., 1978; Schachter *et al*., 1978; Turco and Robbins, 1979; Harpaz and Schachter, 1980a,b). Models to describe oligosaccharide processing have been presented by several authors (Kornfeld *et al*., 1978; Turco and Robbins, 1979; Harpaz and Schachter, 1980a,b; Sharon and Lis, 1981). There is evidence that oligosaccharide processing, as is shown in Fig. 10, also occurs in liver. Using glycopeptides and oligosaccharides, two glucosidases have been detected and partially purified from hepatic endoplasmic reticulum membranes (Grinna and Robbins, 1979; Ugalde *et al*., 1979). Using similar substrates, three mannosidases have been purified and characterized in the hepatic Golgi complex membranes (Tulsiani *et al*., 1977; Opheim and Touster, 1978; Tabas and Kornfeld, 1979); recently, evidence has been provided for a mannosidase activity in rat liver rough endoplasmic reticulum with activity towards high-mannose oligosaccharides (Bischoff and Kornfeld, 1982). Evidence supporting the idea that oligosaccharide processing exists in liver was provided, albeit indirectly, from the work in our laboratory by Friesen and Jamieson (1979, 1980). In this work, the isolation of a high-mannose form of $\alpha_1$-acid glycoprotein was described which provides evidence for reaction 6 in the dolichol cycle (Fig. 9). The protein did not contain glucose suggesting that there is rapid glucose removal after transfer of the oligosaccharide to protein. However, $\alpha_1$-glycoprotein in its native form contains only complex type oligosaccharide chains (Yamashita *et al*.,

Fig. 10. Scheme for processing of oligosaccharide chains of N-linked glycoproteins. Processing begins following transfer of the Glc-containing oligosaccharide from an oligosaccharide-dolichol complex to an asparagine of an asparagine sequon in the polypeptide chain (Figs. 9 and 10, reaction 6). The circled numbers represent the main steps in this process; see text for further details. Numbers in parentheses indicate the nature of the linkages (see Fig. 8). Taken from Jamieson (1983). GlcNAc Tase I, GlcNAc transferase I; GlcNAc Tase II, GlcNAc transferase II.


Dol-P-P
Dol-P-P-oligosaccharide
6
Asn-x-Ser(Thr)
(2) Man-Man
(6) Man
(3) (2) Man-Man
(6)
Asn-(GlcNAc)2-Man
(3) (2) (2) (3) (3) (2) Man-Man-Man-Glc-Glc-Glc
α1→2Glc
Glucosidases 7
(α1→3 Glc)2
Man-Man
Man
Asn-(GlcNAc)2-Man Man-Man
Man-Man-Man
HIGH MANNOSE OLIGOSACCHARIDE
8
(α1→2Man)4
Mannosidase
COMPLEX BIANTENNARY OLIGOSACCHARIDE
Glycosyl transferases
UDP-Gal
CMP-NeuAc
GDP-Fuc
12
Man-GlcNAc
Asn-(GlcNAc)2-Man
Man-GlcNAc
11
GlcNAc Tase II
UDP-GlcNAc
Asn-(GlcNAc)2-Man
Man
Man-GlcNAc
Man
Man
Asn-(GlcNAc)2-Man
Man
Man
GlcNAc Tase I
9
UDP-GlcNAc
Man
Man
Asn-(GlcNAc)2-Man
Man
Man-GlcNAc
10
α1→3; α1→6Man
GlcNAc-dependent
α-mannosidase(s)

1981; Yoshima *et al.*, 1981); thus, oligosaccharide processing of this protein presumably occurs *via* the currently accepted pathway outlined in Fig. 10.

As shown in Fig. 10, the first step in processing is the removal of the terminal α-1,2-linked Glc by glucosidase I; a second enzyme, glucosidase II, then removes the two α-1,3-linked Glc residues (reaction 7). The second stage of processing involves the removal of the four α-1,2-linked Man residues (reaction 8). In rat liver, three distinct enzyme activities, one located in rough endoplasmic reticulum membrane (Bischoff and Kornfeld, 1983) and two located in Golgi membranes (Tabas and Kornfeld, 1979; Tulsiani *et al.*, 1982), are capable of cleaving the α-1,2-linked Man residues. The requirement for the presence of three distinct α-mannosidase activities towards α-1,2-linkages in asparagine-linked oligosaccharide processing remains to be elucidated. The rough endoplasmic reticulum α-mannosidase might be responsible for hydrolyzing specific Man residues from oligosaccharides at specific glycosylation sites on a glycoprotein. For example, since the order of removal of the four α-1,2-linked Man residues from $Man_9GlcNAc_2$ oligosaccharides at $Asn_{402}$ and $Asn_{563}$ of IgM differ (Chapman and Kornfeld, 1979), it could be that one oligosaccharide is processed in the endoplasmic reticulum and the other in the Golgi. Preliminary data suggest that the endoplasmic reticulum and Golgi α-mannosidases generate different oligosaccharide structures from $Man_9GlcNAc_2$ (Tabas and Kornfeld, 1979; Bischoff and Kornfeld, 1983). The primary structure near the glycosylation site influences secondary and tertiary structure of the polypeptide, and this might influence the α-mannosidase activity, and hence the requirement for three distinct α-1,2-mannosidase activities.

Failure to remove the α-1,2-linked Man residues results in the high-mannose structure, as shown in Fig. 5. It is not clear why some glycoproteins do not undergo mannose processing. Studies on the glycosylation of one viral glycoprotein in different cells (Etchison and Holland, 1974; Keegstra _et al._, 1975), or of different viral proteins in one cells type (Sefton, 1976; Schwartz _et al._, 1977; Rosner _et al._, 1980), or comparison of the oligosaccharide chains of an immunoglobulin and a viral glycoprotein glycosylated in the same cell (Weitzman _et al._, 1979), have found that the same protein was glycosylated similarly by different cells, all of which contained the full set of processing enzymes, whereas different proteins were glycosylated differently by the same cell. Thus, these studies suggest that the structure of the polypeptide chain to be glycosylated may determine the processing of the high-mannose chains initially attached.

The $Man_5GlcNAc_2$ structure, which results from the removal of the four α-1,2-linked Man residues, is the starting point for the biosynthesis of all complex structures including the uncommon hybrid structure. A hybrid structure is one having a high-mannose structure on one arm of the core structure,usually one or two Man residues on the Man linked $\alpha 1 \rightarrow 6$ to the β-linked Man, and a complex structure on the other arm of the core (Dorland _et al._, 1979; Liang, 1979). The formation of a particular structure is controlled by a series of specific GlcNAc transferases which recognize specific oligosaccharide structures as acceptors for transfer of GlcNAc. The first reaction involves the enzyme GlcNAc transferase I which is highly specific for the terminal α-1,3-linked Man residue of the core (Fig. 10) (Harpaz and Schachter, 1980a,b; Oppenheimer and Hill, 1981). The action of GlcNAc transferase

I is apparently essential for further processing of the α-1,3-linked and α-1,6-linked Man residues by specific mannosidase(s) (Harpaz and Schachter, 1980b; Tulsiani et al., 1982). The GlcNAc(Man)$_3$GlcNAc$_2$ structure is a substrate for GlcNAc transferase II which initiates the second antenna (Harpaz and Schachter, 1980b). The structure so formed is an excellent substrate for several glycosyl transferases: GlcNAc transferase III can act to form the bisecting structure in which a GlcNAc is attached to the β-linked Man residue of the core (Narasimhan, 1982); GlcNAc transferase IV can act to catalyze the addition of GlcNAc in β-1,4-linkage to the Man residue in α-1,3-linkage to the core to initiate the third antenna (Gleeson et al., 1982, 1983); fucosyl transferase can also act to add fucose in an α-1,6-linkage to the GlcNAc involved in asparagine linkage (Wilson et al., 1976; Schachter et al., 1979). The complex oligosaccharides can be completed by addition of Gal and NeuAc by galactosyl and sialyl transferase enzymes located mainly in the Golgi complex (Schachter, 1978; Schachter and Roseman, 1980). It should be noted that the GlcNAc transferase responsible for initiating the fourth arm of the tetraantennary oligosaccharide structure has yet to be reported, but it clearly must exist to explain the existence of tetraantennary structures on many glycoproteins.

As stated above, the oligosaccharide chains of glycoproteins are synthesized by the sequential action of a series of glycosyl transferases. Glycosyl transferases have been the subject of numerous studies and their activities, properties and functions have been well reviewed (Schachter, 1978; Pierce et al., 1980; Schachter and Roseman, 1980; Beyer et al., 1981; Beyer and Hill, 1982). Glycosyl transferases are defined as a group of enzymes that catalyze the formation of specific

glycosides by transfer of a sugar from a nucleotide sugar donor to an acceptor substrate; the acceptor substrate may be a monosaccharide, oligosaccharide, glycopeptide, glycoprotein, glycolipid or lipid depending on the specificity of the enzyme. A noteworthy exception to the use of a nucleotide sugar as the sugar donor is in the formation of the glycosylamine linkage in which the oligosaccharyldiphosphoryldolichol-protein oligosaccharyltransferase (Das and Heath, 1980) catalyzes the *en bloc* transfer of the G-oligosaccharide from the lipid carrier to an asparagine residue in the protein. Glycosyl transferases may be grouped together and identified on the basis of the sugar transferred from donor to acceptor substrates; thus, sialyl transferases, galactosyl transferases, N-acetylglucosaminyl transferases and others are recognized. The glycosyl transferases involved in glycoprotein biosynthesis are membrane-bound and usually found in the endoplasmic reticulum and Golgi complex (Schachter, 1978; Schachter and Roseman, 1980; Beyer *et al.*, 1981); soluble enzymes in serum and other body fluids have been detected (Wagner and Cynkin, 1971; Kim *et al.*, 1972; Nelson *et al.*, 1973). Many glycosyl transferases have been purified, some to homogeneity, and their enzymic, physical and chemical properties have been established in considerable detail. One interesting property of the glycosyl transferases purified to date is that they are glycoproteins (Beyer *et al.*, 1981). In earlier studies, tissue extracts or subcellular fractions were used to identify the reactants and products of the glycosyl transferase reactions. Despite the problems inherent in employing such impure systems, the characterization of the products and substrate specificities of the reactions demonstrated the remarkable degree of specificity that glycosyl transferases have for their acceptor substrates.

It appears that at least one specific glycosyl transferase is required for the formation of each specific glycosidic linkage found in nature -- the "one enzyme-one linkage" concept (Hagopian and Eylar, 1968). In view of the many different oligosaccharide structures found in glycoproteins and other glycoconjugates, and the many diverse nucleotide sugars, a large number of glycosyl transferases must exist. For example, at least four sialyl transferases would be required to account for the NeuAcα2→6Gal, NeuAcα2→3Gal, NeuAcα2→4Gal and NeuAcα2→6GlcNAc linkages reported in the oligosaccharide chains of N-linked glycoproteins from various sources (Kornfeld and Kornfeld, 1980; Beyer _et al_., 1981). The combined and sequential actions of many glycosyl transferases could effectively result in the synthesis of a variety of oligosaccharide structures. Due to the reasonably strict acceptor substrate specificities, however, the synthesis of only certain oligosaccharides are premitted. For example, the Galβ1→4GlcNAc α2→6 sialyl transferase enzyme purified from rat liver by Paulson's group (Weinstein _et al_., 1982a,b) was found to elaborate with high specificity the sequence NeuAcα2 →6Galβ1→4GlcNAc in N-linked oligosaccharides of glycoproteins (see Fig. 5). Using low molecular weight acceptors, it was found that while the enzyme was active towards Galβ1→4GlcNAc, it showed extremely little or no activity towards Galβ1→3GlcNAc, Galβ1→6GlcNAc, Galβ1→3GalNAc and Galβ1→6GalNAc (Weinstein _et al_., 1982b). Thus, because of the acceptor substrate specificities exhibited by glycosyl transferases, it is possible, in some cases, to deduce how the combined actions of these enzymes lead to the synthesis of well-known structures found in N-linked oligosaccharides of glycoproteins.

An important modification of oligosaccharide processing occurs

during the processing of the carbohydrate chains of lysosomal glycosidases. Several lysosomal enzymes have been found to contain a phosphate group covalently attached to the α-1,2-linked Man residues of the high-mannose structure (Tabas and Kornfeld, 1980; Sly _et al_., 1981). The mannose-6-phosphate group is believed to function as a signal directing the newly synthesized lysosomal enzyme from the Golgi to the lysosome (Rome _et al_., 1979; Sly, 1979; Sly _et al_., 1981). Due to the high-mannose structure of the lysosomal enzymes, Varki and Kornfeld (1980b) suggested that phosphorylation occurs prior to the action of the α-1,2-mannosidases that remove the four α-1,2-linked Man residues (Fig. 10, reaction 8). The enzymes involved in the formation of the mannose-6-phosphate marker have been demonstrated in lymphoma cells, fibroblasts and liver (Reitman and Kornfeld, 1981; Varki and Kornfeld, 1981; Waheed _et al_., 1981a,b). It has been suggested that some aspect of polypeptide structure of the lysosomal enzymes, either an amino acid sequence or a unique conformation, makes them suitable substrates for UDP-GlcNAc: glycoprotein GlcNAc-1-phosphotransferase. This enzyme transfers GlcNAc-1-phosphate from UDP-GlcNAc to hydroxyl groups on C-6 positions of Man residues at terminal or penultimate positions of a high-mannose oligosaccharide chain attached to a lysosomal enzyme (Reitman and Kornfeld, 1981). Subsequently, the GlcNAc is hydrolyzed by a GlcNAc phosphodiesterase leaving a high-mannose oligosaccharide chain with a mannose-6-phosphate recognition marker (Varki and Kornfeld, 1980a,b, 1981; Waheed _et al_., 1981b).

Although the main features of the synthesis and processing of asparagine-linked oligosaccharides are now known, several areas, including the detailed enzymology of the glycosylating and processing

enzymes, the cellular organization of these enzymes, the regulation of the pathway, and factors that control oligosaccharide processing are only beginning to be fully understood.

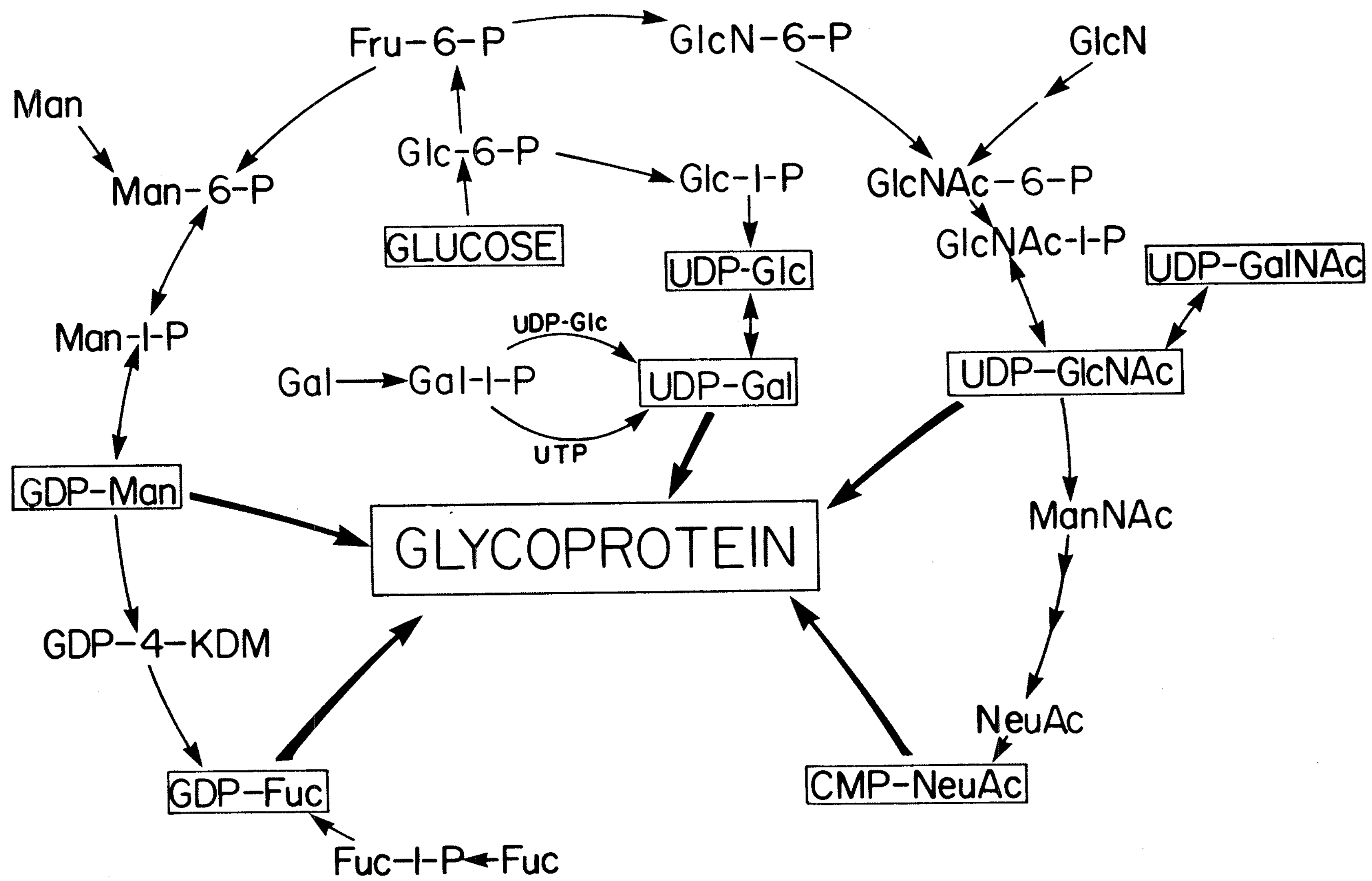
Nucleotide Sugars

It is evident from the previous section on the dolichol cycle and glycosyl transferases, that nucleotide sugars play a central role in the synthesis of oligosaccharide chains of glycoproteins because of their utilization as glycose donors. The pathways of synthesis of the nucleotide sugars important in N-glycosidically linked glycoprotein biosynthesis are shown in Fig. 11. The scheme combines data for many different organisms and tissues as reviewed by Warren (1972) and Schachter and Roden (1973); the validity of the pathways has not been established unequivocally for only one tissue. It is reasonable to assume, however, that the scheme shown in Fig. 11 is a valid representation of nucleotide sugar synthesis in many tissues including liver.

With the possible exception of the sialic acid-activating enzyme, CMP-NeuAc synthase, the enzymes involved in nucleotide sugar synthesis are located in the cytoplasm (Kean, 1970; Warren, 1972; Carey et al., 1980; Coates et al., 1980). A study by Kean (1970) indicated that the nuclear fraction of cells from a variety of tissue sources, including rat liver, was enriched in CMP-NeuAc synthase activity. The CMP-NeuAc synthase levels in other subcellular fractions, including the soluble supernatant, were not reported; thus, although nuclei are enriched in CMP-NeuAc synthase activity, the presence of the enzyme in other cellular locations cannot be ruled out.

The intracellular concentrations of nucleotide sugars are under stringent controls, as indicated by the lack of net accumulation of

Fig. 11. Pathways of formation of nucleotide sugars. Thick, solid arrows indicate precursors of sugars commonly found in oligosaccharide chains of N-linked glycoproteins. Abbreviations: Glc, glucose; Fru, fructose; Man, mannose; GlcN, glucosamine; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; ManNAc, N-acetylmannosamine; NeuAc, N-acetylneuraminic acid; Gal, galactose; Fuc, fucose; KDM, keto-deoxymannose; P, phosphate.




The University of Manitoba LIBRARIES

these precursors in the livers of puromycin-treated rats in which glycoprotein synthesis was blocked (Kornfeld et al., 1964). The obvious point at which the level of any one nucleotide sugar can be controlled is the first enzymic step in the sequence of reactions involved in its synthesis. Thus, the key enzyme in the formation of UDP-GlcNAc, UDP-GalNAc, and CMP-NeuAc from glucose is L-glutamine:D-fructose-6-phosphate aminotransferase, more recently referred to as glucosamine-6-phosphate synthase. This enzyme catalyzes the step in which glucose-6-phosphate is channeled into the hexosamine pool (Fig. 11). The reaction catalyzed by this enzyme involves the transfer of an amide group from glutamine to fructose-6-phosphate, followed by enolization of the resulting Schiff's base, formation of an aldehyde center at the C-1 position and a spontaneous ring expansion from the furanose to the pyranose form (Ghosh et al., 1960). Unlike many other aminotransferases that utilize glutamine, there appears to be no requirement for ATP. However, the reaction cannot be reversed in presence of glucosamine-6-phosphate and glutamate (Ghosh et al., 1960). Extensive studies on glucosamine-6-phosphate synthase have found it to be regulated in a complicated way (Kornfeld et al., 1964; Bates et al., 1966; Kornfeld, 1967; Miyagi and Tsuiki, 1971). Briefly, the enzyme is subject to feedback inhibition by UDP-GlcNAc, but the degree of inhibition is dependent on secondary factors that can alter the binding constant of UDP-GlcNAc to the enzyme; these factors include UTP, AMP, and glucose-6-phosphate. The regulation of glucosamine-6-phosphate synthase activity varies from cell to cell. In normal rat liver, the rate of hexosamine synthesis from glucose is equivalent to less than 0.1% of the rate of glycolysis, whereas it is as high as 15-20% in neonatal rat skin (Hardingham and Phelps, 1968; Winterburn and Phelps,

1971).

UDP-GlcNAc is formed from GlcNAc-1-P and UTP by a pyrophosphorylase (Schachter and Roden, 1973). GlcNAc-1-P can be derived from glucose or from GlcN (Fig. 11). GlcN enters the pathway towards GlcNAc-1-P, and eventually UDP-GlcNAc, by first being acetylated and subsequently phosphorylated by a specific kinase to yield GlcNAc-6-P (Schachter and Roden, 1973). UDP-GalNAc is formed from UDP-GlcNAc by UDP-GlcNAc 4-epimerase (Maley and Maley, 1959). In liver, there is not an appreciable amount of epimerization of UDP-GlcNAc to UDP-GalNAc as the equilibrium has been found to favor the glucosamine derivative (Molnar _et al._, 1964; Robinson _et al._, 1964; Shetlar _et al._, 1964; Macbeth _et al._, 1965; White _et al._, 1965).

Studies with rat liver have shown that CMP-NeuAc inhibits UDP-GlcNAc 2-epimerase, the enzyme catalyzing the biosynthesis of N-acetylmannosamine from UDP-GlcNAc (Kornfeld _et al._, 1964). Since this is the first step in the pathway leading to CMP-NeuAc from UDP-GlcNAc, the activated form of the sialic acid thus exerts a negative feedback on its own synthesis.

The final step in the activation of sialic acid to CMP-NeuAc is catalyzed by CMP-NeuAc synthase (or CMP-sialic acid synthase) (Roseman, 1962; Kean and Roseman, 1966; Schachter, 1978). Unlike other pyrophosphorylases which catalyze the formation of other nucleotide sugars, this enzyme forms CMP-NeuAc from a nucleoside triphosphate and a sugar, NeuAc, rather than with a sugar-phosphate; the resulting nucleotide sugar contains only one phosphate ester linkage instead of two which is found in the other nucleotide sugars. The most common sialic acid is N-acetylneuraminic acid (Schachter, 1978), although N-glycolyl,

N-acetyl-O-diacetyl, and N,O-diacetyl derivatives are well known (Warren, 1963; Cabezas, 1973; Schauer _et al_., 1974). Nucleotide sugars can be formed by CMP-sialic acid synthase for all derivatives of neuraminic acid (Kean, 1970; Schauer and Wember, 1973). Studies by Schauer and Wember (1973) with crude enzyme suggest that a single enzyme is involved in forming the different CMP-sialic acids; this was based on identical pH dependencies and similar kinetic parameters for different sialic acid derivatives. However, absolute determination, if a single enzyme is involved in the synthesis of different CMP-sialic acids, must await purification of CMP-sialic acid synthase(s).

GDP-Man can be formed from glucose _via_ Fru-6-P, Man-6-P, and Man-1-P (Fig. 11). GDP-Man can also be formed from Man which can enter the pathway _via_ Man-6-P (Fig. 11). The formation of GDP-Man from Man-1-P is catalyzed by GDP-Man pyrophosphorylase (Schachter, 1978). The formation of GDP-Fuc from GDP-Man involves the formation of the intermediate GDP-4-keto-6-deoxymannose by GDP-Man oxidoreductase (Ginsburg, 1964). GDP-Fuc can also be formed from Fuc _via_ Fuc-1-P (Fig. 11). This route is catalyzed by Fuc kinase and GDP-Fuc pyrophosphorylase (Schachter and Roden, 1973). At present, little is known concerning the regulation of the intracellular levels of GDP-Man and GDP-Fuc in mammals. However, studies by Kornfeld and Ginsburg (1966) noted that in microorganisms intracellular levels of GDP-Fuc are regulated by feedback inhibition in which the nucleotide sugar inhibits one or two enzymes involved in the pathway for its own synthesis.

UDP-Gal can be formed from glucose _via_ epimerization of UDP-Glc by UDP-Glc 4-epimerase (Feingold, 1972). Gal can also be converted to UDP-Gal (Fig. 11). Gal is first phosphorylated to Gal-1-P, which can

be converted to UDP-Gal either by Gal-1-P uridyltransferase, in a reaction involving UDP-Glc, or by UDP-Gal pyrophosphorylase, in a reaction involving UTP (Fig. 11). Under normal conditions, at least 99% of Gal-1-P is metabolized _via_ the uridyltransferase (Abraham and Howell, 1969).

The intracellular levels of nucleotide sugars may also be controlled by their catabolism. Shoyab and Bachhawat (1967) identified the presence of CMP-NeuAc hydrolase activity in sheep liver and rat tissues which degrades CMP-NeuAc to CMP and NeuAc. A pyrophosphatase with potent nucleotide sugar hydrolase activity has also been detected in various tissues (Sela _et al._, 1972; Evans _et al._, 1973; Mookerjea and Yung, 1975). Although a specific role for these hydrolytic enzymes has not been found, a possible role in regulating intracellular nucleotide sugar levels should not be discounted.

## Transport and Secretion of Glycoproteins

Newly synthesized secretory, membrane and lysosomal proteins, following their segregation within the lumen of the rough endoplasmic reticulum, migrate towards the Golgi apparatus _via_ the smooth endoplasmic reticulum (Whaley and Dauwalder, 1979; Hand and Oliver, 1981; Redman _et al._, 1981; Sly _et al._, 1981). Studies on hepatocytes (Claude, 1970; Ovtracht _et al._, 1973) suggest that smooth tubular continuities may exist between the rough endoplasmic reticulum and the Golgi apparatus; whereas, in most other tissues transfer of proteins from the endoplasmic reticulum to the Golgi apparatus is believed to occur _via_ small vesicles that bud from ribosome-free portions of the endoplasmic reticulum adjacent to the _cis_ face of the Golgi apparatus (Caro and Palade, 1964; Friend, 1965; Jamieson and Palade, 1967; Rothman _et al._,

1980). The Golgi apparatus is a complex structure consisting of _cis_ and _trans_ portions and is the site of post-translational modifications such as trimming of the mannose residues and terminal glycosylation of N-linked oligosaccharide chains (Novikoff, 1976; Farquhar, 1978; Schachter and Roseman, 1980). Transport vesicles usually fuse with the _cis_ Golgi, although these vesicles have been found also to fuse with the _trans_ Golgi or to by-pass the Golgi completely (Hand and Oliver, 1981). Proteins destined for secretion are concentrated and packaged in vesicles or vacuoles at the _trans_ face of the Golgi (Palade, 1975; Cohn and MacGregor, 1981; Hand and Oliver, 1981; Wilson, 1981). After packaging, maturation of the vesicles and their contents occur. This maturation process in hepatocytes includes the conversion of proalbumin to albumin by proteolytic cleavage of the propeptide (Ikehara _et al._, 1976; Redman _et al._, 1978). Maturation has also been found to result in the formation of different types of secretory granules in both liver and parathyroid gland (Schreiber _et al._, 1979; Cohn and MacGregor, 1981; Ledford and Davis, 1983), resulting in their contents being either stored, degraded or secreted. Differences in secretory vesicles might explain the different transit times of proteins through cells (Cohn and MacGregor, 1981). Finally, the secretory vesicle interacts with the microtubular-microfilament system, fuses with the plasma membrane and discharges its contents from the cell.

An additional function of the Golgi apparatus is the targeting of newly synthesized lysosomal glycosidases to lysosomes (Sly _et al._, 1981; Sly and Fisher, 1982; Natowicz _et al._, 1983). Lysosomes are organelles that appear to originate from the smooth membrane tubular network closely associated with the endoplasmic reticulum and Golgi apparatus,

referred to as the Golgi-endoplasmic reticulum-lysosome network (GERL). As mentioned previously, lysosomal enzymes destined for the lysosome contain a mannose-6-phosphate recognition marker. It is believed that receptors in the Golgi apparatus recognize this marker on the proteins, segregate these proteins and transfer them to the lysosomes (Sly and Stahl, 1978; Sly et al., 1979; Gonzalez-Noriega et al., 1980; Sly et al., 1981; Sly and Fischer, 1982; Natowicz et al., 1983).

Clearly, the Golgi complex is an important intracellular organelle for the transport and sorting out of macromolecules that are synthesized in the endoplasmic reticulum of a cell. The involvement of the Golgi complex in glycoprotein synthesis and secretion is of particular importance in view of the numerous biosynthetic reactions involving the oligosaccharide chains of glycoproteins that occur in the complex, and the observations mentioned earlier that acute inflammation is accompanied by elevated acute phase glycoprotein synthesis and substantial liver Golgi proliferation.

## INTRODUCTION TO THE PRESENT WORK

The work presented in this thesis forms part of a study on the acute phase response with particular emphasis on the control of biosynthesis of acute phase glycoproteins by liver. Work conducted in our laboratory the past fifteen years has involved studies on rat $\alpha_1$-acid glycoprotein as a model acute phase glycoprotein which is the major acute phase glycoprotein synthesized by liver (Ashton et al., 1970; Jamieson et al., 1972a,b). This protein was found to increase in content in serum by about six-fold at 48-72 h after induction of inflammation (Jamieson et al., 1972b). The protein was characterized with respect to

its chemical, physical and immunological properties (Jamieson *et al.*, 1972a), and was found to have similar properties to those of human $\alpha_1$-acid glycoprotein, a major acute phase reactant in human serum, whose function has yet to be delineated.

Biosynthetic studies performed *in vivo* indicated that the microsome fraction of liver contained the subcellular sites of biosynthesis of both polypeptide and carbohydrate moieties of $\alpha_1$-acid glycoprotein (Jamieson and Ashton, 1972a). Subsequent quantitative studies revealed that the content of $\alpha_1$-acid glycoprotein associated with the microsome fraction was approximately four-fold greater in animals suffering from induced inflammation (Jamieson and Ashton, 1973a). Increased $\alpha_1$-acid glycoprotein associated with the microsome fraction was believed to result from a greater capacity for synthesis of $\alpha_1$-acid glycoprotein by liver in response to inflammation (Jamieson and Ashton, 1973a). The pathway of secretion of $\alpha_1$-acid glycoprotein from liver in both normal rats and those suffering from inflammation was the same, involving passage of the protein from rough to smooth endoplasmic reticulum and finally to the Golgi complex prior to its appearance in circulating blood (Jamieson and Ashton, 1973b).

The aim of the studies presented in this thesis was to determine the effect of induced inflammation on factors involved in the biosynthesis of the complex type N-linked carbohydrate chains normally found in serum glycoproteins, such as $\alpha_1$-acid glycoprotein. Due to elevated glycoprotein biosynthesis in liver during inflammation, the initial part of this study involved determining the effect of inflammation on the hepatic activities of galactosyl and sialyl transferase, which catalyze the addition of the penultimate and terminal sugars, respectively,

to complex type N-linked oligosaccharide chains. Galactosyl and sialyl transferase activities have also been detected in serum (Hudgin and Schachter, 1971; Kim et al., 1972; Podolsky and Weiser, 1975; Ip and Dao, 1978), and their activities have been found to increase in animals and humans suffering from neoplastic diseases (Kessel and Allen, 1975; Podolsky and Weiser, 1975; Weiser et al., 1976; Bauer et al., 1977). Since the liver is the main source of serum glycoproteins, it has been suggested that it is also the main source of serum glycosyl transferases, particularly sialyl transferase (Bernacki and Kim, 1977; Weiser et al., 1982). Therefore, serum sialyl and galactosyl transferase activities were determined in normal and inflamed rats. Comparisons were made with hepatic enzyme activities in order to determine the origin of the serum glycosyl transferase activities.

Due to the likely increased demand during inflammation for sugars for synthesis of the carbohydrate moieties of glycoproteins, such as $\alpha_1$-acid glycoprotein, hepatic nucleotide sugar pools were measured in normal and inflamed rats. In addition, the activities of key regulatory enzymes, L-glutamine:D-fructose-6-phosphate aminotransferase and UDP-GlcNAc 2-epimerase, and the nonregulatory enzyme, CMP-NeuAc synthase, were determined.

As mentioned previously, recent work in our laboratory has led to the isolation of $\alpha_1$-acid glycoprotein from rough endoplasmic reticulum which contained carbohydrate chains largely unprocessed with respect to mannose, which provided evidence for the last step of the dolichol cycle (Fig. 9, reaction 6) (Friesen and Jamieson, 1979, 1980). Since the synthesis of glycoproteins will depend on a functional dolichol cycle, it was also of interest to examine how the intermediates of the

dolichol cycle could be affected by inflammation when there is enhanced glycoprotein biosynthesis. Thus, experiments concerning the effect of inflammation on the synthesis of lipid-linked saccharides, *in vivo* and *in vitro*, were performed. Attempts were made to characterize isolated hepatic lipid-derived oligosaccharides from normal and inflamed rats by high resolution gel filtration chromatography; identification of the individual oligosaccharide species was made by comparison with known standards. Finally, the effect of inflammation on the activities of the microsome fraction processing enzymes, α-glucosidase and α-mannosidase, was determined.

It was hoped that the results from these studies would provide information to help in understanding the regulatory control of biosynthesis of carbohydrate chains of N-linked serum glycoproteins during the acute phase response.

# EXPERIMENTAL

## MATERIALS

### Radioactive Compounds

L-[4,5-$^{3}$H]Leucine (5 Ci/mmole), D-[6-$^{3}$H]glucosamine (29 Ci/mmole), D-[2-$^{3}$H]mannose (24.3 Ci/mmole), neuramin-[1-$^{3}$H]lactitol (52 mCi/mmole), NaB[$^{3}$H]$_4$ (347 mCi/mmole), D-[1-$^{14}$C]glucosamine (55 mCi/mmole), D-[1-$^{14}$C]mannose (61 mCi/mmole), cytidine 5'-monophosphate-[4,5,6,7,8,9-$^{14}$C]sialic acid (197-247 mCi/mmole), guanosine diphosphate-[U-$^{14}$C] mannose (166-269 mCi/mmole), uridine diphosphate-N-acetyl-D-[U-$^{14}$C] glucosamine (300 mCi/mmole), and uridine diphosphate-[U-$^{14}$C]galactose (340 mCi/mmole) were from Amersham Corp., Oakville, Ont., or New England Nuclear Corp., Lachine, P.Q.

### Sugars, Nucleotide Sugars and Nucleotides

N-Acetyl-D-galactosamine, N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, N-acetylneuraminic acid (Type IV), N-acetylneuraminlactose, D-galactosamine-HCl, D-galactose, D-glucosamine-HCl, D-glucose, D-glucose-6-phosphate (monosodium salt), lactose, maltooligosaccharide, D-mannose, myo-inositol, sucrose, p-nitrophenyl-α-D-glucopyranoside, p-nitrophenyl-α-D-mannoside, cytidine 5'-monophospho-N-acetylneuraminic acid, guanosine 5'-diphospho-D-mannose, uridine 5'-diphospho-N-acetylglucosamine, uridine 5'-diphosphogalactose, adenosine 5'-diphosphate, adenosine 5'-monophosphate, adenosine 5'-triphosphate, cytidine 5'-diphosphate, cytidine 5'-monophosphate, cytidine 5'-triphosphate, guanosine 5'-diphosphate, guanosine 5'-monophosphate, guanosine 5'-triphosphate, uridine 5'-diphosphate, uridine 5'-monophosphate, and uridine 5'-triphosphate were from Sigma Chemical Co., St. Louis, Mo.; all nucleotides were sodium salts; Dextran T70 from Pharmacia, Uppsala, Sweden.

Amino Acids and Proteins

Amino acids (L-configuration), bovine serum albumin, human $\alpha_1$-acid glycoprotein, pyruvate kinase and trypsin were from Sigma Chemical Co., St. Louis, Mo.; pronase B from Calbiochem, La Jolla, Ca.

Chromatographic and Electrophoretic Media

Sephadex G-200 and DEAE-Sephadex A-50 were from Pharmacia (Canada) Ltd., Montreal, P.Q.; CM-cellulose, Dowex 1X8 ($Cl^-$ form, 100-200 mesh) from Sigma Chemical Co., St. Louis, Mo.; Bio-Gel P-4 (minus 400 mesh), acrylamide, N,N'-methylene-bis-acrylamide (Bis), N,N,N',N'-tetra-methylene-diamine (TEMED), ammonium persulfate, and Coomassie Brilliant Blue R-250 from Bio-Rad Laboratories, Richmond, Ca.; 3% SP2330 on 100/120 Supelcoport, from Supelco Inc., Bellefont, Pa; Ampholine Carrier Ampholytes from LKB Produkter AB, Stockholm-Bromma 1, Sweden.

Chemicals

Trizma Base (Tris), imidazole, sodium cacodylate, 2(N-morpholino)-ethane sulfonic acid (MES), ethylenediaminetetraacetic acid (EDTA), dithiothreitol, Triton X-100, p-dimethylaminobenzaldehyde, penicillin G, streptomycin sulfate, puromycin dihydrochloride, and phosphoenol-pyruvate were from Sigma Chemical Co., St. Louis, Mo.; picric acid was from BDH, Poole, England; Noble agar from Difco Laboratories, Detroit, Mich.; 2-aminobiphenyl hydrogen oxalate, Pierce Chemical Co., Rockford, Il.; Aqueous Counting Scintillant (ACS) from Amersham Corp, Oakville, Ont.; turpentine oil (double rectified) from Fisher Scientific Co., Toronto, Ont. Other chemicals were of analytical grade obtained from local suppliers.

## METHODS

## Physical and Chemical Methods

### Determination of radioactivity

Radioactivity was determined with an LKB-Wallac RackBeta model 1215 liquid scintillation counter fitted with an automatic external standard which corrected for quenching to calculate disintegrations per minute (dpm). The LKB counter generated quench correction curves from data obtained using the Hat-Trick Calibration Kit 1210-126, Fisher Scientific Co., Toronto, Ontario. The channel gate setting for $^{14}C$ was 20-165; the gate setting for $^{3}H$ was 8-130. For double label counting of $^{3}H$ and $^{14}C$ the gates were set at 16-100 (channel 1) and 100-165 (channel 2), respectively. Aqueous solutions (up to 1 ml containing 3 mg protein or 0.2 ml aqueous solutions of nucleotide sugars, monosaccharides and oligosaccharides) were counted following the addition of 10 ml ACS cocktail. Radioactivity in sugar-lipid complexes (see below) was determined by first drying the samples under $N_2$ at 40 $^{o}C$, then adding 10 ml ACS cocktail. For measurement of radioactivity in sialyl and galactosyl transferase assays (see below), samples (up to 0.40 ml containing 0.5 mg protein) were spotted on 2.5 cm diameter discs of Whatman No. 1 filter paper and counted with 10 ml ACS as above. For measurement of radioactivity following separation of isomers of sialyllactose (see below) 0.5 x 6 cm strips of Whatman 3 MM filter paper cut from the chromatogram were counted with 10 ml ACS cocktail as above, or with 4 ml ACS cocktail using Omni-Vials (Wheaton Scientific Co., Millville, N.J.). Paper chromatograms were scanned for radioactivity using a Packard radiochromatogram scanner model 7220.

Gas liquid chromatography of alditol acetate derivatives of sugars

Gas liquid chromatography of alditol acetate derivatives of sugars (prepared as described below) was performed using a Perkin-Elmer Sigma 2B gas liquid chromatograph fitted with a flame ionization detector. Glass columns 1.8 m long and 2 mm I.D. were packed with 3% SP2330 on 100/120 Supelcoport. The temperature program was set for 6 min at 180 $^{o}C$ followed by 2 min at 220 $^{o}C$ at a rate of 1 $^{o}C$/min, and finally for 15 min at 250 $^{o}C$ at a rate of 5 $^{o}C$/min. Injection port temperature was 220 $^{o}C$ and the detector temperature was 285 $^{o}C$. The carrier gas was $N_2$ at a flow rate of 20 ml/min. Peak areas were determined by a Perkin-Elmer M-2 integrator.

Alditol acetate derivatives of sugars were prepared using a method based on that described by Torello _et al_. (1980). Reactions were performed in 1 ml Reacti-vials (Pierce Chemical Co., Rockford, IL.). Sugars were reduced by adding 0.5 ml of a solution containing 2 mg/ml $NaBH_4$ in 1 M ammonium hydroxide and letting stand for 1 h at room temperature. Glacial acetic acid was added dropwise to destroy excess $NaBH_4$ and the samples were taken almost to dryness under $N_2$ at 65 $^{o}C$. Boric acid was removed as methyl borate by addition of 0.85 ml methanol:benzene (5:1, v/v), heating at 90 $^{o}C$ for 5 min and then evaporating to dryness under $N_2$ at 65 $^{o}C$; the methanol:benzene mixture was then replaced with anhydrous methanol and the procedure was repeated five times. Acetylation was performed by addition of 0.75 ml acetic anhydride and heating at 100 $^{o}C$ for 70 min; the samples were taken to dryness at 38 $^{o}C$ under $N_2$. Samples were desalted by adding 0.5 ml chloroform and partitioning against 5 x 0.5 ml water; chloroform was removed at 38 $^{o}C$ under $N_2$. The residue was

then dissolved in 10-30 μl chloroform and stored in vacuo over $P_2O_5$ until required. Samples of 1-2 μl were analyzed by gas liquid chromatography as described above.

Polyacrylamide gel electrophoresis

Discontinuous polyacrylamide disc gel electrophoresis was performed on basic gels as described by Davis (1964), using a Bio-Rad model 155 electrophoresis cell. The resolving gel consisted of 6% acrylamide and 0.16% bisacrylamide in 0.75 M Tris, pH 8.9. The stacking gel consisted of 3% acrylamide and 0.08% bisacrylamide in 0.10 M Tris, pH 6.7. Aqueous protein samples were mixed with an equal volume of 0.20 M Tris/10% glycerol/0.1% bromophenol blue, pH 6.7, and 30-60 μl samples applied to the tops of the gels. Electrophoresis tank buffer was 5 mM Tris-37 mM glycine, pH 8.3. A potential was applied to maintain a current of 2-3 mA/gel with 5.5 mm diameter tube gels. Electrophoresis was performed at 4 $^{o}$C and continued until the tracking dye migrated 90% of the length of the gel or 6 cm.

Isoelectric focusing

For isoelectric focusing, a 110 ml isoelectric focusing column (LKB 8100-10) and a gradient mixing device (LKB 8121) obtained from LKB Produckter AB, Stockholm-Bromma 1, Sweden, were used. The temperature was maintained at 2 $^{o}$C by circulating 10% ethanol from a Haake FKN Circulating Bath with a Haake 4391 Line Heater (Haake, Karlsruhe, West Germany) through the cooling jacket of the column. Isoelectric focusing of $\alpha_1$-acid glycoprotein was performed on a pH 1-3 gradient as described by Jamieson *et al*. (1972a) as a final purification step of the protein isolated from rat serum (see below). A

dense electrode solution containing 0.15 ml concentrated sulfuric acid, 16 ml glycerol, and 4 ml distilled water was added to the anode at the bottom of the column. A glycerol gradient containing the pH 1-3 gradient system and 5-8 carrier ampholytes was slowly introduced into the column using the LKB gradient device. The dense gradient solution contained 0.1 g monochloroacetic acid, 0.1 g dichloroacetic acid, 0.1 g orthophosphoric acid, 35 ml glycerol, and 20 ml distilled water. The light gradient solution contained 0.1 g acetic acid, 0.1 g formic acid, 0.05 g aspartic acid, 0.05 g glutamic acid, 0.30 ml Ampholine Carrier Ampholytes, pH 5-8, and 60 ml distilled water. After about 50 ml of the gradient had entered the column, the sample, 30 mg protein, dissolved in 1 ml distilled water was added to the light gradient solution. When the column had filled, the light electrode solution consisting of 0.1 M sodium hydroxide was added to the cathode at the top of the column. During isoelectric focusing, the potential applied to the column was increased from 150 V to 600 V while keeping the current below 15 mA. The column was run until the current dropped below 1 mA. Fractions of 1 ml were collected. The material absorbing at 280 nm and which focused at pH 2.95 was pooled, dialyzed exhaustively against water, and lyophilized.

Preparation of Dowex ion-exchangers

In all cases, Dowex 1-chloride form resin was washed with 3 x 20 volumes of 1 M hydrochloric acid, then exhaustively with deionized water before use. Dowex 1-carbonate was prepared from Dowex 1-chloride by successive treatments with 20 volumes of 1 M sodium hydroxide and 1 M sodium carbonate. After each treatment the resin was washed with

20 volumes of deionized water. Dried Dowex 1-carbonate and dried Dowex 50-hydrogen were prepared immediately before use by washing with deionized water, then with acetone and air-dried (Spivak and Roseman, 1966). Dowex 1-phosphate was prepared according to the method of Paulson et al. (1977). Dowex 1-phosphate was prepared from Dowex 1-chloride by successive treatments with 20 volumes of 1 M sodium hydroxide, 1 M phosphoric acid, and 1 M sodium phosphate buffer, pH 6.8. After each treatment, the resin was washed with 20 volumes of deionized water.

Ultrafiltration and Other Methods

Concentration of protein solutions by ultrafiltration was performed with Amicon stirred cells fitted with UM10 or PM10 ultrafiltration membranes from Amicon Canada Ltd., Oakville, Ont. Routine and concentration dialysis against buffer or deionized water was performed at 4 $^{o}$C using Spectrapor dialysis membranes from Spectrum Medical Industries Inc., Los Angeles, Ca.

Absorbances in the visible and ultraviolet (UV) regions of the spectrum were measured with a Carl Zeiss PMQ 2 spectrophotometer. Measurements of pH were made with a Radiometer model 28 pH meter or a Fisher Accumet model 610A pH meter. Amino sugars were analyzed using an NC-2P Technicon Amino Acid Analyzer System, Technicon Instruments, Tarrytown, N.Y. Protein was assayed as described by Lowry et al. (1951), but with modified reagents and volumes described by Miller (1959); bovine serum albumin was used as the standard.

Immunological Methods

Antisera to rat $\alpha_1$-acid glycoprotein and rat serum proteins were prepared as described by Simkin *et al*. (1964b) using male full lop albino rabbits. An emulsion of 0.75 mg antigen, 0.25 ml sterile 0.15 M sodium chloride and 0.4 ml Freund's complete adjuvant was injected intramuscularly, one-half of the dose into each thigh of the rabbit. Six days later, a second preparation containing 1.25 mg antigen with Freund's complete adjuvant was injected as before. After a further 22 days, a series of intravenous injections of a coprecipitate of antigen with aluminum hydroxide was given. To prepare the coprecipitate, 0.1 ml 1 M sodium bicarbonate was added to neutralize a mixture of 0.4 ml 10% (w/v) potassium alum ($Al_2(SO_4)_3$, $K_2SO_4 \cdot 24H_2O$) and 6 mg antigen in 0.2 ml water. The neutralized solution was stored at 2 $^{o}$C for 16 h after which the precipitate was resuspended in 0.4 ml 0.15 M sodium phosphate buffer, pH 7.4, containing 0.01% thimerosal. Samples of 0.05 ml, 0.10 ml, and 0.25 ml of this suspension were injected intravenously on alternate days. The rabbits were bled 5 days after the final injection. The blood was allowed to clot at room temperature for 1 h, centrifuged at 2000 rpm (3000 $g_{av}$) for 20 min, and the cleared supernatant taken as antiserum. Samples of antiserum were stored at -20 $^{o}$C until required.

Double diffusion analyses of test samples and antisera were performed as described by Ouchterlony (1953). The medium contained 1.25% Noble agar, 0.15 M sodium chloride, and 0.01% sodium azide. Gels were 2 mm thick and wells were cut using a Bio-Rad Template System and Universal Puncher (Bio-Rad Laboratories, Richmond, Ca.). Plates were allowed to develop at room temperature in a humidity chamber.

Isolation of β-Galactosidase from Jack Bean Meal

The isolation of β-galactosidase from Jack Bean Meal (Sigma Chemical Co., St. Louis, Mo.) was based on a method described by Li and Li (1972). A suspension of Jack Bean Meal in 3 l deionized water was stirred at room temperature for 1 h. The suspension was strained through several layers of cheesecloth and the turbid filtrate adjusted to pH 5.5 with 1.5 M sodium citrate buffer, pH 2.7. The solution was then centrifuged at 5000 rpm (4080 $g_{av}$) for 30 min in the Sorvall RC2-B centrifuge fitted with the GSA rotor. The volume of the supernatant was measured and solid ammonium sulfate was added to 30% saturation. After standing at room temperature for 2 h, the precipitate was removed by centrifugation as above. Solid ammonium sulfate was again added to the supernatant to 60% saturation. The solution was allowed to stand overnight at 4 $^{o}$C, then centrifuged as above. The supernatant was aspirated and discarded; the precipitate was dissolved in 125 ml 0.1 M sodium phosphate buffer, pH 7.0. To this crude enzyme fraction, 95% ethanol was added dropwise at room temperature to a final concentration of 25%. The mixture was cooled to -10 $^{o}$C and centrifuged at -10 $^{o}$C as above. The supernatant was warmed to room temperature and adjusted to pH 4.9 with 1.5 M sodium citrate buffer, pH 2.7. The mixture was cooled to -10 $^{o}$C, kept at this temperature overnight, then centrifuged at -10 $^{o}$C as above. The precipitate was dissolved in 10 ml 0.1 M sodium phosphate buffer, pH 7.0. This fraction was applied to a 5 x 50 cm column of Sephadex G-200 equilibrated with 0.1 M sodium phosphate buffer, pH 7.0. The column was eluted with the same buffer. The fractions containing β-galactosidase activity (as determined using the p-nitrophenyl assay

system - see below) were pooled and precipitated by reverse dialysis against saturated ammonium sulfate at 4 $^{o}$C overnight. The precipitate, obtained by centrifugation as previously described, was dissolved in 10 ml 0.005 M sodium phosphate buffer, pH 7.0, to give a β-galactosidase-rich fraction.

This fraction was dialyzed exhaustively against 0.05 M sodium phosphate buffer, pH 7.0, and then applied to a 5 x 30 cm column of DEAE-Sephadex A-50 which had been equilibrated with 0.05 M sodium phosphate buffer, pH 7.0. β-Galactosidase was eluted from the column with starting buffer. The fractions containing only β-galactosidase activity were pooled, the volume reduced by concentration dialysis against 0.05 M sodium phosphate buffer, pH 7.0, and stored at -20 $^{o}$C until required. The solution of β-galactosidase was free of other glycosidase activities as determined with various p-nitrophenyl glycosides as substrates as described by Li and Li (1972).

## Treatment of Rats for Preparation of Serum and Liver

Male Long-Evans hooded rats of 200-250 g body weight were purchased locally and were maintained on a diet of Purina Laboratory Chow and tap water *ad libitum*; the animals were maintained under constant light conditions. Inflammation was induced by a subcutaneous injection of 0.5 ml turpentine per 100 g body weight into the dorsolumbar region as described by Ashton *et al*. (1970); rats used as controls received injections of sterile 0.15 M sodium chloride. Rats were starved for 16 h prior to sacrifice which was between 9 and 11 a.m. to minimize any changes that might occur due to diurnal effects. Rats were killed by a blow on the head and blood was collected by severing the jugular veins. Blood was allowed to clot for 10 min at room temperature

and serum was prepared by centrifuging at 5000 $g_{av}$ for 20 min in a bench centrifuge. Unless otherwise stated, livers were perfused *in situ via* the portal vein with ice-cold 0.15 M sodium chloride, rapidly excised and transferred to beakers containing ice-cold 0.15 M sodium chloride.

For *in vivo* studies on the incorporation of radioactive sugars into hepatic sugar-lipid complexes and protein (see below), rats received intravenous injections of radioactive sugars in 0.2 ml 0.15 M sodium chloride into the femoral vein.

## Isolation of $\alpha_1$-Acid Glycoprotein from Rat Serum

Rat serum $\alpha_1$-acid glycoprotein was prepared by a method based on the procedures of Simkin *et al.* (1964a) and Jamieson *et al.* (1972a). Rats suffering from inflammation for 48 h were used since serum levels of $\alpha_1$-acid glycoprotein were highest at this time after inflammation (Jamieson and Ashton, 1973a). Volumes of 50-100 ml serum were diluted with 9 volumes of 0.15 M sodium chloride and the solution stirred rapidly with a magnetic stirrer at room temperature. The solution was then made to 0.6 M perchloric acid by dropwise addition of 1.8 M perchloric acid, allowed to stand for 10 min, and then centrifuged at 5000 rpm (4080 $g_{av}$) for 20 min at 4 $^{o}$C in the Sorvall RC2-B centrifuge fitted with the GSA rotor. The supernatant, referred to as the perchloric acid-soluble (PCA-soluble) fraction, was decanted and neutralized to pH 6.5 with 2 M sodium hydroxide. The solution was dialyzed exhaustively against water, concentrated by ultrafiltration using a PM10 Amicon membrane, and lyophilized. The $\alpha_1$-acid glycoprotein in the PCA-soluble fraction was further purified by ion-exchange chromatography on CM-cellulose. CM-cellulose was suspended

in 0.05 M sodium acetate buffer, pH 4.9, packed into a 2.5 x 45 cm column, and equilibrated with the same buffer. A sample of 250 mg PCA-soluble fraction was dissolved in 10 ml equilibrating buffer, applied to the column, and eluted with the equilibrating buffer at a flow rate of 20 ml/h. Protein was detected in the eluate by monitoring the absorbance at 254 nm with an LKB Uvicord II. Material that absorbed at 254 nm was pooled, dialyzed against water, lyophilized, and designated fraction CMC-1. $\alpha_1$-Acid glycoprotein, the major component in CMC-1, was further purified by isoelectric focusing as previously described. This material was pure $\alpha_1$-acid glycoprotein as judged by double diffusion analysis against anti-whole rat serum and anti-$\alpha_1$-acid glycoprotein as previously described.

Preparation of Asialo- and Asialoagalacto-$\alpha_1$-Acid Glycoprotein.

For the preparation of asialo-$\alpha_1$-acid glycoprotein, 36 mg of rat or human $\alpha_1$-acid glycoprotein was dissolved in 6.0 ml 0.025 M sulfuric acid and heated in a glass-stoppered tube at 80 $^{o}$C for 1 h. The hydrolysate was dialyzed exhaustively against distilled water, and then lyophilized.

For the preparation of rat asialoagalacto-$\alpha_1$-acid glycoprotein, 20 mg of asialo-$\alpha_1$-acid glycoprotein, prepared as above, was dissolved in 6.0 ml sodium citrate buffer, pH 3.5, containing 3 units of β-galactosidase activity (see below for definition of a unit of glycosidase activity). The solution was incubated at 37 $^{o}$C for 24 h. The release of galactose was determined quantitatively by the method of Gordon _et al._ (1956). This method involved collecting aliquots of the hydrolysate mixture at several times during the incubation and spotting each sample on Whatman 3 MM paper along with known amounts (up to 40 μg) of galactose.

The chromatogram was then developed by descending chromatography with isopropanol:pyridine:water:glacial acetic acid (8:8:4:1, v/v) for 5 h. The chromatogram was air-dried, sprayed with 0.1 M 2-aminobiphenyl hydrogen oxalate (1.69 g 2-aminobiphenyl and 1.26 g oxalate dihydrate dissolved in a solution of 5 ml glycerol, 10 ml water and 84 ml acetone), and placed in an oven at 110 $^{o}C$ for 5 min for color development. Galactose appeared as yellow-brown spots. These spots were cut out, eluted with 5 ml methanol, and the absorbances read at 400 nm.

Preparation of Liver Slices

Liver slices were prepared according to the method of Jamieson et al. (1975). Liver slices were cut by hand on a template which had a groove 7 mm wide and 0.36 mm deep (Hultin et al., 1960; Simkin and Jamieson, 1967). Ice-cold 0.15 M sodium chloride was used as medium during preparation of slices. Slices were transferred to beakers containing ice-cold 0.15 M sodium chloride and washed by gentle agitation for 20 min. The sodium chloride solution was then replaced with ice-cold incubation medium, which had been purged with $O_2/CO_2$ (95:5) for 1 h; washing was continued for 10 min. The washing procedure with incubation medium was repeated twice. The composition of the incubation medium was based on that described by Marsh and Drabkin (1958), and contained: 77 mM KCl, 39 mM NaCl, 32.5 mM $NaHCO_3$, 3.1 mM $MgSO_4$, 0.6 mM $KH_2PO_4$, 1.3 mM $CaCl_2$, and 25 mM glucose; 25 mg penicillin G and 25 mg streptomycin sulfate were added per litre. Samples of 1-5 g wet weight slices were transferred to 50 ml or 100 ml Erlenmeyer flasks and incubated under a constant flow of $O_2/CO_2$ (95:5) for 1-6 h at 37 $^{o}C$ with gentle shaking. Each flask contained 5 ml of medium and 0.1 ml

of a stock amino acid solution per gram of slices. The final concentration of each L-amino acid present in each flask was approximately twice that present in blood (Clemens and Korner, 1970; Woloski et al., 1983b). These concentrations (in µmoles per 100 ml) were: alanine, 96; arginine, 44; asparagine, 7.6; aspartic acid, 7.6; cysteine, 7.6; glutamic acid, 3.8; glutamine, 38; glycine, 86; histidine, 18; isoleucine, 18; leucine, 34; lysine, 96; methionine, 14; phenylalanine, 16; proline, 48; serine, 48; threonine, 58; tryptophan, 13.8; tyrosine, 18; valine, 40. In some experiments radiolabeled sugars and amino acids were added. Other additions (see Results) included tunicamycin, proteases, puromycin, cycloheximide, and colchicine. After incubation, samples were chilled in ice, and the medium was aspirated and then centrifuged at 40000 rpm (106000 $g_{av}$) for 1 h in a Beckman L5-50 ultracentrifuge equipped with a 50 Ti rotor. The clear supernatant was either used immediately for determination of enzyme activity (see below) or stored at -20 $^{o}$C until required. Liver slices were washed three times with 5 ml ice-cold 0.25 M sucrose and then subjected to subcellular fractionation or homogenized in 15 ml ice-cold 0.25 M sucrose and used directly (see below).

Subcellular Fractionation

Unless otherwise stated, a Beckman L5-50 ultracentrifuge equipped with 50 Ti and 60 Ti fixed angle rotors and an SW27.1 swinging bucket rotor was used for all subcellular fractionation procedures. Livers were homogenized in a Potter-Elvehjem homogenizer fitted with a motor-driven polytetrafluoroethylene pestle (Jamieson and Ashton, 1973); the pestle rotated at 2000 rpm. Unless otherwise stated, livers were previously perfused with ice-cold 0.15 M sodium chloride as described

above. All procedures were performed at 0-4 $^{o}$C.

Preparation of microsome fraction and cell sap

Liver was homogenized with 3 volumes of 0.25 M sucrose using eight up and down strokes of a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 13500 rpm (10000 $g_{av}$) for 20 min in a 60 Ti rotor. The supernatant was aspirated and centrifuged at 40000 rpm (112000 $g_{av}$) for 90 min in a 60 Ti rotor. The supernatant or cell sap fraction was aspirated and collected; the pelleted material consisted of the microsome fraction.

In some experiments cell sap was concentrated tenfold using an Amicon ultrafiltration cell fitted with a PM10 membrane and then reconstituted to its original volume with 0.25 M sucrose. This was done to remove endogenous small molecular weight constituents to produce a preparation rich in the high molecular weight components.

For experiments on measurement of hepatic nucleotide sugars or enzymes of nucleotide sugar metabolism, cell sap was prepared by a method based on that of Bley *et al*. (1973). Livers were perfused *in situ* with ice-cold 0.154 M KCl-1 mM EDTA adjusted to pH 7.5 with sodium hydroxide, followed by homogenization with 2 volumes of perfusion buffer using five up and down strokes of a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 13000 rpm (10000 $g_{av}$) for 10 min in the Sorvall RC2-B centrifuge fitted with the SS-34 rotor. The supernatant was removed and centrifuged at 40000 rpm (106000 $g_{av}$) for 90 min in a 50 Ti rotor. The supernatant or cell sap fraction was collected. For assay of glucosamine-6-phosphate synthase activity (see below) homogenates were fractionated as above, but the homogenizing medium also contained 12 mM glucose-6-phosphate to prevent conversion

of fructose-6-phosphate to glucose-6-phosphate by phosphoglucose isomerase (Bley *et al*., 1973; Miyagi and Tsiuki, 1971).

Preparation of rough and smooth membrane fractions

The fractionation procedure for the preparation of hepatic rough and smooth membrane fractions was a modification of that described by Dallner (1963). A 60 Ti rotor was used throughout. Liver was homogenized with 3 volumes of 0.25 M sucrose using eight up and down strokes of a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 13500 rpm (10000 $g_{av}$) for 20 min to remove large granular material; CsCl was added to the supernatant to a final concentration of 15 mM CsCl and 15 ml samples layered on 10 ml 1.3 M sucrose containing 15 mM CsCl and centrifuged at 40000 rpm (112000 $g_{av}$) for 150 min. The sediment was taken as the rough membrane fraction. To prepare a smooth membrane fraction, material at and above the interface formed between the 0.25 M and 1.3 M sucrose solutions was aspirated and $MgCl_2$ added to a final concentration of 10 mM; an equal volume of 0.25 M sucrose was added and the solution centrifuged at 40000 rpm (112000 $g_{av}$) for 150 min. Both rough and smooth membrane fractions were resuspended in 20 ml 0.15 M Tris-HCl buffer, pH 8.0, containing 5 mM $MgCl_2$ and centrifuged at 40000 rpm (112000 $g_{av}$) for 90 min. This step was included to minimize contamination by pyrophosphatase (Dallner, 1974). The sedimented material was suspended by gentle homogenization in an appropriate volume of deionized water.

Preparation of Golgi-enriched fraction

Golgi-enriched fractions were prepared according to the methods of Schachter *et al*. (1970) and Hudgin *et al*. (1971). Liver was

homogenized with 4 volumes of 0.5 M sucrose in medium A (0.1 M Tris-HCl, pH 7.6; 0.01 M $MgCl_2$; 1% Dextran T70); only five up and down strokes of the homogenizer were employed. Aliquots of 8 ml were layered on a discontinuous sucrose density gradient consisting of (from bottom to top) 10 ml 1.7 M sucrose in medium A, 8 ml 1.3 M sucrose in medium A, and 8 ml 0.7 M sucrose in medium A. Centrifugation was at 24000 rpm (70000 $g_{av}$) for 45 min in an SW27.1 rotor. The Golgi-enriched fraction at the interface between the 0.7 M and 1.3 M sucrose layers was collected, diluted four-fold with 0.25 M sucrose, and centrifuged at 40000 rpm (112000 $g_{av}$) for 90 min in a 60 Ti rotor. The supernatant was removed and the sedimented Golgi-enriched fraction was resuspended by gentle homogenization in an appropriate volume of deionized water.

Preparation of lysosome - enriched fraction

The fractionation procedure for the preparation of a lysosome-enriched fraction was performed according to the method of Kaplan and Jamieson (1977). Liver was homogenized with 3 volumes of 0.25 M sucrose using five up and down strokes of a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 2000 rpm (500 $g_{av}$) for 10 min in a Sorvall RC2-B centrifuge fitted with an SS-34 rotor. A maximum amount of supernatant was removed and the lysosome - enriched fraction was prepared by centrifuging at 22000 rpm (33000 $g_{av}$) for 7.5 min in a 60 Ti rotor. The supernatant was removed and the sedimented material was washed by resuspension in 10 ml 0.25 M sucrose and centrifuging as above. The pellet of lysosome - enriched material was resuspended in 10 ml 0.25 M sucrose.

Incorporation of Radioactive Sugars into Sugar-Lipid Complexes and Protein.

Incorporation of radioactive sugars into sugar-lipid complexes and protein derived from hepatic rough membrane fractions was studied *in vivo* and in two types of *in vitro* assays, one with and one without a cell-free protein synthesizing system.

In vivo studies

For *in vivo* incorporation studies, rats were given injections of 10 μCi [$^{14}$C]mannose and 20 μCi [$^{3}$H]glucosamine as previously described. Rats were sacrificed at appropriate times and hepatic rough membrane fractions prepared as previously described.

In vitro studies

Two types of *in vitro* systems were used. For incorporation studies in absence of a cell-free protein synthesizing system (see below), the assay was that described by Nilsson *et al*. (1978). The incubation mixture contained (in a total volume of 400 μl): 30 mM Tris buffer, pH 7.8; 10 mM $MnCl_2$; 4 mM AMP; 2.5 mM EDTA; 25 μM UDP-GlcNAc; 1.0 nmole GDP-[$^{14}$C]Man (80 mCi/mmole); and, 1.5 mg rough fraction protein. In some experiments, Triton X-100 (0.1% final concentration) was added. Incubations were performed at 30 $^{o}$C for up to 30 min. For incorporation studies in presence of a cell-free protein synthesizing system, a method based on that described by Simkin and Jamieson (1967) was used. The incubation mixture contained (in a total volume of 400 μl): 50 mM Tris buffer, pH 7.8; 50 mM KCl; 5 mM $MgCl_2$; 2.5 mM $MnCl_2$; 2.5 mM dithiothreitol; 0.1% Triton X-100; 2 mM ATP; 0.5 mM GTP; 25 μg pyruvate kinase (400 units/mg); 25 mM phosphoenolpyruvate; 25 μM UDP-GlcNAc; 0.4-0.6 nmole GDP-[$^{14}$C]Man (80-269 mCi/mmole); 0.4 nmole [$^{3}$H]leucine

(5 Ci/mmole); 1.0 mg cell sap protein and 1.6 mg rough membrane fraction protein. In some experiments, part of the cell sap was replaced with a high molecular weight-enriched fraction of cell sap prepared as previously described. Incubations were at 25 $^{o}$C for up to 120 min.

Extraction of Sugar-Lipid Complexes from Liver

For *in vivo* and *in vitro* studies on the incorporation of radioactive sugars into sugar-lipid complexes and protein in hepatic rough membrane fractions, an extraction procedure based on that described by Nilsson *et al*. (1978) was used. The procedure is outlined in Fig. 12. This procedure provides for the sequential extraction of Man-P-Dol and $(GlcNAc)_{1-2}$-P-P-Dol, termed Lipid I, oligosaccharide-P-P-Dol ($(Glc)_{0-3}$-$(Man)_{1-9}$-$(GlcNAc)_2$-P-P-Dol), termed Lipid II, and protein (Nilsson *et al*., 1978; Ronin and Bouchilloux, 1978; Coolbear *et al*., 1979; Ravoet *et al*., 1981) from hepatic rough membrane fractions.

For the isolation of oligosaccharide-lipid complexes from whole livers or liver slices for the purpose of chromatographic analysis (see below), the following procedure was employed. Microsome fractions or rough membrane fractions, prepared from 5-8 g liver as previously described, were homogenized with 20 ml chloroform:methanol (2:1, v/v), allowed to stand at room temperature for 15 min, centrifuged, and the supernatant discarded. This step was repeated twice. The pellet was then homogenized with 20 ml chloroform:methanol:water (1.0:1.0:0.3, v/v/v) centrifuged, and the supernatant collected. This step was repeated twice. The supernatants were pooled, then rotary evaporated to dryness to collect the oligosaccharide-lipid complexes.

Figure 12. Procedure for extraction of sugar-lipid complexes and protein from hepatic rough membrane fractions. See text for compositions of Lipid I and Lipid II. Abbreviation: MeOH, methanol.

Incubation stopped with 3 ml $CHCl_3$:MeOH (2:1, v/v) incubate at 40 °C for 20 min and centrifuge

upper layer discarded; lower $CHCl_3$ layer washed with 1.5 ml "upper phase" -- $CHCl_3$:MeOH:$H_2O$ (3:48:47, v/v/v) -- and centrifuged

upper layer discarded; "$CHCl_3$" layer transferred to a new tube

insoluble material washed 3 times with 1.5 ml "upper phase" as above

pellet extracted twice with 3 ml $CHCl_3$:MeOH (2:1, v/v) as above —— pool extracts → Lipid I

pellet extracted 3 times with $CHCl_3$:MeOH:$H_2O$ (1.0:1.0:0.3, v/v/v) —— pool extracts → Lipid II

pellet washed 5 times with $H_2O$; protein dissolved in 1 ml 1 M NaOH at 55 °C for 2 h

"$CHCl_3$" extract washed 4 times with "upper phase" as above

Lipid I
- solvent evaporated under $N_2$ at 40 °C

Lipid II
- solvent evaporated under $N_2$ at 40 °C

Labeling and Chromatographic Analysis of Oligosaccharides Derived from Oligosaccharide-Lipid Complexes.

Labeling of hepatic lipid-derived oligosaccharides was performed either by reduction with $NaB[^3H]_4$ or by incorporation of radioactive sugars.

For treatment with $NaB[^3H]_4$, oligosaccharide-lipid complexes were isolated from liver as described above and the oligosaccharide released by dilute acid hydrolysis as described by Turco and Robbins (1979). This involved dissolving the oligosaccharide-lipid complexes in a few drops of n-propyl alcohol followed by the addition of 1.0 ml 0.02 M hydrochloric acid and heating at 100 $^oC$ for 20 min. After cooling, an equal volume of chloroform was added to partition the lipid. The upper aqueous phase was transferred to a 1 ml screw-top vial and dried under $N_2$ at 60 $^oC$. The oligosaccharides were then treated with $NaB[^3H]_4$ according to the method of Li and Kornfeld (1978). A 0.4 ml volume of 0.1 M sodium hydroxide containing 120 nmoles $NaB[^3H]_4$ (223 mCi/mmole) was added to each sample. Each vial was capped, vortexed, and allowed to stand at room temperature for 3 h followed by an additional hour at 30 $^oC$. Glacial acetic acid was added dropwise to destroy excess $NaBH_4$. The samples were dried under $N_2$ at 60 $^oC$. Boric acid was removed as methyl borate by repeated evaporations (five times) with methanol.

Radioactive sugars were incorporated in a liver slice system. Liver slices, 5 g, were incubated for 2 h as described above. Either 20 μCi [$^{14}$C]mannose and 50 μCi [$^3$H]glucosamine, or 50 μCi [$^3$H]mannose and 10 μCi

$[^{14}C]$glucosamine, were added to each flask at the start of the incubation and again after 1 h. In some experiments, the incubation medium contained 0.1 mM puromycin. Oligosaccharide-lipids were isolated and oligosaccharides prepared by mild acid hydrolysis as described above.

The radiolabeled oligosaccharides were analyzed by gel filtration chromatography. The oligosaccharides were dissolved in 0.25 ml 0.5 M acetic acid and applied to a 1 x 210 cm column of Bio-Gel P4 (minus 400 mesh) equilibrated with 0.5 M acetic acid. A flow rate of 4 ml/h was maintained by use of a peristaltic pump. Fractions of 0.4 ml (8 drops) were collected. Aliquots of 100-200 μl were removed for determination of radioactivity.

Isolation and Analysis of Hepatic Nucleotide Sugars

Nucleotide sugars were isolated from rat liver by a procedure involving precipitation of protein with picric acid as described by Stein and Moore (1954) followed by chromatography on Dowex 1-chloride resin as described by Molnar *et al*. (1964). Samples of 1 g wet weight liver were homogenized with 10 ml 1% picric acid using 10 strokes of a Potter-Elvehjem homogenizer at 2000 rpm. Homogenates were then centrifuged at 6500 rpm (5000 $g_{av}$) for 10 min in a Sorvall RC2-B centrifuge fitted with an SS-34 rotor. In some experiments $[^{14}C]$-labelled nucleotide sugars were added (typically 0.1 nmol, 30 nCi) prior to homogenization to test for recoveries following ion-exchange chromatography. Samples of 8 ml supernatants were applied to 1.2 x 4 cm columns of Dowex 1-chloride which had previously been equilibrated with 1 M hydrochloric acid and washed exhaustively with deionized water. Columns were then eluted with 40 ml 0.04 M hydrochloric acid to remove amino acids, neutral sugars and amino sugars (Molnar *et al*.,

1964; Woloski et al., 1983b) and then with 40 ml 0.25 M hydrochloric acid to elute nucleotide sugars. Based on recoveries from radioactivity, not less than 90% of the nucleotide sugars in the homogenates were eluted with 0.25 M hydrochloric acid. The 0.25 M hydrochloric acid effluent was taken to dryness by rotary evaporation and used for analyses of UDP-N-acetylhexosamines, UDP-Gal and GDP-Man (see below). CMP-NeuAc was analyzed (see below) from a high-speed supernatant of 4 g liver prepared as described above.

The dried 0.25 M HCl effluents from Dowex columns (see above) were redissolved in 2 ml water; 1.5 ml were removed for analysis of UDP-N-acetylhexosamines and the remainder was used for analysis of GDP-Man and UDP-Gal. For analysis of UDP-GlcNAc and UDP-GalNAc, the sample was adjusted to 2 M hydrochloric acid by addition of 1.5 ml 4 M hydrochloric acid and hydrolysed in vacuo at 100 $^{o}$C for 3 h to release free amino sugars (Molnar et al., 1964); samples were dried in a desiccator in vacuo over NaOH. Samples were dissolved in 1 ml water and appropriate volumes analyzed in a NC-2P Technicon Amino Acid Analyzer System. To test for recovery after hydrolysis, samples of 100 nmoles UDP-GlcNAc, UDP-GalNAc, glucosamine-HCl, galactosamine-HCl, either alone or as mixtures, were analyzed as above. Recoveries were not less than 90%.

For analyses of GDP-Man and UDP-Gal, 0.5 ml 0.04 M hydrochloric acid and 100 nmoles myo-inositol were added to the 0.5 ml sample of the 0.25 M hydrochloric acid column effluent that remained after removal of a sample for hexosamine analyses (see above). Nucleotide sugars were hydrolyzed at 100 $^{o}$C for 20 min to release free sugars (Strominger, 1962). The samples were applied directly to columns of

Dowex 1-chloride as above and neutral sugars eluted with 40 ml 0.04 M hydrochloric acid; the effluent was taken almost to dryness by rotary evaporation, transferred to 1 ml Reacti-vials (Pierce Chemical Co., Rockford, IL.), dried under $N_2$ at 65 $^{o}$C and the sugars analyzed by gas liquid chromatography as described above.

Analysis of CMP-NeuAc was performed on hepatic cell sap, prepared according to Bley et al. (1973) as described above, using a method based on that described by van den Eijnden and van Dijk (1972). Samples of 38 nCi CMP-[$^{14}$C]NeuAc (247 mCi/mmole) were added to 1 ml volumes of high-speed supernatant in ice and the entire solution was applied to a 0.5 x 40 cm origin on Whatman 3 MM paper (46 x 57 cm) and descending chromatography performed at 4 $^{o}$C for 24 h using 95% ethanol: 0.6 M $NH_4OH$ (7:3, v/v) as solvent. The band containing CMP-NeuAc was detected under UV light and in some experiments by radiochromatogram scanning. Under the conditions used CMP-NeuAc was well separated from NeuAc. The CMP-NeuAc was eluted from the chromatogram with water and the eluate lyophilized. The eluate typically contained 90% of the radioactivity applied to the chromatogram. The lyophilized material was dissolved in 0.5 ml water and suitable volumes removed for counting and determination of NeuAc by the thiobarbituric acid method of Warren (1959). This method includes a hydrolysis step which releases NeuAc from CMP-NeuAc in situ. This involved removal of a 0.2 ml sample to which was added 0.1 ml 0.2 M sodium metaperiodate in 9 M phosphoric acid. The mixture was allowed to stand for 20 min. A 1 ml volume of 10% sodium arsenite in 0.5 M sodium sulfate-0.1 M sodium hydroxide was added and the mixture vortexed until the yellow-brown color disappeared. A sample of 3 ml 0.6% thiobarbituric acid in 0.5 M sodium sulfate was added to each tube and the mixture was heated in a boiling water bath

for 15 min, cooled, and the chromophore extracted with 4.3 ml cyclohexanone. The organic layer was separated by centrifugation at 2000 rpm in a bench centrifuge and the absorbance read at 549 nm. It should be noted that according to Warren (1959), μmoles NeuAc = 0.075 x absorbance at 549 nm is valid only for values between 0.01 and 0.06 μmoles NeuAc.

In all experiments involving recovery of sugars, results were corrected for losses based on the recoveries of radioactivity obtained following the isolation procedures, or in the case of gas liquid chromatography, by using myo-inositol as an internal standard.

Enzyme Assays

In the enzyme assays, unless otherwise noted, the conditions used were such that product formation was linear with respect to incubation time and amount of enzyme protein in the incubation mixture.

Enzymes of nucleotide sugar metabolism

Enzyme activities were measured in liver cell sap prepared according to Bley *et al*. (1973) as described above.

Glucosamine-6-phosphate synthase (EC 5.3.1.19; L-glutamine:D-fructose-6-phosphate aminotransferase) activity was measured according to Kornfeld (1967). Each incubation mixture contained: 12 mM glutamine, 6 mM fructose-6-phosphate, 1 mM EDTA, 40 mM sodium phosphate buffer, pH 7.5, and about 1 mg cell sap protein containing 12 mM glucose-6-phosphate (see above) in a total volume of 0.5 ml. Samples were incubated at 37 °C for 60 min and the reactions stopped by heating in a boiling water bath for 2 min. Protein was removed by centrifugation at 2000 rpm for 10 min in a bench top centrifuge and 0.2 ml samples of

supernatant were analyzed for glucosamine-6-phosphate by a modification of the Morgan-Elson method as described by Ghosh et al. (1960) scaled down to a final volume of 3.6 ml. Samples of 0.2 ml were treated first with 0.05 ml saturated sodium bicarbonate, then with 0.05 ml ice-cold, 5%, aqueous acetic anhydride. After vigorous mixing, the tubes were maintained at room temperature for 3 min, then heated in a boiling water bath for 3 min to destroy excess acetic anhydride, and cooled to room temperature. The samples were then treated with 0.1 ml 0.8 M sodium borate, pH 9.0, vortexed, and heated in a boiling water bath for 3 min. After cooling, the samples were treated with 3.0 ml of Ehrlich's reagent (1.0 g p-dimethylaminobenzaldehyde added to 1.25 ml 10 M hydrochloric acid and then diluted to 100 ml with glacial acetic acid). The samples were vortexed, incubated at 37 $^{o}$C for 20 min, cooled, and the absorbances measured at 585 nm. Glucosamine was used as the standard; results were corrected for the lower color constant of glucosamine-6-phosphate as described by Ghosh et al. (1960). Controls consisted of heat inactivated enzyme. One unit of glucosamine-6-phosphate synthase activity is defined as 1 nmole glucosamine-6-phosphate formed per 60 min.

UDP-GlcNAc 2-epimerase (EC 5.1.3.14) activity was assayed according to Sommar and Ellis (1972). Each incubation mixture contained: 0.5 μmoles UDP-GlcNAc, 12.5 μmoles $MgSO_4$, 50 μmoles Tris buffer, pH 7.5, and about 1 mg cell sap protein in a total volume of 0.25 ml. Samples were incubated at 37 $^{o}$C for 20 min and the reaction was stopped by heating in a boiling water bath for 2 min. The reaction product, N-acetyl-D-mannosamine, was determined by a modification of the Morgan-Elson method described by Spivak and Roseman (1966). This involved the addition of 150 mg each of dried Dowex 1-carbonate and dried

Dowex 1-hydrogen to each sample followed by 0.75 ml water. The addition of the resins was required to prevent interference with the color reaction by salts such as ammonium sulfate (Spivak and Roseman, 1966). The samples were allowed to stand at room temperature for 10 min, and the protein and resin removed by centrifugation at 2000 rpm for 10 min in a bench top centrifuge. To 0.4 ml supernatant, 0.1 ml water was added, followed by 0.125 ml 0.8 M potassium borate. The tubes were heated in a boiling water bath for 12 min, then chilled in ice. Ehrlich's reagent (see above), 3 ml, was added, the mixtures incubated at 37 $^{o}$C for 10 min, and then cooled to room temperature. Absorbances were determined at 590 nm. The standard was N-acetyl-D-mannosamine treated with Dowex resins as above. Controls consisted of heat inactivated enzyme. One unit of UDP-GlcNAc 2-epimerase activity is defined as 1 nmole N-acetyl-D-mannosamine formed per 20 min.

CMP-NeuAc synthase (EC 2.7.7.43) activity was assayed by the procedure of Kean and Roseman (1966). Each incubation mixture contained: 2.5 μmoles NeuAc, 2.5 μmoles CTP, 10 μmoles $MgCl_2$, 90 μmoles Tris buffer, pH 9.0, and about 1.5 mg cell sap protein in a total volume of 0.5 ml. Incubations were at 37 $^{o}$C for 20 min. Free NeuAc was destroyed with 0.075 ml 2.5 M $NaBH_4$ for 15 min at room temperature. After the addition of 0.075 ml acetone and standing for 15 min, CMP-NeuAc was assayed by measuring NeuAc with the Warren method (Warren, 1959) as described above for the determination of CMP-NeuAc content in liver. Controls consisted of heat inactivated enzyme. One unit of CMP-NeuAc synthase activity is defined as 1 nmole CMP-NeuAc formed per 20 min.

Sialyl and galactosyl transferases

Sialyl and galactosyl transferase activities were assayed in serum, liver homogenates, liver slice homogenates and liver slice medium which were prepared as described above.

Sialyl transferase activities were assayed by a method based on that described by Baxter and Durham (1979). The acceptor for sialyl transferase assays was rat asialo-$\alpha_1$-acid glycoprotein, except in some kinetic studies when human asialo-$\alpha_1$-acid glycoprotein was used. The standard assay for serum and liver slice medium contained: 250 μg acceptor, 7.5 μmoles imidazole buffer, pH 7.0, 2 nmoles CMP-[$^{14}$C] NeuAc (15 nCi/nmole), 25 μl serum or liver slice medium in a final volume of 0.15 ml. Incubations were typically for 20-30 min at 37 $^{o}$C. The incubation mixture for liver sialyl transferase was as described above except that 5 nmoles CMP-NeuAc (12 mCi/mmole), 1% Triton X-100,, and 0.1-0.2 mg liver protein were used. Assays of liver, medium and serum sialyl transferase activities were also performed as described above but using up to 20 nmoles CMP-[$^{14}$C]NeuAc (5 mCi/mmole) and 1 mg rat asialo-$\alpha_1$-acid glycoprotein.

In experiments to determine the nature of the linkages formed by sialyl transferase activities in serum, liver slice medium and liver homogenates, lactose was used as acceptor to produce 6'-($\alpha 2 \rightarrow 6$) and 3'-($\alpha 2 \rightarrow 3$) isomers of sialyllactose. The procedure was based on that described by Hudgin and Schachter (1972) and by Weinstein _et al_. (1982). Reaction mixtures were as described above except that 0.25 M lactose, 1 nmole of CMP-[$^{14}$C]NeuAc (247 mCi/mmole) and 80 μl serum, medium or liver homogenate were used and incubations were for 16 h. At the end of the incubation ethanol was added to 50% (v/v) to precipitate

protein; protein was sedimented at 8000 $g_{av}$ in an Eppendorf centrifuge for 5 min and washed with 1 ml 50% aqueous ethanol. Supernatants and wash solutions were pooled and dried under $N_2$ at 40 °C. To remove unreacted CMP-[$^{14}$C]NeuAc, each sample was dissolved in 1 ml ice-cold 5 mM sodium phosphate buffer, pH 6.8, and applied to a 0.5 x 2 cm column of Dowex 1-phosphate form resin (Paulson et al., 1977). The effluents were collected and each column washed with an additional 1 ml phosphate buffer. Pooled column effluents were taken to dryness under $N_2$ at 40 °C. The residues were dissolved in 30 µl water and appropriate volumes spotted on Whatman 3 MM paper (47 x 56 cm) along with a standard mixture containing 3' and 6' isomers of neuraminlactose and neuramin-[1-$^3$H]lactitol. Chromatograms were developed for 5-7 days by descending chromatography in ethyl acetate:pyridine:water (12:5:4, v/v/v). Radioactive regions of the chromatogram were located using a radiochromatogram scanner and then quantitated by cutting strips from the chromatogram for determination of radioactivity by liquid scintillation counting as described above. The 3' and 6' isomers of neuraminlactose were visualized as described by Kobata and Ginsburg (1969) and Warren (1960). The standards were first hydrolyzed by dipping the paper strip in 0.01 M sulfuric acid-acetone (1:10) and placing the paper in a moisture-saturated oven at 90 °C for 25 min. To detect the released sialic acid, the paper was sprayed with a solution of 0.05 M sodium periodate in 0.025 M sulfuric acid. After 15 min, a solution of ethylene glycol:acetone:concentrated sulfuric acid (50:50:0.3, v/v/v) was sprayed onto the paper. After 10 min the paper was sprayed with an aqueous solution of 6% sodium 2-thiobarbiturate. Red spots appeared after heating in an oven at 100 °C for 10 min. The spots gave a red

fluorescence under UV light. The 3' and 6' isomers of neuramin-[1-$^{3}$H] lactitol, located by scanning as described above, were found to co-chromatograph with corresponding neuraminlactose isomers.

The electrophoretic behaviour of sialyl transferase was determined according to the method of Sadler et al. (1979a, 1979b). Discontinuous polyacrylamide gel electrophoresis on up to 5 μl serum, 35 μl liver slice medium and 35 μl liver homogenate was performed as described above. Gel sections, 2 mm, were cut into quarter sections, placed in tubes and protein eluted into 150 μl 0.1 M sodium cacodylate buffer, pH 5.5, containing 0.1% bovine serum albumin for 1 h at room temperature. An aliquot was removed and assayed for sialyl transferase activity. The incubation mixture contained: 250 μg rat protein acceptor, 0.2 nmole CMP-[$^{14}$C]NeuAc (125 nCi/nmole), 15 μmoles sodium cacodylate buffer, pH adjusted to the same concentration that was used for imidazole buffer (see above), and 80 μl eluant. Incubations were for 10 h at room temperature.

Galactosyl transferase activities were assayed according to Fraser and Mookerjea (1976) using asialoagalacto-$\alpha_1$-acid glycoprotein as acceptor. The assay system contained: 250 μg acceptor, 2 μmoles $MnCl_2$, 0.1 μmole dithiothreitol, 3 nmoles UDP-[$^{14}$C]Gal (10 mCi/mmole), 15 μmoles 2(N-morpholino)ethane sulfonate buffer, pH 6.8, 400 nmoles CDP-choline to inhibit nonspecific pyrophosphatase activity, and 25 μl serum or liver slice medium or 0.1-0.2 mg liver homogenate protein. For liver homogenates, the incubations also contained 1% Triton X-100. Incubations were at 37 $^{o}$C for 20 min for all enzyme sources.

Except when lactose was used as acceptor, assays of sialyl and galactosyl transferase activities were stopped by transferring to ice;

samples of 40 μl were then immediately spotted on 2.5 cm diameter circles of Whatman No. 1 filter paper which were rapidly transferred to a container of 10% trichloroacetic acid. The circles were washed three times with 10% trichloroacetic acid followed by washes with ethanol:ether (2:1, v/v) and ether (Baxter and Durham, 1979). After drying, samples were counted in a liquid scintillation counter with 10 ml ACS cocktail as described above.

In all assays, endogenous enzyme activities were determined using controls in which the addition of appropriate acceptor was omitted; these values were subtracted from the corresponding exogenous acceptor values. In order to determine the stability of sialyl and galactosyl transferase activities during incubation in the liver slice experiments, additional controls were included. These involved the addition of sialyl and galactosyl transferase activities in the form of 1.3 ml rat serum to flasks containing either 3.7 ml of medium alone, or 3.7 ml of medium with 1 g control or 48 h experimental liver slices. Samples of medium were removed from enzyme assays after 6 h of incubation. Other controls were designed to determine if serum, medium or liver contained small molecular weight factors that could stimulate or inhibit sialyl transferase activities. In these experiments samples of serum, medium and liver homogenates were heated in a boiling water bath for 1 min to precipitate protein. Protein was removed by centrifuging at 8000 $g_{av}$ for 2 min in an Eppendorf centrifuge and suitable volumes of supernatants were added to assays for determination of sialyl transferase activities in serum, medium and liver from control and experimental rats.

α-Glucosidase and α-mannosidase

Hepatic α-glucosidase and α-mannosidase activities were assayed by a method based on that described by Kaplan and Jamieson (1977). α-Glucosidase and α-mannosidase activities in the rough and smooth membrane fractions and Golgi complex were measured at pH 6.5 and 5.5, respectively, which were the experimentally obtained pH optima values for the microsome fraction enzyme activities (see Figs. 53-54). Lysosomal α-glucosidase and α-mannosidase activities were measured at pH 4.2 (Brown *et al.*, 1972) and pH 4.6 (Opheim and Touster, 1978), respectively. Incubation mixtures normally contained: 0.4 ml McIlvaine buffer (0.1 M citric acid adjusted to the appropriate pH value with 0.2 M $Na_2HPO_4$); 0.1% Triton X-100; 2 mM p-nitrophenyl-α-D-glucopyranoside and p-nitrophenyl-α-D-mannoside; and 0.3 mg enzyme protein, in a final volume of 0.5 ml. Incubations were at 37 $^oC$ for 30 min. Incubations were stopped by addition of 1.5 ml 0.2 M glycine adjusted to pH 10.2 with sodium hydroxide. Samples were centrifuged at 8000 $g_{av}$ for 5 min in an Eppendorf centrifuge and absorbances read at 400 nm. One unit of enzyme activity is defined as the amount of enzyme which hydrolyzes 1 nmole p-nitrophenyl glycoside per min at 37 $^oC$; the molar extinction coefficent of p-nitrophenol under the assay conditions used is 1.77 x $10^4$ $M^{-1}cm^{-1}$.

# RESULTS

## THE EFFECT OF INFLAMMATION ON SIALYL AND GALACTOSYL TRANSFERASE ACTIVITIES IN RAT LIVER AND SERUM

Before assay of sialyl transferase activity, linearity was established for all enzyme sources, including liver, liver slice medium and serum, with respect to time (Fig. 13) and amount of enzyme protein (Fig. 14). Assays were also performed under saturating conditions of acceptor protein (Fig. 15). Using both saturating and subsaturating levels of CMP-NeuAc, no change was observed in the ratios of specific radioactivities of the acceptor protein between experimental and control samples for all enzyme sources. Thus, for routine work, assays were performed using subsaturating levels of CMP-NeuAc (see Experimental) in order to conserve material. Under the routine assay conditions, less than 5% of the acceptor protein and CMP-NeuAc substrates were consumed in the reaction. The use of smaller assay volumes was not attempted in order to avoid the inaccuracies inherent in the use of very small volumes.

The effect of inflammation on liver and serum sialyl and galactosyl transferase activities is shown in Figs. 16 and 17. Both hepatic enzyme activities showed a maximum increase at 48 h following onset of inflammation when both enzyme activities increased to about three times control values (Fig. 16). Although inflammation caused increases of both enzyme activities in liver, only sialyl transferase activity increased significantly in serum. Figure 17 shows that serum sialyl transferase activity increased rapidly following inflammation reaching a maximum at 48 h after inflammation when the activity was about five times the control value. In contrast, serum galactosyl transferase activity increased by only about 50% at 48 h after inflammation (Fig. 17).

As mentioned in the Introduction, several workers have suggested

Fig. 13. Linearity of sialyl transferase activity with time. Panel A, liver (●, O); panel B, liver slice medium from a 3 h slice incubation (■, □) and serum (▲, Δ). Closed and open symbols represent control and 48 h experimental samples, respectively. Enzyme activity is expressed as pmoles NeuAc transferred per ml medium or serum, or per mg liver protein, using 250 μg acceptor protein. Results are from a typical experiment; reproducibility was within ±10%.

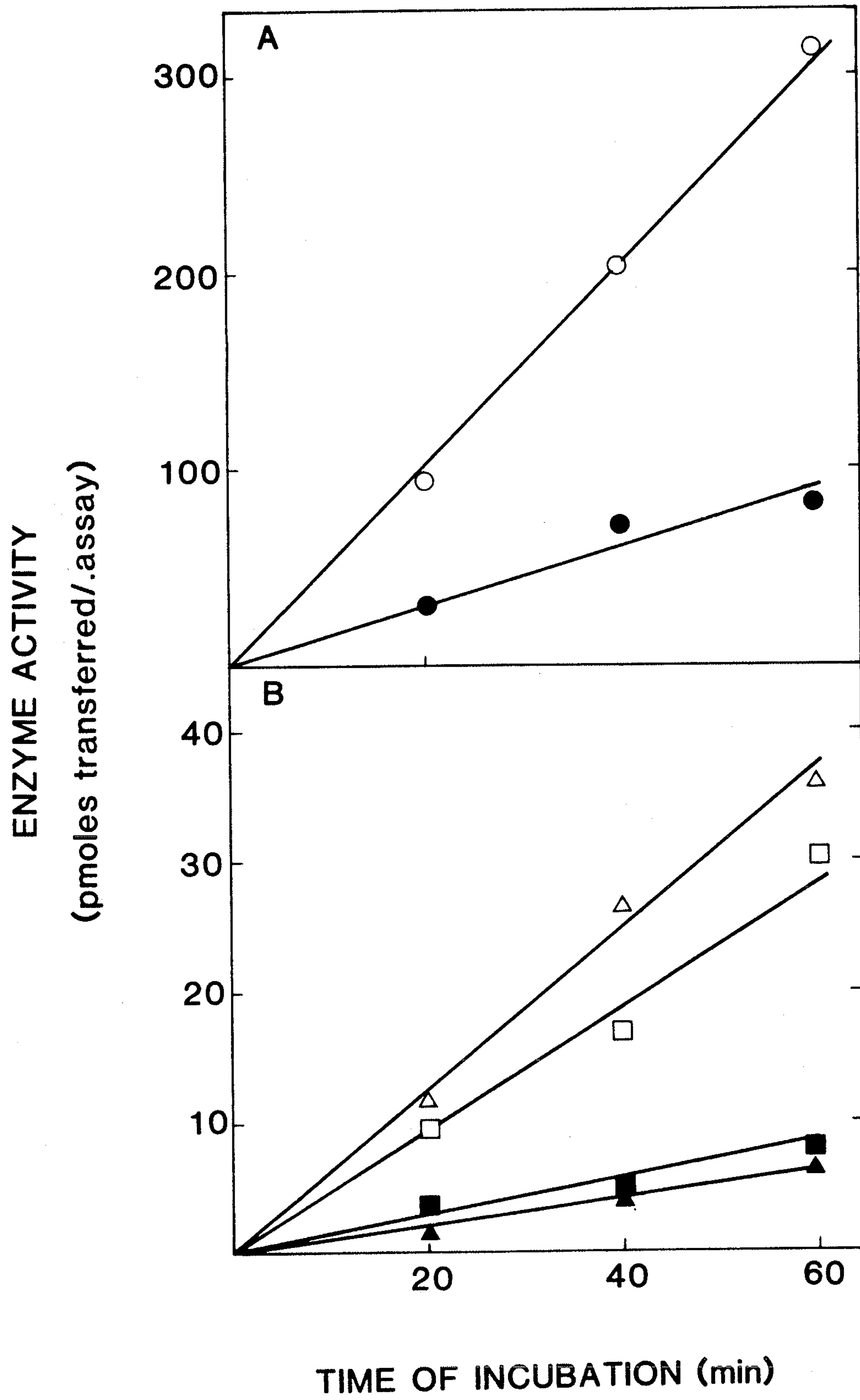
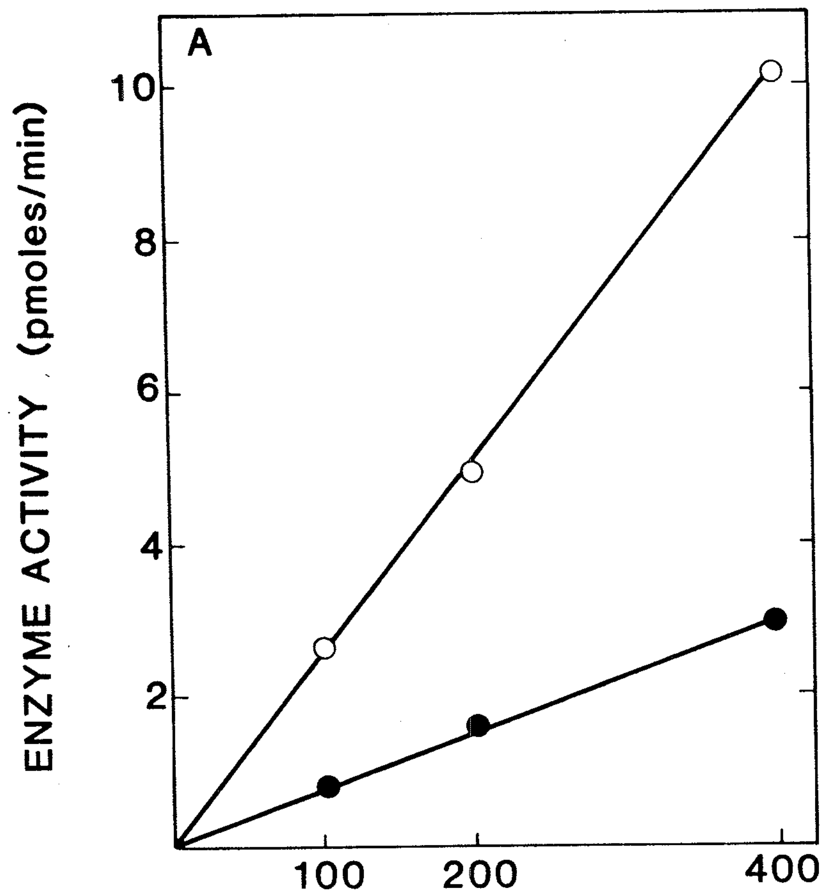
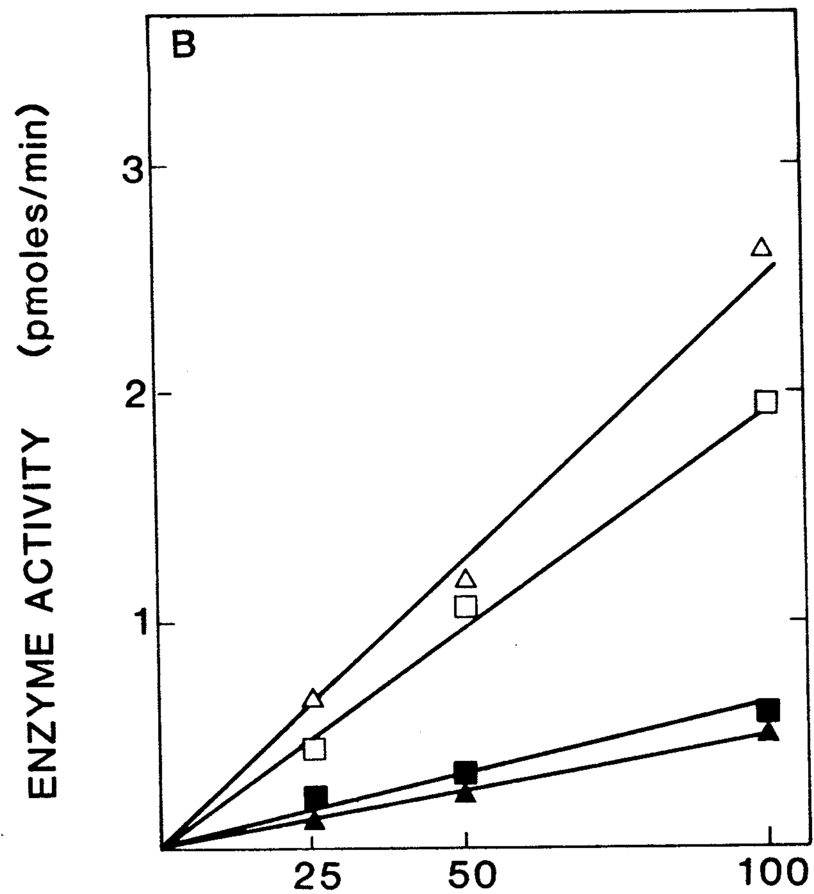

A
B
300
200
100
40
30
20
10
20
40
60
ENZYME ACTIVITY
(pmoles transferred/.assay)
TIME OF INCUBATION (min)

Fig. 14. Sialyl transferase activity as a function of amount of enzyme protein. Panel A, liver (●, O); panel B, liver slice medium from a 3 h slice incubation (■, □) and serum (▲, Δ). Closed and open symbols represent control and 48 h experimental samples, respectively. Enzyme activity is expressed as pmoles NeuAc transferred per min using 250 μg acceptor protein. Results are from a typical experiment; reproducibility was within ±10%.




AMOUNT OF ENZYME

($\mu$g protein) ($\mu$l)

Fig. 15. Effect of amount of acceptor protein on sialyl transferase activities. Results shown are from 48 h experimental samples: liver (●); liver slice medium (■) from a 3 h slice incubation; and serum (▲). Corresponding controls (not shown) gave curves which also plateaued at 200-250 μg acceptor protein, but exhibited lower enzyme activities (see Figs. 13-14). Enzyme activity is expressed as pmoles NeuAc transferred per min per ml medium or serum, or per mg liver protein. Results are from a typical experiment; reproducibility was within ±10%.

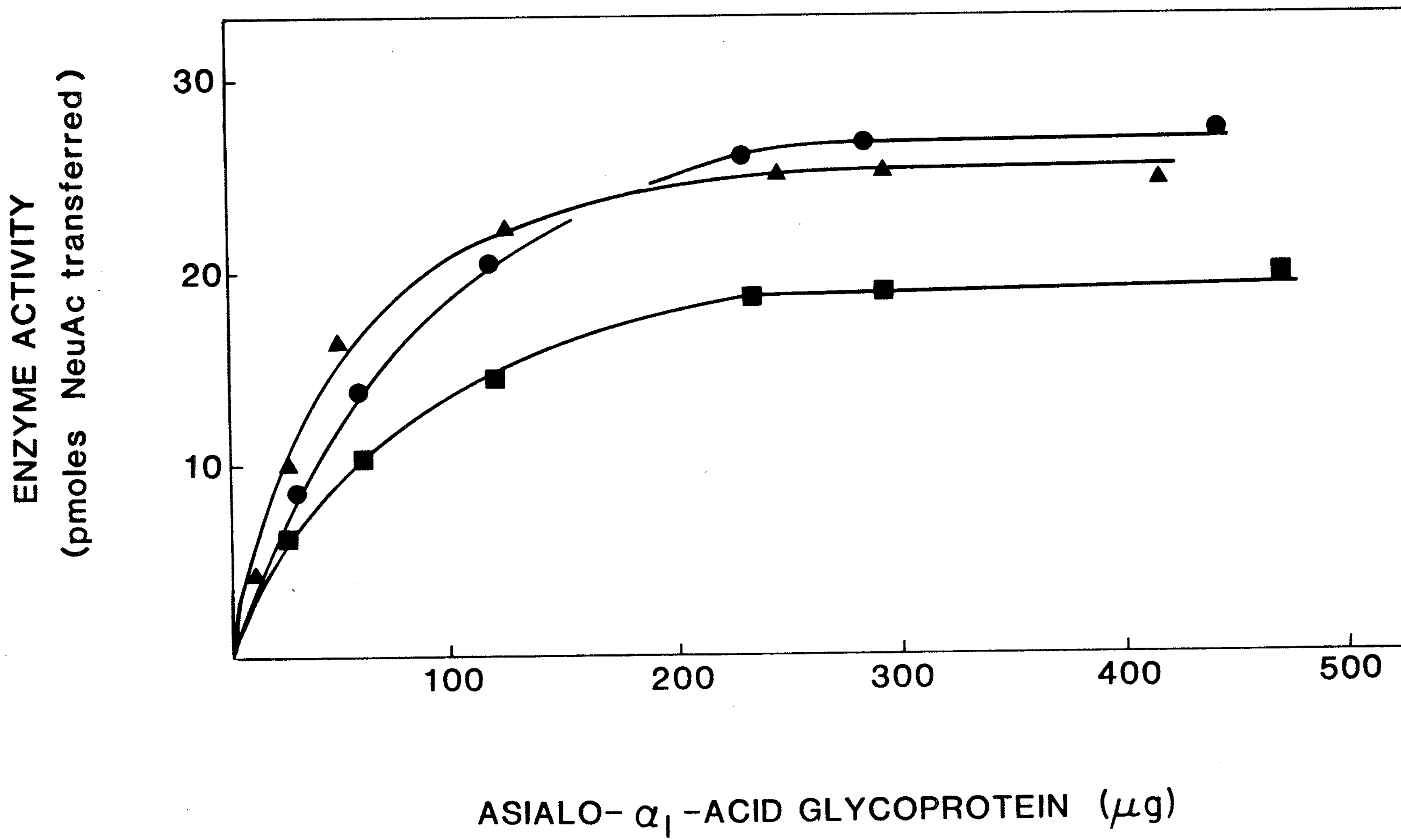

30
20
10
ENZYME ACTIVITY
(pmoles NeuAc transferred)
100
200
300
400
500
ASIALO- $\alpha_1$ -ACID GLYCOPROTEIN ($\mu$g)

Fig. 16. Effect of inflammation on liver sialyl and galactosyl transferase activities. Sialyl transferase (●), galactosyl transferase (■). Enzyme activities are expressed at pmoles sugar transferred per min per mg liver protein with 250 μg appropriate acceptor (see Experimental). Results are the means of 5-10 analyses; the bars represent the range of standard deviations of the means.

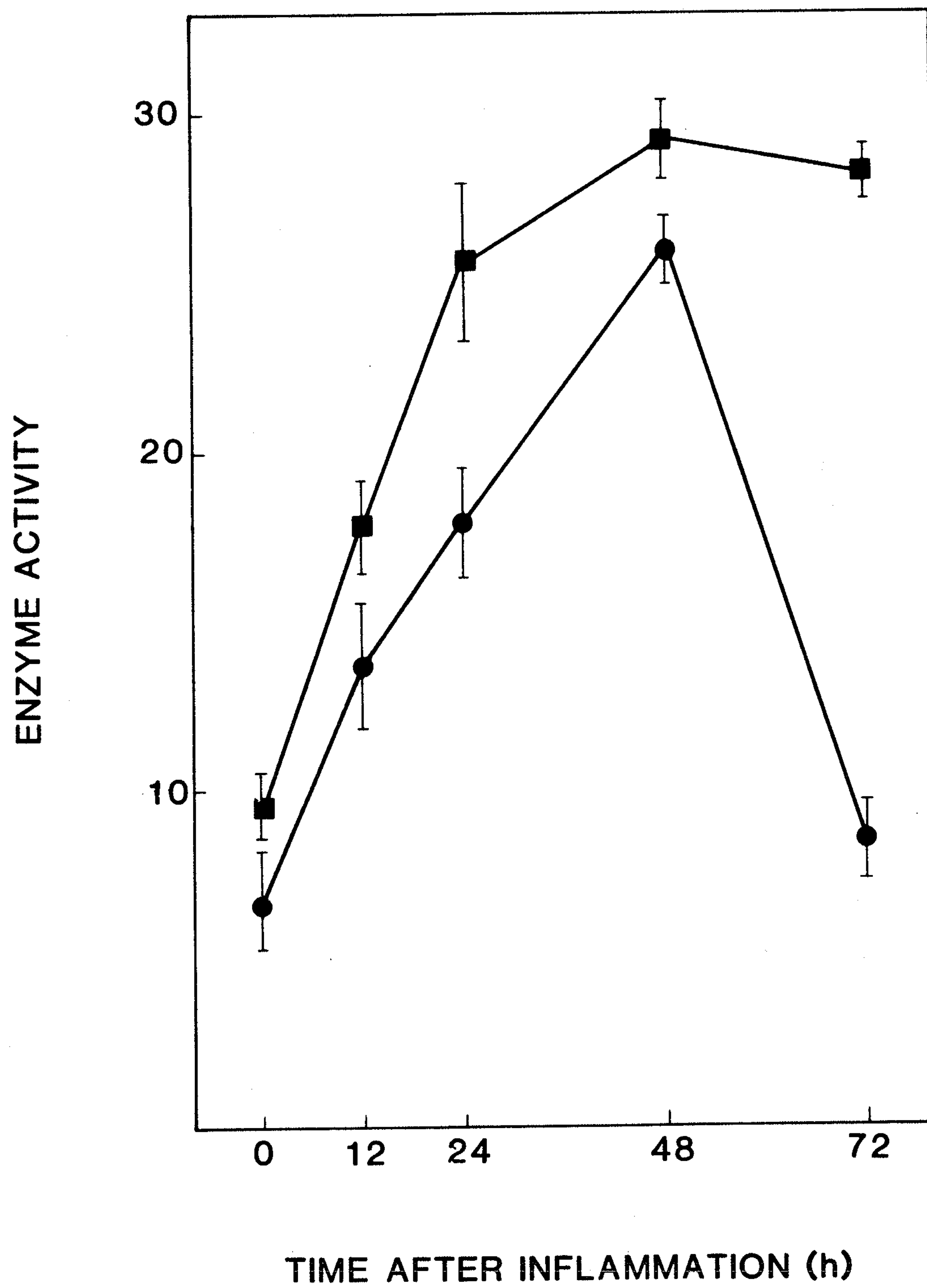
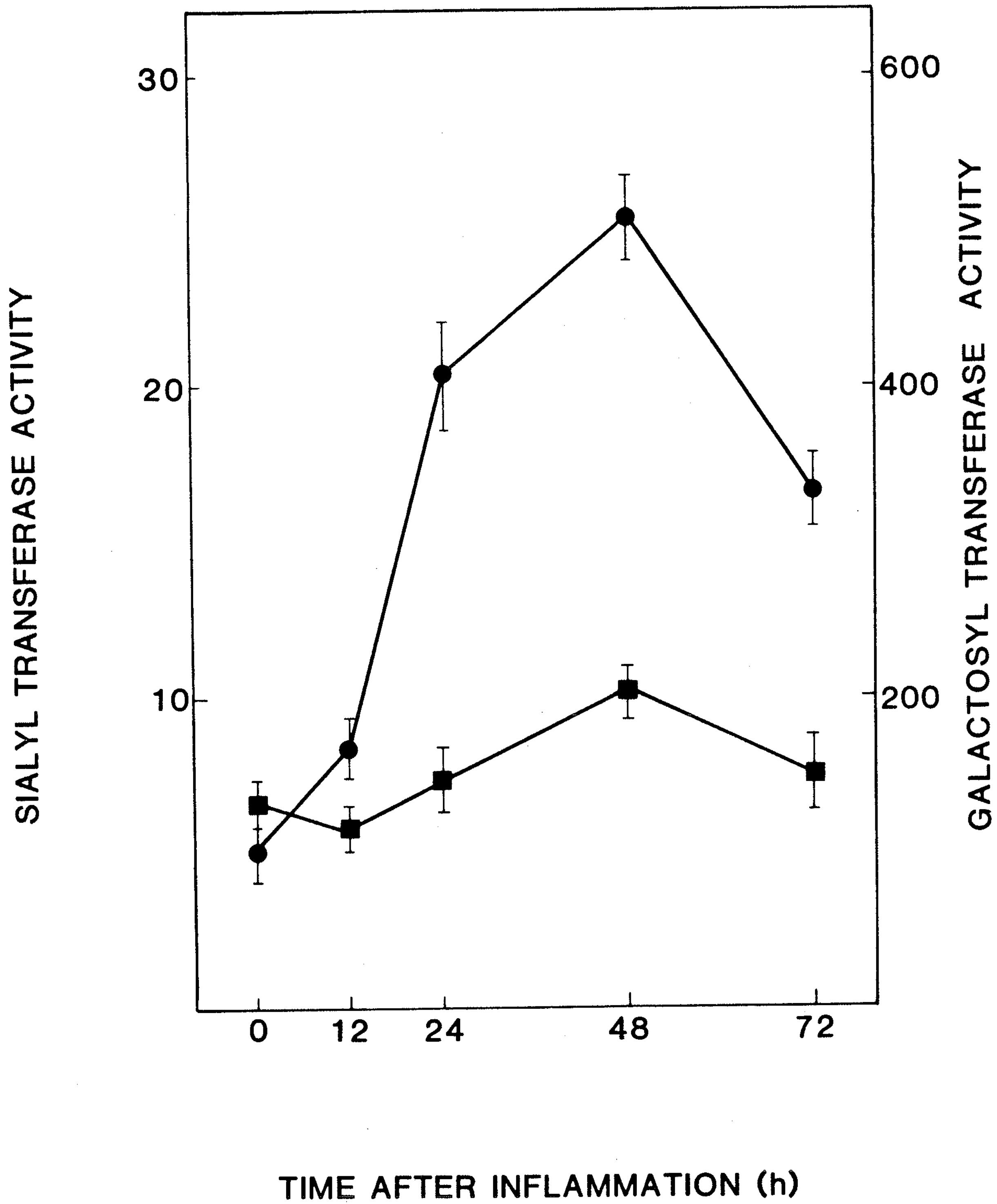

30
20
10
ENZYME ACTIVITY
0
12
24
48
72
TIME AFTER INFLAMMATION (h)

Fig. 17. Effect of inflammation on serum sialyl and galactosyl transferase activities. Sialyl transferase (●), galactosyl transferase (■). Enzyme activities are expressed as pmoles sugar transferred per min per ml serum with 250 μg appropriate acceptor (see Experimental). Results are the means of 5-10 analyses; the bars represent the range of standard deviations of the means.


30
20
10
600
400
200
SIALYL TRANSFERASE ACTIVITY
GALACTOSYL TRANSFERASE ACTIVITY
0
12
24
48
72
TIME AFTER INFLAMMATION (h)

that liver is the source of the elevated glycosyl transferase activities found in serum in inflammatory conditions although no direct proof of this idea has been offered. In order to test this idea directly, a liver slice system used previously in this laboratory to study the rates of synthesis of $\alpha_1$-acid glycoprotein and albumin (Jamieson et al., 1975) was used to examine the effect of inflammation on the release of sialyl and galactosyl transferase from liver. In order to minimize contamination of liver slice medium with residual serum sialyl and galactosyl transferase activities in these experiments, livers were exhaustively perfused prior to preparation of slices and then slices were carefully washed. Previous experience in our laboratory (Jamieson et al., 1975) has shown that perfusion coupled with washing essentially eliminates contamination of medium by residual blood proteins. The results from slice experiments in which sialyl and galactosyl transferase activities were monitored in medium using liver slices from control and experimental rats are shown in Fig. 18. Sialyl transferase activity in medium was substantially elevated when liver slices from inflamed rats were used with the maximum elevation occurring at 48 h following onset of inflammation (Fig. 18). Figure 19 shows that there was a linear increase in medium sialyl and galactosyl transferase activities up to about 6 h of incubation. These results suggest that the liver slice system was functionally stable up to 6 h of incubation, as was found in the earlier work on $\alpha_1$-acid glycoprotein and albumin synthesis (Jamieson et al., 1975), and that the appearance of sialyl and galactosyl transferase activities in medium was not due to cellular disruption during incubation.

Taken at face value, the above results strongly suggest that sialyl transferase, but not galactosyl transferase is released from liver

Fig. 18. Effect of inflammation on sialyl and galactosyl transferase activities in liver slice medium. Sialyl transferase (●), galactosyl transferase (■). Slice incubations were for 3 h. Enzyme activities are expressed as pmoles sugar transferred per min per ml medium with 250 μg approapriate acceptor (see Experimental). Results are the means of 5-10 analyses; the bars indicate the range of standard deviations of the means.

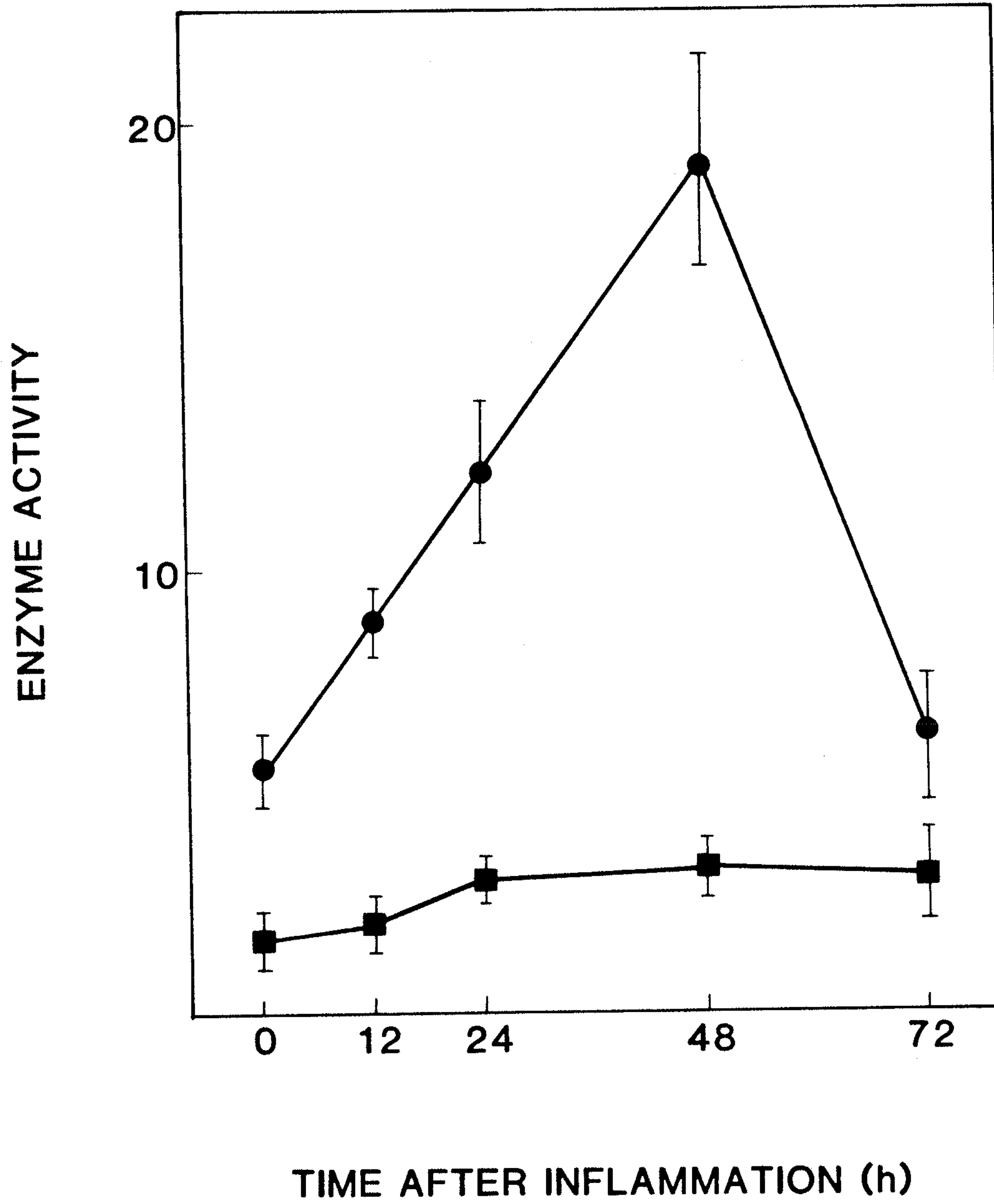

ENZYME ACTIVITY
20
10
0
12
24
48
72
TIME AFTER INFLAMMATION (h)

Fig. 19. Effect of liver slice incubation time on release of sialyl and galactosyl transferase activities into medium. Sialyl transferase (O, ●), galactosyl transferase (□, ■). Open and closed symbols represent control and 48 h experimental samples, respectively. Enzyme activity is expressed as in Fig. 18. Results are the means of 3-5 analyses; the bars represent the range of standard deviations of the means.

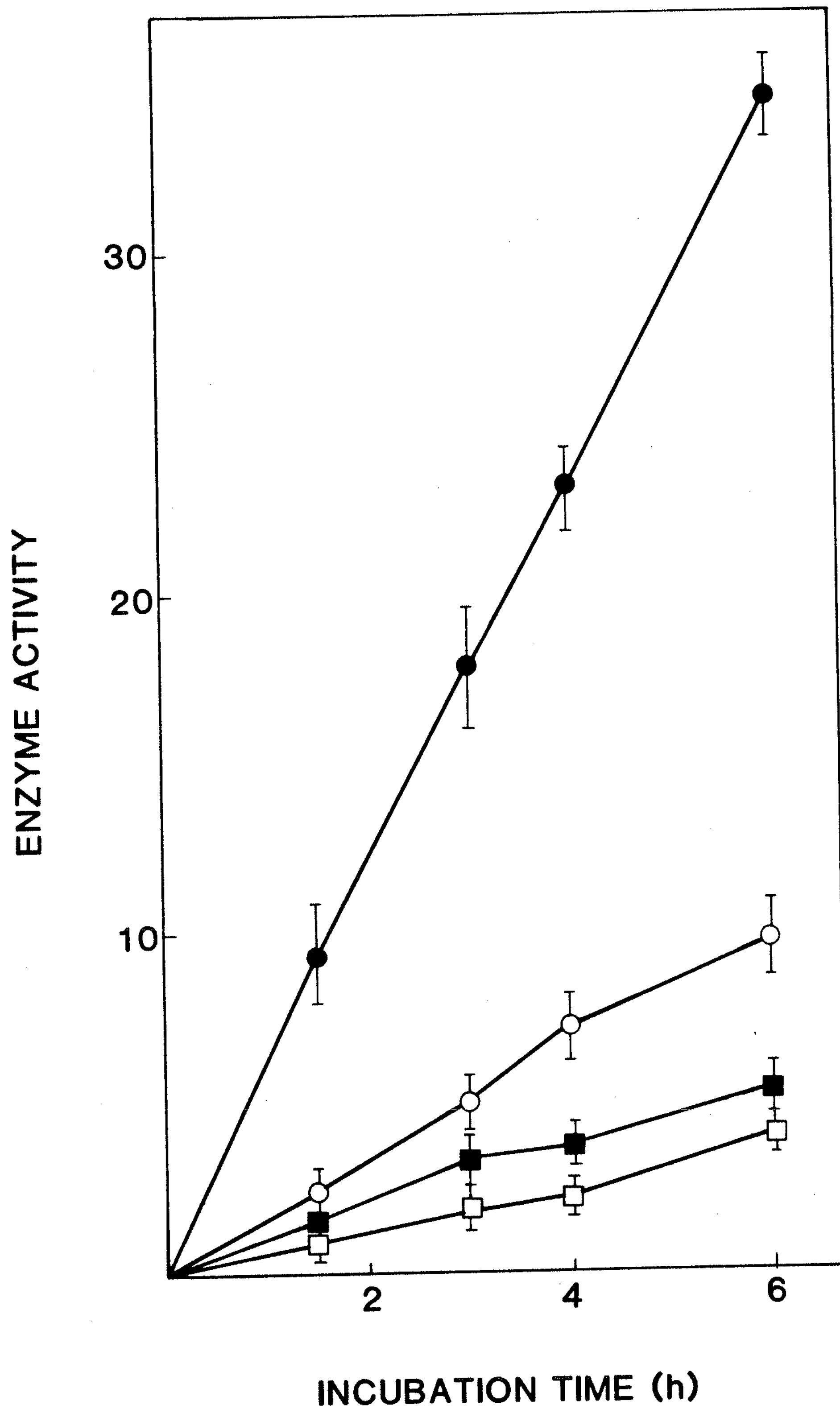

ENZYME ACTIVITY
30
20
10
2
4
6
INCUBATION TIME (h)

Table 3. Stability of hepatic sialyl and galactosyl transferase activities in liver slice incubations.

| Time of Incubation (h) | Enzyme activity | | | |
|---|---|---|---|---|
| | Sialyl transferase | | Galactosyl transferase | |
| | Control | 48 h Inflamed | Control | 48 h Inflamed |
| | Ratio incubated/unincubated | | | |
| 1.5 | 0.95 ± 0.08 | 1.03 ± 0.10 | 0.96 ± 0.07 | 1.02 ± 0.08 |
| 3.0 | 0.96 ± 0.09 | 0.98 ± 0.05 | 0.94 ± 0.10 | 0.96 ± 0.07 |
| 4.0 | 0.97 ± 0.06 | 0.94 ± 0.08 | 0.95 ± 0.09 | 0.95 ± 0.03 |
| 6.0 | 0.92 ± 0.06 | 0.93 ± 0.05 | 0.94 ± 0.07 | 0.93 ± 0.06 |

Values for enzyme activities of unincubated livers are given in Fig. 16. Values represent the means and standard deviations of the means of 3-6 analyses.

Table 4. Stability of sialyl and galactosyl transferase activities in medium in liver slice incubations.

| Enzyme Source | Enzyme activity | | | |
|---|---|---|---|---|
| | Sialyl transferase | | Galactosyl transferase | |
| | Control | 48 h Inflamed | Control | 48 h Inflamed |
| Serum (fresh) | 5.2±0.5 | 26.5±1.9 | 146.5±11.1 | 208.0±16.8 |
| Serum (37 °C) | 5.0±0.4 | 24.3±2.2 | 139.1±12.1 | 198.1±11.7 |
| Serum + medium | 5.0±0.5 | 24.8±1.7 | 142.0±6.6 | 197.7±13.1 |
| Serum + slice assay | 4.9±0.5 | 24.1±2.0 | 138.2±9.3 | 201.3±14.9 |

Sialyl and galactosyl transferase activities are expressed as pmoles sugar transferred per min per ml serum using 250 μg of appropriate acceptor substrate (see Experimental). Serum (fresh) represent enzyme activities prior to addition to incubation flasks; serum (37 °C) represents enzyme activities after incubation of an identical serum sample under slice incubation conditions for 6 h prior to assay; serum + medium represents assay after 6 h incubation in presence of slice medium alone; and serum + slice assay represents assay in presence of 1 g liver slices. Corrections were made for enzyme activities released from liver slices during the 6 h slice incubations. Results shown represent the means and standard deviations of the means of 4 analyses. See text for further details.

following inflammation. However, other explanations are possible. For example, galactosyl transferase could be undergoing catabolism in medium leading to lower activities; there could be inhibitors present; or the enzyme could be unstable for a variety of reasons. Control experiments were carried out to investigate some of these possibilities. Table 3 shows that the activities of both sialyl and galactosyl transferase in liver slices from control and 48 h experimental rats remained relatively constant throughout the 6 h slice incubation. The stabilities of sialyl and galactosyl transferase activities in medium were determined in an experiment in which sialyl and galactosyl transferase activities were added to liver slice incubations in the form of serum from control and 48 h experimental rats (see Experimental). Table 4 shows that there was little or no change in either sialyl or galactosyl transferase activity in medium containing serum in 6 h liver slice incubations. There was also little or no change in either glycosyl transferase activity in serum or serum plus medium, but without liver slices, incubated at 37 $^{o}$C for 6 h (Table 4). These results suggest that sialyl and galactosyl transferase activities in medium are a valid representation of release from the slices, and that the lower activities in medium from control rats cannot be due to elevated catabolism of medium enzymes.

The above results suggest that the elevated levels of sialyl transferase activity in liver, liver slice medium, and serum in experiments with inflamed rats are due to an increased amount of enzyme. However, several workers (Shah and Raghupathy, 1977; IP and Dao, 1978; Kessel _et al_., 1978) have shown that nucleotides and other low molecular weight factors, such as divalent cations, can influence sialyl transferase activities of serum and liver. The results of studies on the

effect of nucleotides on sialyl transferase activity, presented later in this thesis, indicate that nucleotides can indeed affect the enzyme activity in liver, medium and serum. In order to determine if endogenous levels of nucleotides, divalent cations, or other low molecular weight factors were responsible for the elevated sialyl transferase activities in liver, medium and serum from experiments with inflamed rats, the following approach was used. Samples of liver homogenate, medium and serum from experiments with control and 48 h inflamed rats were incubated in a boiling water bath for 1 min to precipitate protein; the resulting supernatants were taken as a source of low molecular weight material. Appropriate aliquots of control and 48 h experimental low molecular weight-enriched fractions were added to the corresponding untreated 48 h experimental and control samples, respectively, and the effect on sialyl transferase activities tested. Table 5 shows that regardless of enzyme source, sialyl transferase activities did not change significantly in the presence of added low molecular weight factors. These results indicate that endogenous levels of low molecular weight factors in liver, medium and serum were insufficient to cause the observed changes in sialyl transferase activities found in inflammation.

The results from the preceding studies suggest that sialyl transferase is preferentially released from liver when compared with galactosyl transferase, and that the increased sialyl transferase activity found in liver, medium and serum from experiments with inflamed rats is due to an increase in the amount of the enzyme. Based on the assumption that enzyme activity can be equated with enzyme protein, a calculation can be made of the amount of sialyl and

Table 5. Effect of endogenous, heat stable, low molecular weight factors on liver, medium and serum sialyl transferase activities.

| Enzyme source | Enzyme activity: Control | Enzyme activity: 48 h Inflamed |
|---|---|---|
| | Ratio treated/untreated | |
| Liver | 1.12 ± 0.05 | 1.20 ± 0.10 |
| Medium | 0.86 ± 0.07 | 0.83 ± 0.08 |
| Serum | 0.96 ± 0.09 | 0.84 ± 0.09 |

Control samples of liver, medium and serum were assayed in the presence of equal volumes of preparations of low molecular weight fractions from 48 h experimental samples of liver, medium and serum, respectively; similarly, 48 h samples were assayed in the presence of low molecular preparations from control samples. Results shown are the means and standard deviations of the means of 3 analyses. See Experimental for preparation of low molecular weight fractions.

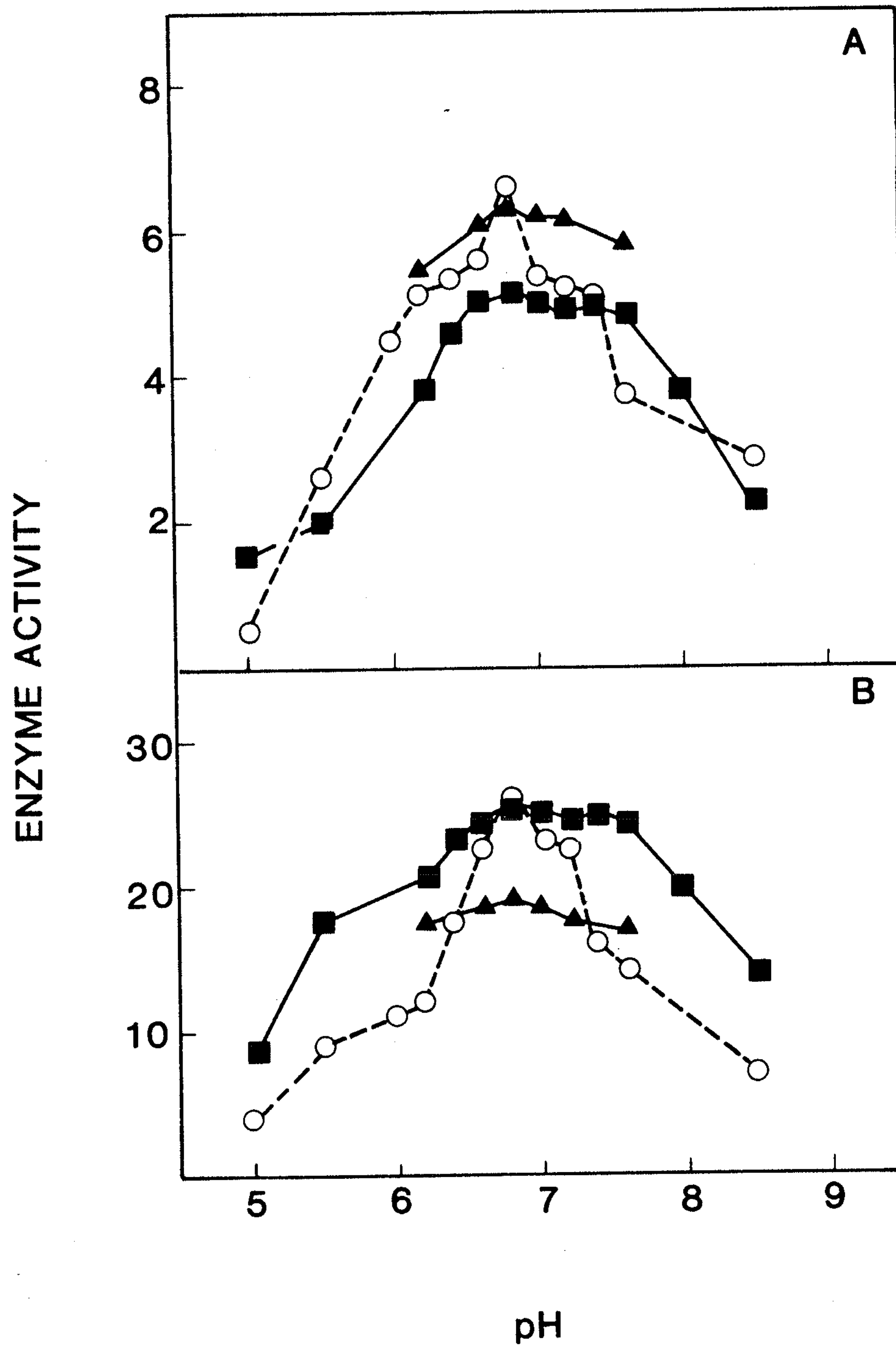
galactosyl transferase released from liver in a typical slice experiment. Over a 3 h incubation period, slice medium contains about 5% of the sialyl transferase activity of liver, whereas it contains only about 0.5% of the activity of liver galactosyl transferase. It is more difficult to estimate what proportion serum sialyl transferase represents of the total liver sialyl transferase activity since serum activity represents an accumulation of sialyl transferase over a long period. However, in view of the similar increases found for medium and serum sialyl transferase after inflammation, it is likely to be of the same magnitude.

Kinetic Properties of Sialyl Transferase in Liver, Medium and Serum

Due to the apparent preferential release of sialyl transferase compared to galactosyl transferase from liver, coupled with the enzyme's substantial increase in activity in liver, medium and serum from experiments with inflamed rats, a series of experiments was performed to compare some of the kinetic properties of sialyl transferase in liver, medium and serum. These included the determination of optimum pH and apparent $K_m$ values, and the effect of nucleotides on sialyl transferase activity.

Optimum pH values for sialyl transferase in liver, medium and serum from experiments with control and 48 h inflamed rats as source of enzyme are shown in Fig. 20. The curves in Fig. 20 indicate that liver sialyl transferase activities from both control and experimental rats had fairly sharp pH optima at 6.8, while medium and serum sialyl transferase activities from experiments with control and 48 h inflamed rats had a range of pH optima from 6.6-7.6. These results indicate that the pH optima for all six sources of enzyme were not substantially different.

Fig. 20. The effect of pH on sialyl transferase activity. Liver (O), medium (▲) and serum (■); panel A, control samples; panel B, 48 h experimental samples. The buffers used (50 mM final concentration) were as follows: pH 5.0-5.5, potassium acetate; pH 6.0, 2-(N-morpholino)ethanesulfonic acid; pH 6.2-7.6, imidazole; pH 8.0-8.5, Tris. Enzyme activities of liver, serum and medium are expressed as in Figs. 16, 17 and 18, respectively. Results represent the means of 3-5 analyses; reproducibility was within ±10%.


A
B
ENZYME ACTIVITY
8
6
4
2
30
20
10
5
6
7
8
9
pH

Apparent $K_m$ values for sialyl transferase using liver, medium and serum from experiments with control and 48 h inflamed rats as source of enzyme are given in Table 6. Values of apparent $K_m$ determined with respect to the substrates CMP-NeuAc and rat and human asialo-$\alpha_1$-acid glycoprotein were very close; there was also little difference in apparent $K_m$ values for the six activities with human asialo-$\alpha_1$-acid glycoprotein as acceptor (Table 6). Apparent $K_m$ values with CMP-NeuAc as substrate were also similar for the six enzyme activities, except for the serum enzyme from 48 h inflamed rats whose $K_m$ value was always found to be slightly lower than the values found for the other enzyme activities (Table 6).

As previously mentioned, low molecular weight factors, such as nucleotides, can affect sialyl transferase activity. Indeed, studies by several workers have indicated that nucleotides have the ability to stimulate or inhibit a number of glycosyl transferase activities from a variety of sources (Bella and Kim, 1971; Jabbal and Schachter, 1971; Wagner _et al_., 1971; Bernacki, 1975; Mookerjea and Jung, 1975; Kuhlenschmidt _et al_., 1976; Shah and Raghupathy, 1977; Klohs _et al_., 1979). For example, uracil, cytosine, adenine and guanine nucleotides have been shown to cause inhibition of human and rat serum sialyl transferase activities (Shah and Raghupathy, 1977; Klohs _et al_., 1979), whereas uracil nucleotides have been shown to stimulate sialyl transferase activity in rat liver homogenates (Bernacki, 1975; Shah and Raghupathy, 1977). Thus, in order to further compare the properties of sialyl transferase in liver, medium and serum, the effect of nucleotides on sialyl transferase activity from these enzyme sources was examined. The effects of UMP, UDP, UTP and CTP concentrations on liver and serum

Table 6. Apparent $K_m$ values for sialyl transferase.

| Enzyme source | Substrate: apparent $K_m$ (μM) | | |
|---|---|---|---|
| | | Asialo-$\alpha_1$-acid glycoprotein | |
| | CMP-NeuAc | Rat | Human |
| Liver: control | 25.0 ± 3.1 | 12.0 ± 1.9 | 1.4 ± 0.1 |
| 48 h inflamed | 32.1 ± 2.8 | 10.0 ± 2.2 | 2.3 ± 0.4 |
| Serum: control | 26.8 ± 3.2 | 10.0 ± 1.7 | 2.1 ± 0.2 |
| 48 h inflamed | 16.0 ± 1.0 | 7.1 ± 1.6 | 1.2 ± 0.1 |
| Medium: control | 27.0 ± 0.7 | 7.6 ± 1.6 | 1.2 ± 0.1 |
| 48 h inflamed | 26.4 ± 1.7 | 8.3 ± 1.2 | 1.4 ± 0.1 |

$K_m$ values using asialoglycoproteins were calculated according to Wilkinson (1961) assuming a molecular weight of 34600 for the rat asialoprotein (Jamieson et al., 1972a) and 37000 for the human asialoprotein (Yoshima et al., 1981); it should be noted that on average there will be 5.5 acceptor positions/mole for the rat protein and 17.0 acceptor positions/mole for the human protein (based on the average number of Galβ1→4GlcNAc linkages at the non-reducing end of asialo-oligosaccharide chains; see Yoshima et al., 1981). Medium samples are from 3 h slice incubations. Apparent $K_m$ values represent the means and standard deviations of the means of 3-5 analyses.

sialyl transferase activities from control and 48 h inflamed rats are shown in Fig. 21. Of the uracil nucleotides, UTP was found to be the most potent inhibitor of sialyl transferase activities in all sources of enzyme over the range of concentration tested, followed by, in decreasing order, UDP and UMP (Fig. 21). CTP was the most potent inhibitor of sialyl transferase activities in all four sources of enzyme. CTP caused about 90% inhibition of enzyme activity at 0.25 mM, the lowest concentration used, whereas a UTP concentration of 1.0 mM was required to obtain 90% inhibition (Fig. 21). A variety of nucleotides at 1.0 mM concentration were evaluated for their effects on liver, medium and serum sialyl transferase activities from experiments with control and 48 h inflamed rats. Table 7 summarizes the results. All of the nucleotides tested in this study inhibited sialyl transferase activities from all six sources of enzyme. All cytosine nucleotides (CMP, CDP and CTP) were potent inhibitors of sialyl transferase activities (Table 7). Regardless of the nucleotide, the magnitude of enzyme inhibition was proportional to the number of phosphate moieties in the nucleotide. Thus, increasing the number of phosphate groups in the nucleotide caused a concomitant increase in the ability of that nucleotide to inhibit sialyl transferase activity, regardless of enzyme source (Table 7).

Thus, the results from the studies on the kinetic properties of sialyl transferase in liver, medium and serum indicate that the enzymes from these sources have very similar properties.

Fig. 21. The effect of UMP (▲), UDP (■), UTP (●), and CTP (O) on liver and serum sialyl transferase activities. Panels A and B, 48 h experimental and control sera, respectively; panels C and D, 48 h experimental and control livers, respectively. Values for 100% enzyme activities of control and experimental liver and serum are given in Fig. 16 and 17, respectively. Results represent the means of 3-5 analyses; reproducibility was within ±10%.

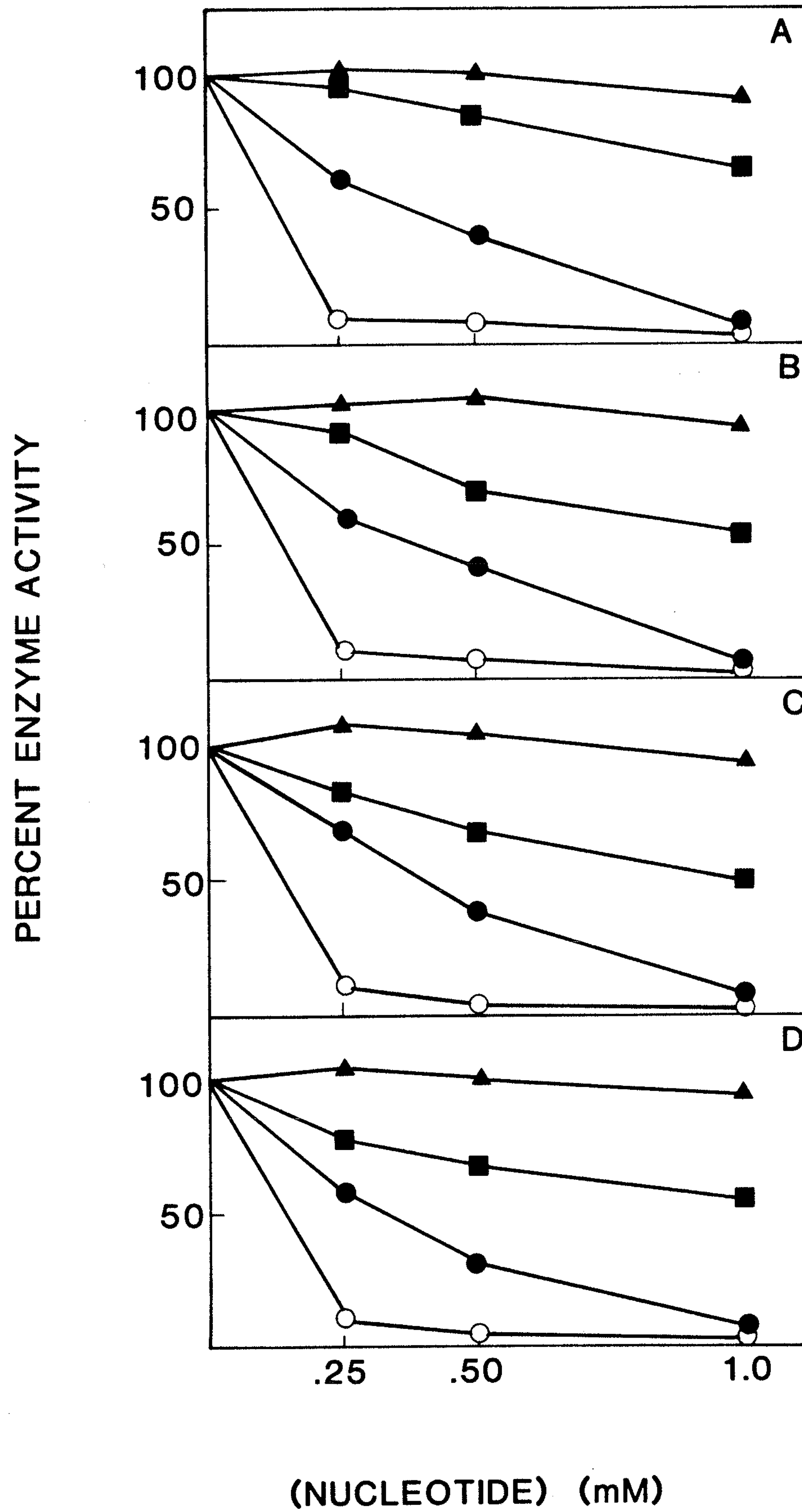

A
B
C
D
100
50
PERCENT ENZYME ACTIVITY
.25
.50
1.0
(NUCLEOTIDE) (mM)

Table 7. Effect of nucleotides on liver, medium and serum sialyl transferase activities

| Nucleotide (1.0 mM) | Percent inhibition of enzyme activity: Liver Control | Liver 48 h | Medium Control | Medium 48 h | Serum Control | Serum 48 h |
|---|---|---|---|---|---|---|
| AMP | 6 | 8 | 6 | 5 | 5 | 5 |
| CMP | 82 | 87 | 86 | 87 | 88 | 85 |
| GMP | 17 | 19 | 15 | 11 | 9 | 14 |
| UMP | 4 | 12 | 10 | 5 | 9 | 4 |
| ADP | 77 | 70 | 60 | 56 | 45 | 70 |
| CDP | 92 | 91 | 88 | 94 | 92 | 94 |
| GDP | 67 | 50 | 61 | 54 | 43 | 65 |
| UDP | 46 | 38 | 55 | 47 | 36 | 50 |
| ATP | 96 | 97 | 92 | 96 | 90 | 92 |
| CTP | 99 | 99 | 99 | 99 | 99 | 99 |
| GTP | 87 | 86 | 90 | 90 | 89 | 91 |
| UTP | 93 | 92 | 93 | 96 | 94 | 96 |

Values represent the means of 3-5 analyses; reproducibility was within ±10%. Medium samples are from 3 h slice incubations.

Characterization of the Sialyl Transferase Released from Liver in Inflammation

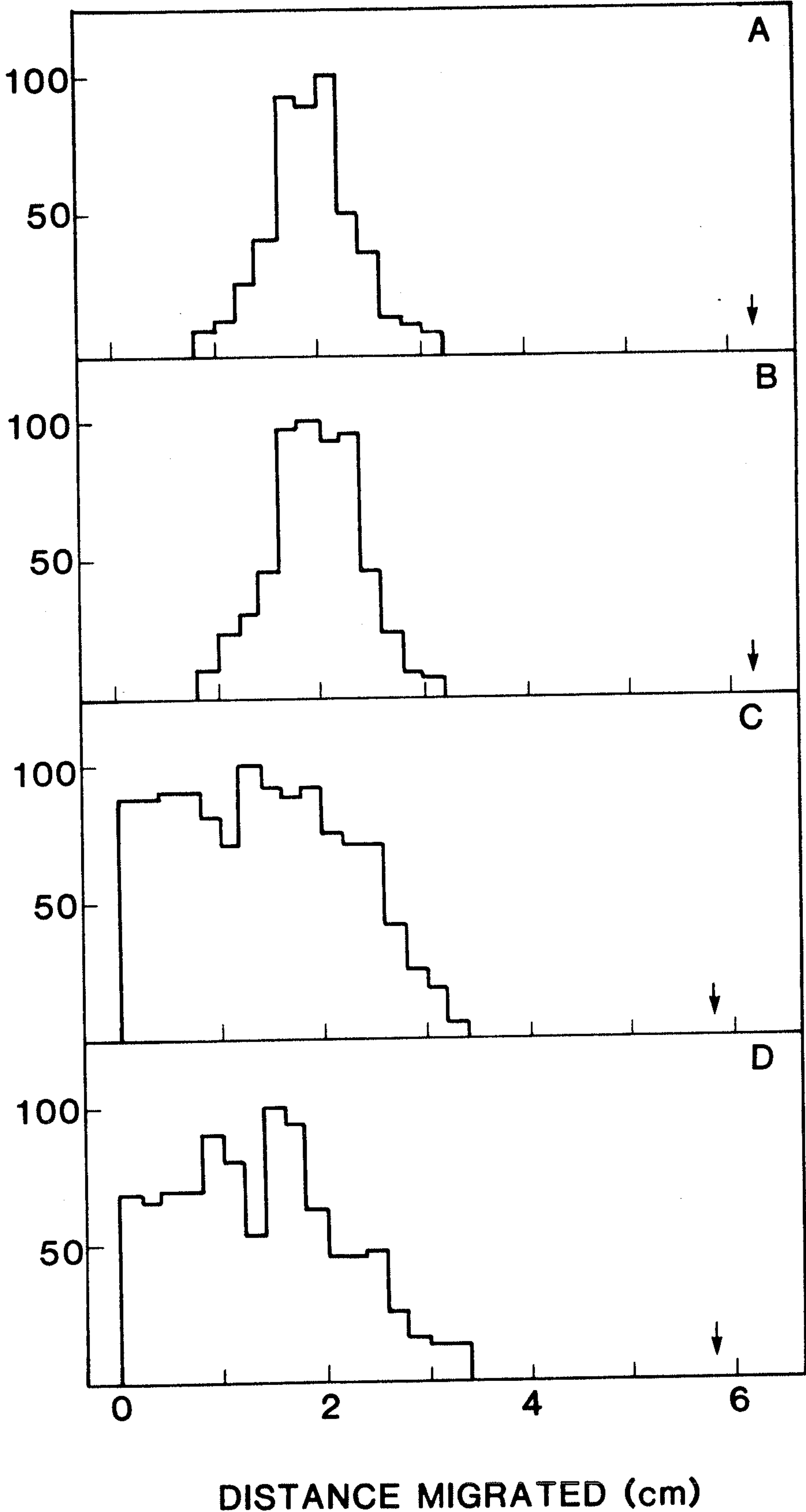
Liver appears to contain at least six sialyl transferases. The two enzymes responsible for elaborating the NeuAc$\alpha$2→6Gal$\beta$1→4GlcNAc and NeuAc$\alpha$2→3Gal$\beta$1→3(4)GlcNAc sequences of asparagine-linked oligosaccharide chains of glycoproteins, such as rat $\alpha_1$-acid glycoprotein (Yoshima et al., 1981), have recently been purified and characterized by Paulson's group (Weinstein et al., 1982a,b). Rat $\alpha_1$-acid glycoprotein has also been reported to contain the sequence NeuAc$\alpha$2→3Gal$\beta$1→3(NeuAc$\alpha$2→6)GlcNAc (Yoshima et al., 1981), but the enzyme capable of synthesizing the NeuAc$\alpha$2→6GlcNAc linkage has not yet been detected. Three other liver sialyl transferases have been described, but these enzymes are involved in ganglioside synthesis and in synthesis of the O-linked oligosaccharide chains of fetuin (Keenan et al., 1974; Spiro and Bhoyroo, 1974; Eppler et al., 1980a,b). In the case of the sialyl transferases characterized by Paulson's group (Weinstein et al., 1982a,b) the $\alpha$2→6 enzyme had a high specificity for substrates containing the Gal$\beta$1→4GlcNAc sequence to form NeuAc$\alpha$2→6Gal$\beta$1→4GlcNAc. The other enzyme utilized substrates containing Gal$\beta$1→3GlcNAc, Gal$\beta$1→4GlcNAc or Gal$\beta$1→3(NeuAc$\alpha$2→6)GlcNAc, in all cases linking NeuAc$\alpha$2→3 to the terminal Gal. Both enzymes showed low activities towards lactose with the $\alpha$2→3 and $\alpha$2→6 sialyl transferases forming the 3' and 6' isomers of sialyllactose, respectively. Neither enzyme utilized O-linked oligosaccharides of glycoproteins as substrates.

In the studies outlined above, the high molecular weight acceptors, rat and human asialo-$\alpha_1$-acid glycoprotein, were used to assay enzyme. However, the rat protein can accept NeuAc linked in three different ways to the oligosaccharide chains (Yoshima et al., 1981). In order to

identify the sialyl activities in liver, medium and serum from experiment with control and inflamed rats, two approaches were used. In the first approach, an attempt was made to identify the enzymes in liver, medium and serum based on their electrophoretic mobilities. In the second approach, lactose was used as an acceptor substrate and the products of the reaction were analyzed to identify the linkage specificities of the enzymes from the various sources.

Polyacrylamide gel electrophoresis has been used to separate galactosyl transferases in serum of cancer patients (Podolsky and Weiser, 1975). This approach was therefore applied to the sialyl transferases in preparations of liver, medium and serum from experiments with control and 48 h inflamed rats. The results of polyacrylamide gel electrophoresis are shown in Fig. 22. Both medium samples, from experiments with control and 48 h inflamed rats, contained one broad band of sialyl transferase activity which migrated between 1.5 cm and 2.5 cm from the top of the resolving gel (Fig. 22). Both control and 48 h experimental liver samples contained two major broad bands of enzyme activity; a slow moving band migrated between 0 cm and 1 cm from the top of the resolving gel, and a fast band migrated between 1.2 cm and 2.6 cm (Fig. 22). Several attempts to determine the electrophoretic behavior of control and 48 h experimental serum sialyl transferases were unsuccessful. These experiments were complicated by the high protein content of serum which limited the amount of enzyme activity which could be subjected to electrophoresis. Attempts to fractionate the serum to alleviate this problem resulted in a substantial loss of enzyme activity. This was not surprising since many glycosyl transferases have been found to be quite unstable after partial purification (Beyer _et al_., 1981). As a result

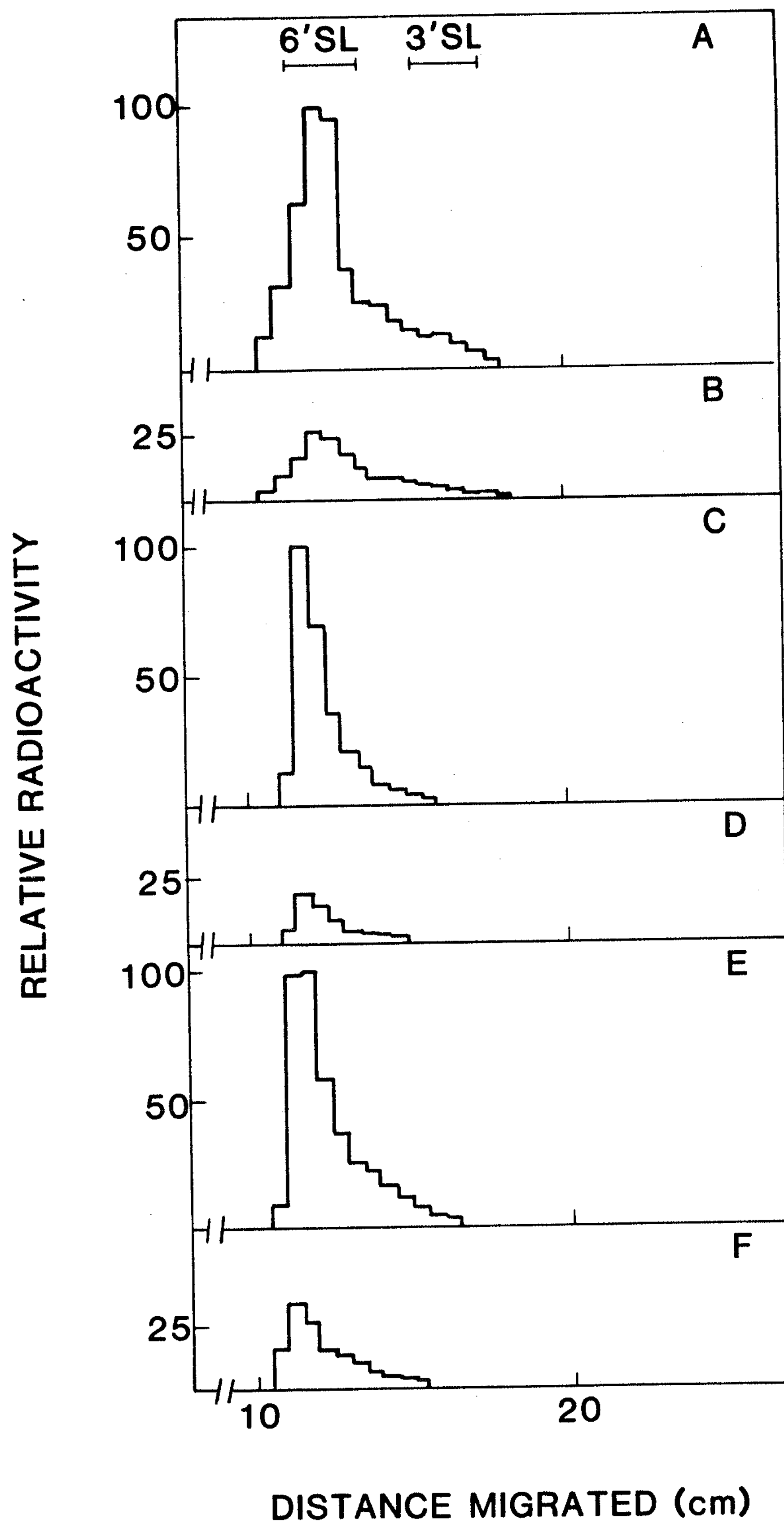
Fig. 22. Polyacrylamide gel electrophoresis patterns of liver slice medium and liver sialyl transferases in experiments with control and 48 h inflamed rats. Resolving gels were 5.5 x 75 mm. Panels A and B, experimental and control medium, respectively; panels C and D, experimental and control liver, respectively. Results are expressed as relative dpm [$^{14}C$] using the maximum dpm from each sample as 100%. The 100% values for panels A, B, C, and D were 974, 297, 917, and 396 dpm, respectively. Arrow indicates dye front. See Experimental for details.


A
B
C
D
100
50
0
2
4
6
PERCENT MAXIMUM SIALYL TRANSFERASE ACTIVITY
DISTANCE MIGRATED (cm)

of these observations, this procedure was abandoned in favor of the second approach involving the use of lactose as an acceptor.

The linkage specificities of the sialyl transferase activities present in liver, medium and serum from experiments with control and 48 h inflamed rats were determined using lactose as acceptor substrate; the reaction products were identified following five days of descending paper chromatography. In these experiments, neither lactose nor CMP-[$^{14}$C]-NeuAc was limiting so that semi-quantitative information was obtained on the relative amounts of sialyllactose isomers formed in the reaction. The results of these experiments are shown in Fig. 23. Although radiochromatogram scans were made, numerical data could only be obtained by cutting up the chromatograms and counting in a liquid scintillation counter; therefore, the results are presented in the form of histograms. Both 6'-($\alpha$2→6) and 3'-($\alpha$2→3) isomers of sialyllactose were detected as reaction products using liver as enzyme source. The 6'-($\alpha$2→6) isomer of sialyllactose was easily identified as the major reaction product with the 3'-($\alpha$2→3) isomer appearing as a shallow peak or plateau region migrating ahead of the 6'-($\alpha$2→6) isomer (Fig. 23). Liver from 48 h experimental rats formed larger amounts of both sialyllactose isomers under the assay conditions used (Fig. 23). Medium from slice experiments showed that 6'-($\alpha$2→6) sialyllactose was the main or only reaction product with medium from experiments with slices from 48 h inflamed rats forming larger amounts of reaction product (Fig. 23). The results obtained with serum as enzyme source were very similar to those found with liver slice medium; the 6'-($\alpha$2→6) isomer of sialyllactose was the major reaction product with serum from 48 h inflamed rats producing much larger amounts of reaction product (Fig. 23). Clearly, the major form of sialyl transferase in serum from

Fig. 23. Sialyllactose isomers produced by liver, serum and liver slice medium sialyl transferases in experiments with control and 48 h inflamed rats; 6'SL shows the position of the 6'(NeuAcα2→6Galβ1→4Glc) isomer, and 3'SL shows the position of the 3'(NeuAcα2→3Galβ1→4Glc) isomer. Chromatography was for five days (see Experimental). Panels A and B, experimental and control liver, respectively, using 1 mg liver protein; panels C and D, experimental and control serum, respectively, using 80 μl serum; and panels E and F, experimental and control medium, respectively, using 80 μl medium. Chromatograms were cut into 0.5 cm widths and counted in a liquid scintillation counter. Results are expressed as relative dpm [$^{14}C$] using the maximum dpm from experimental samples as 100% for each pair. The 100% values for liver, serum and medium were 26,700, 29,643 and 36,200 dpm, respectively. See Experimental for further details.


6'SL
3'SL
A
B
C
D
E
F
100
50
25
100
50
25
100
50
25
10
20
RELATIVE RADIOACTIVITY
DISTANCE MIGRATED (cm)

inflamed rats was the α2→6 enzyme which was the same activity as that found in elevated levels in medium from liver slice experiments. This strongly suggests a hepatic origin for the elevated sialyl transferase activity in serum from inflamed rats.

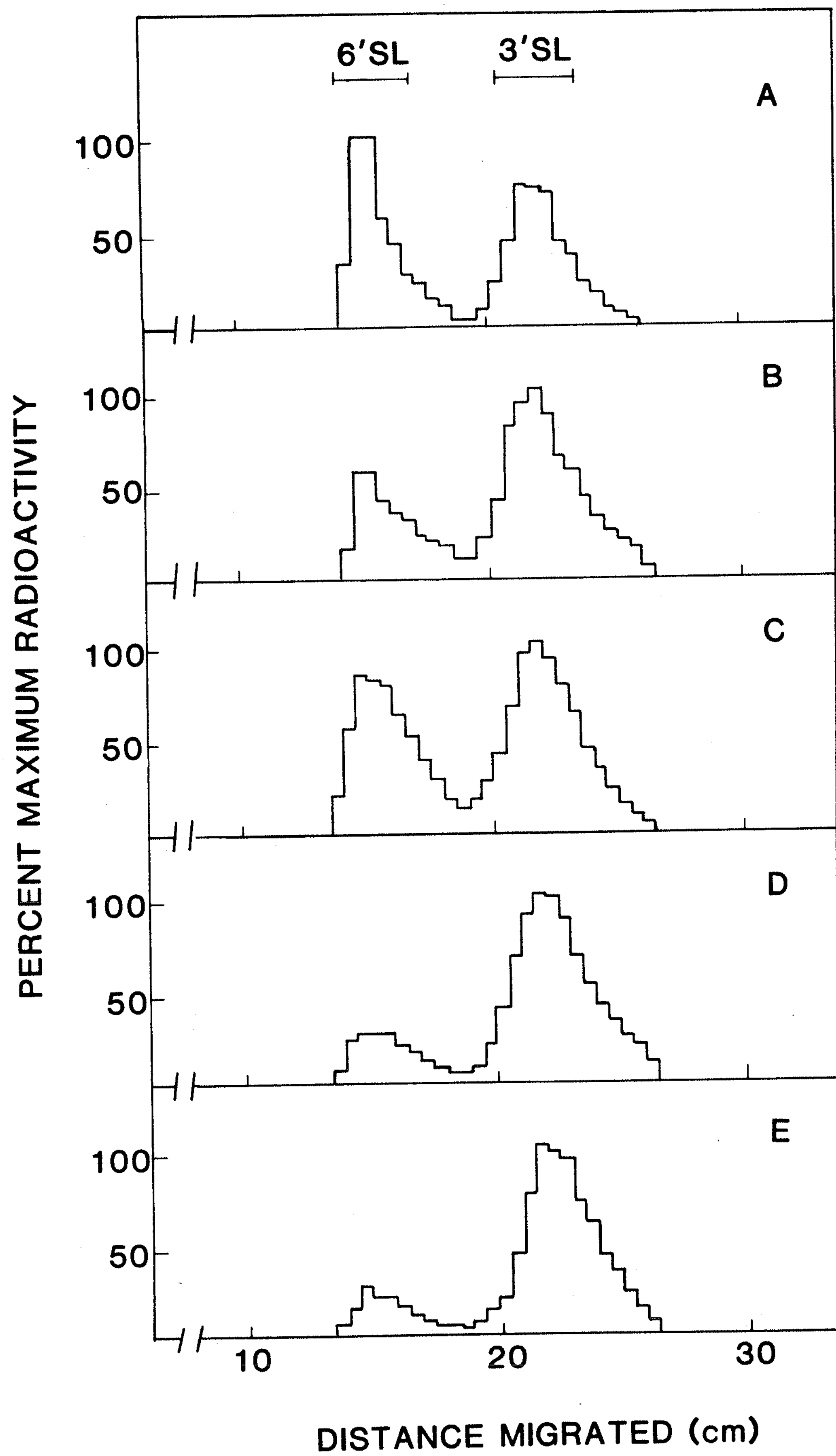
Elevations in serum sialyl transferase activity have been found in cancer patients (Kessel and Allen, 1975; Kessel *et al.*, 1976, 1978; Henderson and Kessel, 1977; Ip and Dao, 1978; Silver *et al.*, 1979). In view of the results obtained in the present work, it was of interest to determine the nature of the elevated serum sialyl transferase activity in humans suffering from cancer. Human serum samples from metastatic cancer patients were kindly supplied by the Manitoba Cancer Institute; suitable control samples were provided by normal, healthy volunteers. Serum sialyl transferase activities from normal individuals and cancer patients using rat asialo-$\alpha_1$-acid glycoprotein as acceptor substrate are shown in Table 8. Sialyl transferase activities were elevated two- to three-fold in serum from cancer patients compared to controls. Lactose was used as before to determine linkage specificities. The results are shown in Fig. 24. Unlike rat serum (see Fig. 23), both 3'-(α2→3) and 6'-(α2→6) isomers of sialyllactose were produced by the enzyme activities from normal and pathological sera (Fig. 24). In contrast to rat serum, the 3'-(α2→3) isomer of sialyllactose was the major product formed by the two normal human serum samples studied (Fig. 24). However, as in the rat system, it was the α2→6 enzyme which increased significantly in pathological conditions, suggesting that this enzyme may also be responsive to inflammation in humans. These experiments were not continued further because they did not represent the prime objective of the work. However, it is clear

Table 8. Human serum sialyl transferase activities in normal individuals and metastatic cancer patients.

| Serum sample | Sialyl transferase activity |
|---|---|
| Control 1 | 19.7 |
| 2 | 21.3 |
| Experimental 1 | 31.3 |
| 2 | 44.7 |
| 3 | 36.8 |
| 4 | 44.7 |
| 5 | 38.2 |
| 6 | 63.6 |
| 7 | 51.4 |
| 8 | 40.0 |
| 9 | 59.7 |
| 10 | 53.4 |

Enzyme activity is expressed as pmoles NeuAc transferred per min per ml serum with 250 μg rat asialo-$\alpha_1$-acid glycoprotein as acceptor substrate. Each assay was performed in triplicate; reproducibility was within ±10%.

Fig. 24. Sialyllactose isomers produced by human serum sialyl transferases from normal individuals and patients with neoplastic diseases. Chromatography was for seven days. Panels A, B, and C, pathological sera -- experimental samples 8, 2 and 3 from Table 8, respectively; panels D and E, normal sera -- control samples 1 and 2 from Table 8, respectively. Results are expressed as relative dpm [$^{14}C$] using the maximum dpm from each sample as 100%. The 100% values for panels A, B, C, D and E were 7640, 3818, 8498, 8774 and 7624 dpm, respectively. See Table 8 and Experimental for further details.


6'SL
3'SL
A
B
C
D
E
100
50
10
20
30
PERCENT MAXIMUM RADIOACTIVITY
DISTANCE MIGRATED (cm)

that a detailed study of the human sialyl transferase activities would be of interest; in addition, sera from other species should be examined to determine the relative abundances of the α2→3 and α2→6 activities.

Nature of the Release of Sialyl Transferase from Liver

Results from the present work suggest that the elevated serum sialyl transferase activity in inflamed rats is of hepatic origin. Elevated hepatic and serum activities following inflammation, coupled with increased release of the enzyme activity from liver slices prepared from inflamed rats, suggest that the α2→6 sialyl transferase is behaving like an acute phase reactant. As previously mentioned in the Introduction, acute phase proteins are synthesized on bound polyribosomes and are transported via the channels of the rough and smooth endoplasmic reticulum and Golgi complex whereupon they enter secretory vesicles; these vesicles eventually fuse with the plasma membrane and release their contents, secretory proteins, outside the cell. Since liver sialyl transferases are membrane-bound enzymes located mainly in the Golgi complex, it was of interest to examine the nature of the release of α2→6 sialyl transferase from liver. Thus, an attempt was made to obtain information on release of sialyl transferase from liver by studying the effect of various compounds that can effect synthesis and secretion of liver from proteins. Compounds examined were colchicine, puromycin, cycloheximide, tunicamycin, trypsin and pronase B.

Numerous studies involving liver and other tissues have implicated the microtubular system in secretory processes (Lacy et al., 1968; Williams and Wolff, 1972; Le Marchand et al., 1973, 1974; Stein and Stein, 1973; Ehrlich et al, 1974; van Obberghen et al., 1974, Feldmann and Maurice, 1975; Feldmann et al., 1975; Redman et al., 1975; Banerjee

*et al.*, 1976; Patzelt *et al.*, 1977; Reaven and Reaven, 1980). In these studies, colchicine, an alkaloid that interferes with microtubular function (Wilson *et al.*, 1974), when applied to various cells in culture resulted in inhibitory effects on secretion or transport of molecules into medium. The effect of colchicine on the release of sialyl transferase activities from liver into medium in slice experiments with control and 24 h inflamed rats is shown in Table 9. Colchicine, although not significantly affecting liver sialyl transferase activities, caused a decrease in both control and experimental medium enzyme activities. Control medium sialyl transferase activities decreased about 30% in the present of $1.0 \times 10^{-5}$ M colchicine; no further decrease in enzyme activity was observed as the colchicine concentration was increased to $5.0 \times 10^{-5}$ M (Table 9). Experimental medium sialyl transferase activities decreased from 23% to 45% as the colchicine concentration was increased from $1.0 \times 10^{-5}$ M to $5.0 \times 10^{-5}$ M (Table 9). These results suggest the possible involvement of the microtubular system in the release of sialyl transferase from liver.

In order to determine whether the release of sialyl transferase from liver was dependent on the synthesis of protein *de novo*, slice experiments were performed in the presence of puromycin and cycloheximide, inhibitors of protein synthesis (Pestka, 1977). Since glycosyl transferases are believed to be glycoproteins containing N-linked oligosaccharide chains (Beyer *et al.*, 1981; Fujita-Yamaguchi and Yoshida, 1981), experiments were also performed in the presence of tunicamycin, an inhibitor of N-linked glycosylation (Schwartz and Datema, 1982), to determine the consequences of inhibiting protein glycosylation on liver and medium sialyl transferase activities. Results from these experiments

Table 9. Effect of colchicine on release of sialyl transferase into slice medium.

| Colchicine concentration (M) | Sialyl transferase activity | | | |
|---|---|---|---|---|
| | Control | | 24 h Inflamed | |
| | Liver | Medium | Liver | Medium |
| 0 | 8.31±0.60 | 3.69±0.21 | 12.93±0.91 | 6.42±0.43 |
| $1.0 \times 10^{-5}$ | 8.30±0.42 | 2.58±0.14 | 13.32±1.02 | 4.96±0.28 |
| $2.5 \times 10^{-5}$ | 8.13±0.44 | 2.86±0.20 | 12.93±0.75 | 4.01±0.20 |
| $5.0 \times 10^{-5}$ | 8.60±0.35 | 2.75±0.17 | 12.83±0.65 | 3.54±0.15 |

Liver and medium enzyme activities are expressed in Figs. 16 and 18, respectively; medium is from 2 h slice incubations. Results are the means and standard deviations of the means of 3-4 analyses.

are given in Table 10. Little or no change was observed in liver and medium sialyl transferase activities from slice experiments with control and 12 h inflamed rats, when slice incubations contained puromycin, cycloheximide or tunicamycin. In some of these experiments, liver slice incubation medium was supplemented with [$^{3}$H]glucosamine (0.4 μCi/ml medium) to follow incorporation into glycoprotein oligosaccharide chains. The specific radioactivities of total liver and medium proteins were determined using the trichloroacetic acid precipitation method of Jamieson and Ashton (1973b). The effect of puromycin and tunicamycin on [$^{3}$H]glucosamine incorporation into 12 h experimental liver and medium proteins is shown in Table 11. Both puromycin and tunicamycin caused a decrease of about 50% in the specific radioactivities of liver and medium proteins (Table 11). The above results suggest that the release of sialyl transferase activity from liver did not depend on protein synthesis _de novo_ or on the glycosylation of N-linked glycoproteins.

Glycosyl transferase activities have been shown to be localized on the surface of some cell types including rat liver cells (Pricer and Ashwell, 1971; Pierce _et al_., 1980). Experiments were performed to determine if medium sialyl transferase activity was due to cell surface shedding of the enzyme by partial proteolytic degradation (Magee _et al_., 1973; Powell and Brew, 1974; Magee _et al_., 1976; Sadler _et al_., 1979). Table 12 shows the effect of low levels of trypsin and pronase B added to liver slice incubations. While having little or no effect on liver sialyl transferase activity, both proteases substantially decreased medium enzyme activities probably by proteolytic degradation of the soluble sialyl transferases (Table 12).

Table 10. Effect of puromycin, cycloheximide and tunicamycin on liver slice and medium sialyl transferase activities.

| Addition: concentration | | Sialyl transferase activity | | | |
|---|---|---|---|---|---|
| | | Control | | 24 h Inflamed | |
| | | Liver | Medium | Liver | Medium |
| None | - | 6.20±0.51 | 6.10±0.46 | 13.2±0.65 | 8.41±0.75 |
| Puromycin: | $10^{-5}$ M | 6.71±0.44 | 6.40±0.42 | 13.9±0.80 | 8.77±0.72 |
| | $10^{-4}$ M | 6.83±0.49 | 6.18±0.51 | 13.8±0.66 | 8.55±0.62 |
| Cycloheximide: | $10^{-4}$ M | 6.82±0.40 | 6.31±0.44 | 13.9±0.85 | 9.21±0.83 |
| Tunicamycin: | 5 μg/ml | 5.93±0.37 | 5.78±0.41 | 13.0±0.78 | 7.86±0.71 |

Liver and medium enzyme activities are expressed as in Figs. 16 and 18, respectively; medium and liver samples are from 3 h slice incubations. Results are the means and standard deviations of the means of 3-4 analyses.

Table 11. Effect of puromycin and tunicamycin on [$^{3}H$]glucosamine incorporation into total liver and medium proteins.

| Addition to liver slice assay | Specific radioactivity (nCi/mg protein) | |
|---|---|---|
| | Liver | Medium |
| None | 0.82 ± 0.090 | 6.6 ± 0.71 |
| Puromycin ($10^{-4}$ M) | 0.37 ± 0.041 | 3.7 ± 0.30 |
| Tunicamycin (5 μg/ml) | 0.42 ± 0.032 | 3.4 ± 0.21 |

Medium and liver samples are from 3 h incubations with liver slices prepared from 12 h inflamed rats. Results represent the means and standard deviations of the means of 4-6 analyses.

Table 12. Effect of trypsin and pronase B on sialyl transferase activity in a liver slice system.

| | Enzyme activity[a] | | | |
|---|---|---|---|---|
| | Control | | 48 h Inflamed | |
| | Liver | Medium | Liver | Medium |
| Protease | Ratio treated/untreated | | | |
| Trypsin (5 μg/ml medium) | 1.06 | 0.21 | 0.91 | 0.28 |
| Pronase B (5 μg/ml medium) | 0.93 | 0.13 | 1.05 | 0.16 |

[a]Liver and medium samples are from 3 h slice incubations. Results represent the means of 2 analyses; reproducibility was within ±10%.

## STUDIES ON THE EFFECT OF INFLAMMATION ON RAT LIVER NUCLEOTIDE SUGAR POOLS

As stated in the Introduction, nucleotide sugars serve as the glycosyl donors for complex carbohydrate assembly; the transfer of a sugar from its nucleotide derivative to an acceptor molecule is catalyzed by an appropriate glycosyl transferase. In the preceding study, hepatic glycosyl transferase activities were found to increase following onset of inflammation, probably in response to increased hepatic glycoprotein biosynthesis (Kushner, 1982; Jamieson *et al.*, 1983). The availability of nucleotide sugars in liver is obviously essential for complex carbohydrate biosynthesis in both normal and inflamed rats. In order to help determine the relationship between nucleotide sugar and glycoprotein biosynthesis during inflammation, two studies were performed. The first involved studies aimed at determining the effect of inflammation on pools of those nucleotide sugars of importance for oligosaccharide chain assembly of N-linked glycoproteins. Since nucleotide sugar biosynthesis is under regulatory control, the second study involved attempts to determine how two key enzymes of nucleotide hexosamine metabolism were altered in inflammation. In addition, a nonregulatory enzyme was also examined in these studies.

### Liver Nucleotide Sugar Pools

#### UDP-N-acetylhexosamines

The hepatic levels of UDP-GlcNAc and UDP-GalNAc were determined using an amino acid analyzer after acid hydrolysis of the nucleotide sugars to release free amino sugars (see Experimental); amino sugars were determined colorimetrically by post-column coupling to ninhydrin.

The result of a typical sample run on the amino acid analyzer is shown in Fig. 25. It is worthwhile to note that the amino sugars were well separated from any contaminating ninhydrin-positive molecules (Fig. 25).

The effect of experimental inflammation on hepatic levels of UDP-N-acetylhexosamines is shown in Fig. 26. Control rat liver contained 141 nmoles of UDP-N-acetylhexosamines per g wet weight liver composed of 95 nmoles UDP-GlcNAc and 46 nmoles UDP-GalNAc. Inflammation caused a rapid rise in UDP-N-acetylhexosamine pools reaching a maximum at 8 h after inflammation when the pool sizes were about twice those found in controls. This was followed by a rapid decline reaching a value of about 50% above controls at 12 h after inflammation; a second minor peak was observed at 16 h after inflammation before a steady state was established where UDP-N-acetylhexosamine pools were about 50% above control values (Fig. 26). Figure 26 also shows that the response of UDP-GlcNAc to inflammation paralleled that of UDP-GalNAc although the pool of UDP-GlcNAc was about twice that of UDP-GalNAc. This observation was not surprising since it is known that UDP-GlcNAc 4-epimerase which forms UDP-GalNAc from UDP-GlcNAc maintains steady state levels of these nucleotide sugars such that UDP-GlcNAc levels are about twice those of UDP-GalNAc (Molnar _et al_., 1964).

GDP-Man, UDP-Glc, UDP-Gal and CMP-NeuAc

The hepatic levels of UDP-Gal, UDP-Glc and GDP-Man were determined by gas liquid chromatography (see Experimental). A typical chromatogram obtained using this method of carbohydrate analysis is shown in Fig. 27. As shown in Fig. 27, the alditol acetate sugar derivatives are well resolved. Hepatic pool sizes of CMP-NeuAc were determined

Fig. 25. Chromatogram of acid-hydrolyzed UDP-N-acetylhexosamines, for the determination of UDP-GlcNAc and UDP-GalNAc, on a Technicon Amino Acid Analyzer System model NC-2P. Nucleotide sugars were isolated from 1 g wet weight of liver from a 12 h inflamed rat; 0.73 ml of a 1.0 ml extract was chromatographed. Abbreviations: GlcN, glucosamine; GalN, galactosamine; $NH_3$, ammonia.

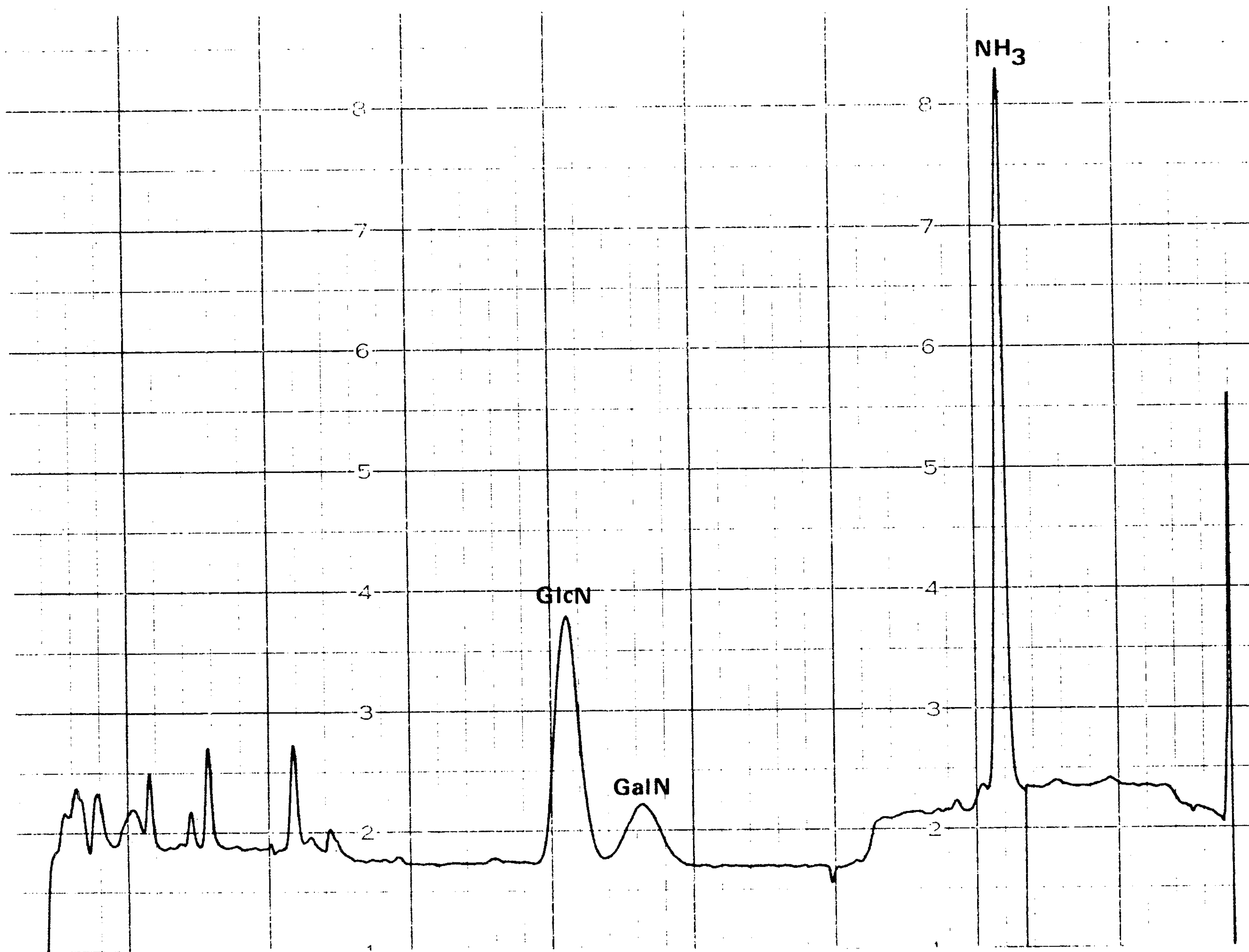

$NH_3$
GlcN
GalN
8
7
6
5
4
3
2

Fig. 26. Effect of inflammation on hepatic levels of UDP-GlcNAc, - ● - , and UDP-GalNAc, - ▲ - , as determined by chromatography on the amino acid analyzer system. Results are the means of 4-6 analyses; the bars represent the range of standard deviations of the means.

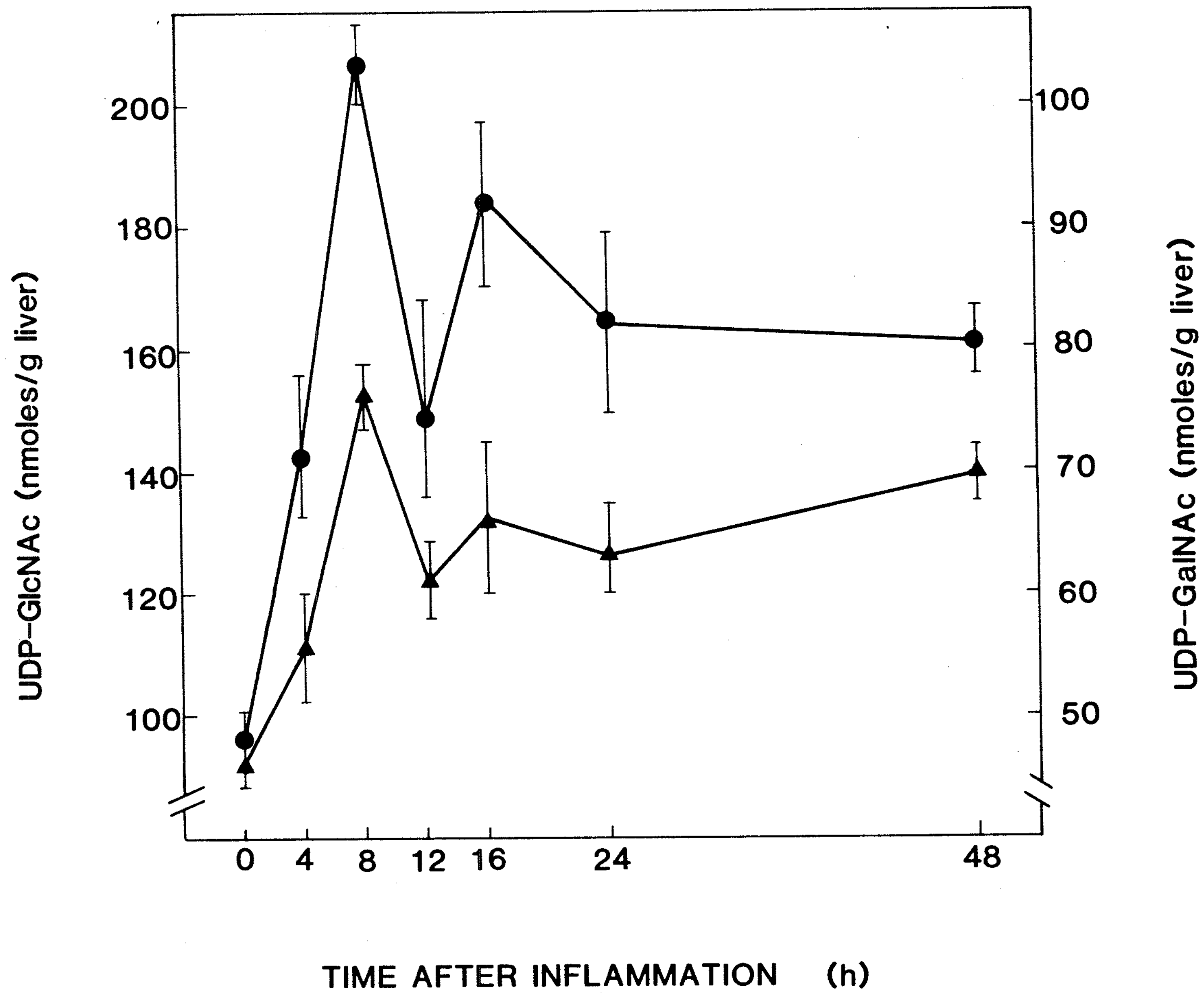
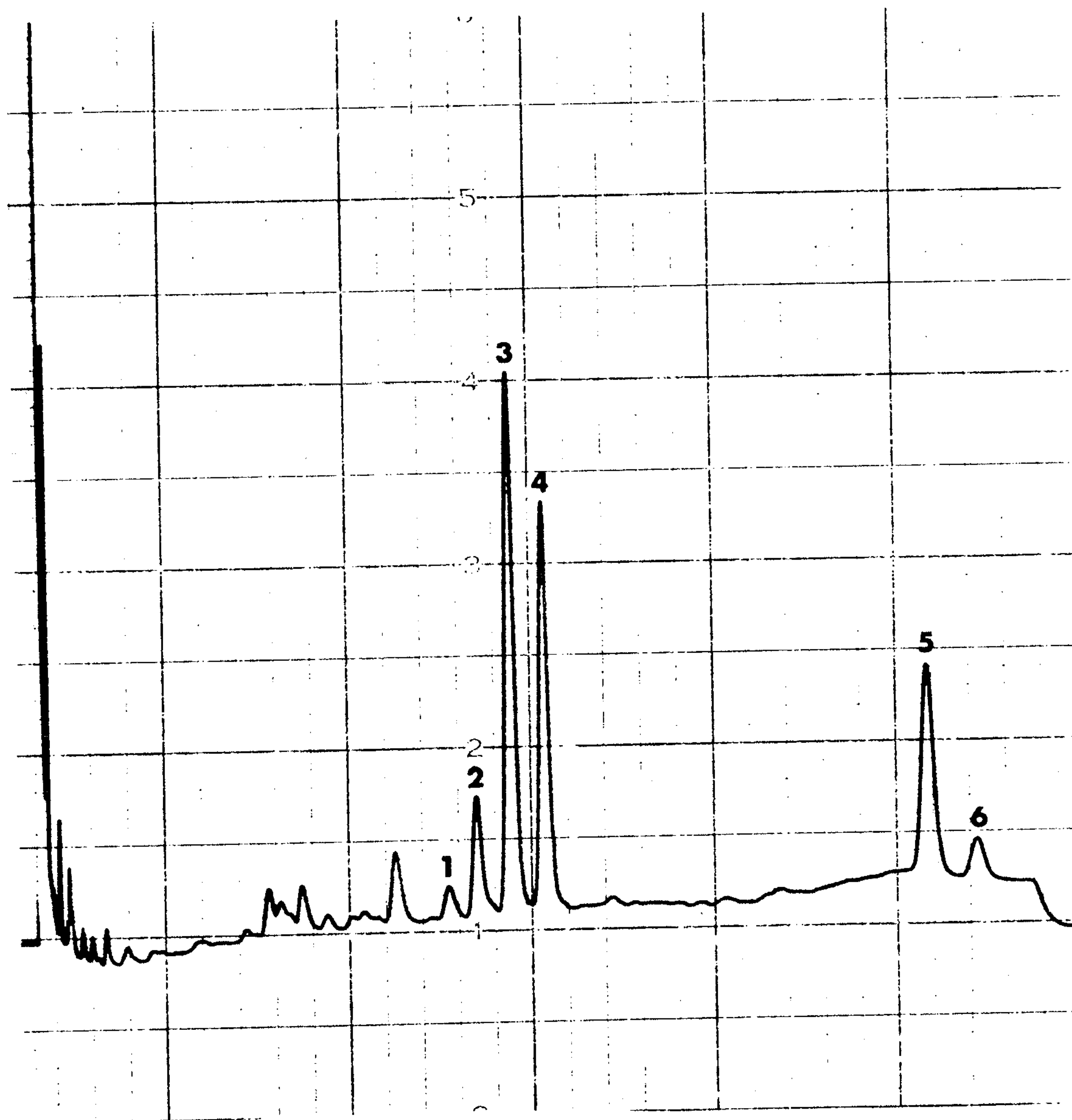

200
180
160
140
120
100
UDP-GlcNAc (nmoles/g liver)
100
90
80
70
60
50
UDP-GalNAc (nmoles/g liver)
0
4
8
12
16
24
48
TIME AFTER INFLAMMATION (h)

Fig. 27. Chromatogram of alditol acetate sugar derivatives. Acid hydrolyzed nucleotide sugars were analyzed on a Perkin-Elmer Sigma 2B gas liquid chromatograph equipped with a flame-ionization detector and a 1.8 m x 2 mm glass column containing 3% SP2330 on 100-200 mesh Supelcoport; results are from a typical control liver. Numbered peaks: 1, mannose; 2, galactose; 3, glucose; 4, myo-inositol (internal standard); 5, N-acetylglucosamine; 6, N-acetylgalactosamine.


1
2
3
4
5
6

following descending paper chromatography by the colorimetric method of Warren (1959; see Experimental).

Figure 28 shows that the control level of GDP-Man was 4.8 nmoles/g wet weight liver. Inflammation resulted in a rapid increase to about 70% above control values within 4 h after inflammation; there was then a rapid decline to about half control values at 12 h followed by a second peak at 24 h before returning close to control values at 48 h after inflammation. Although the levels of GDP-Man were much lower than those found for UDP-N-acetylhexosamines (Fig. 26), the response did bear a resemblance to that found with UDP-N-acetylhexosamines. For example, all three nucleotide sugars increased rapidly to about twice control values at short times after inflammation (i.e., at 4 h for GDP-Man and 8 h for UDP-N-acetylhexosamines (Figs. 26 and 28), declined at 12 h after inflammation and then increased again at longer times after inflammation (see Figs. 26 and 28).

The effects of inflammation on hepatic levels of CMP-NeuAc and UDP-Gal are shown in Fig. 29. The control level of CMP-NeuAc was 49 nmoles/g wet weight liver, but this was reduced by about 30% at 8-12 h after inflammation; there was then a rapid increase to give values slightly above control values at 24-48 h after inflammation. The response of UDP-Gal to inflammation seemed to parallel that of CMP-NeuAc (Fig. 29). The control level of UDP-Gal was 31 nmoles/g wet weight liver. Like CMP-NeuAc the level of UDP-Gal declined at short times after inflammation before increasing rapidly to just above control values at 16-48 h after inflammation (Fig. 29).

The effect of inflammation on hepatic levels of UDP-Glc is shown in Fig. 30. The control level was 133 nmoles/g wet weight liver, but

Fig. 28. Effect of inflammation on hepatic levels of GDP-Man. Results are the means of 4-8 analyses; the bars represent the range of standard deviations of the means.

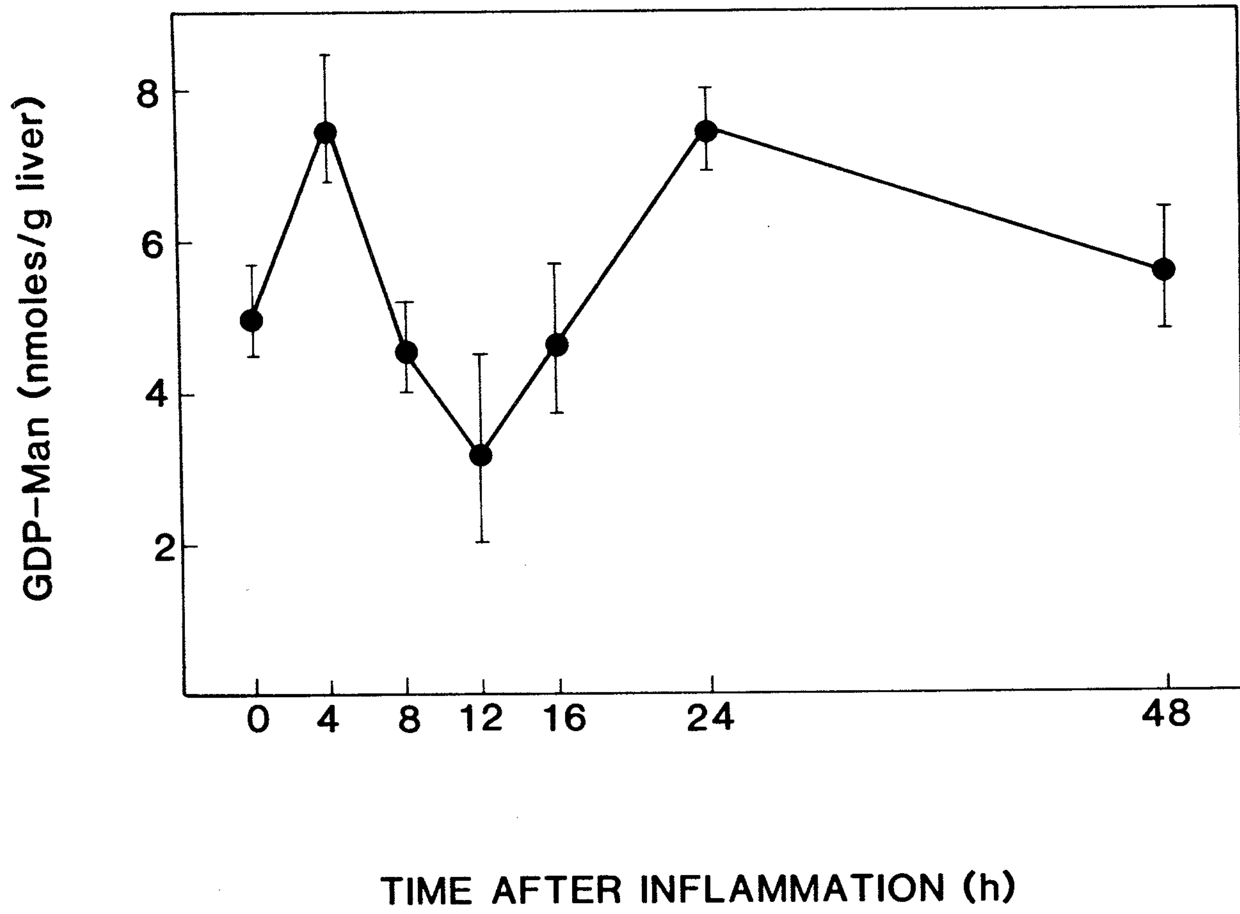
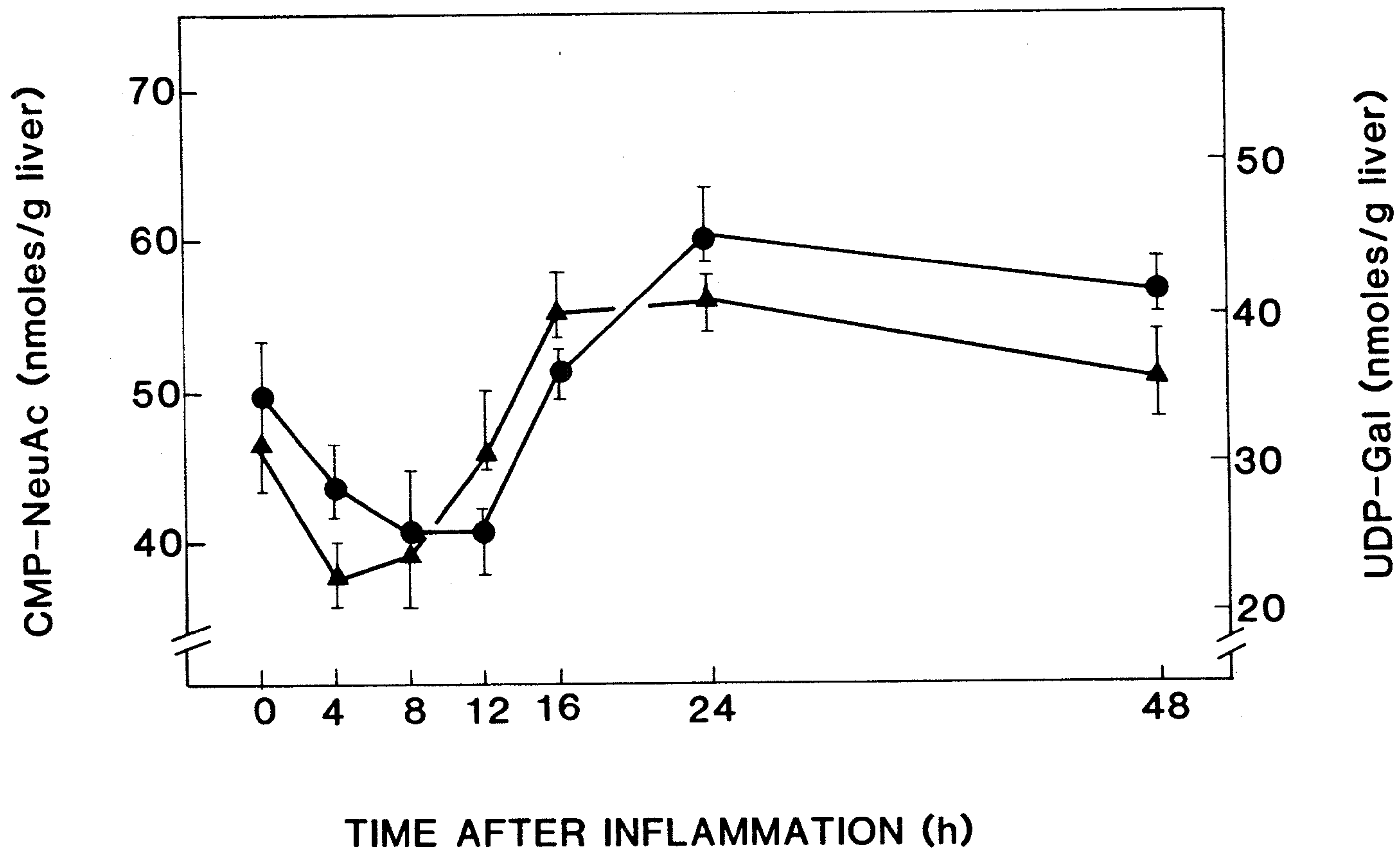
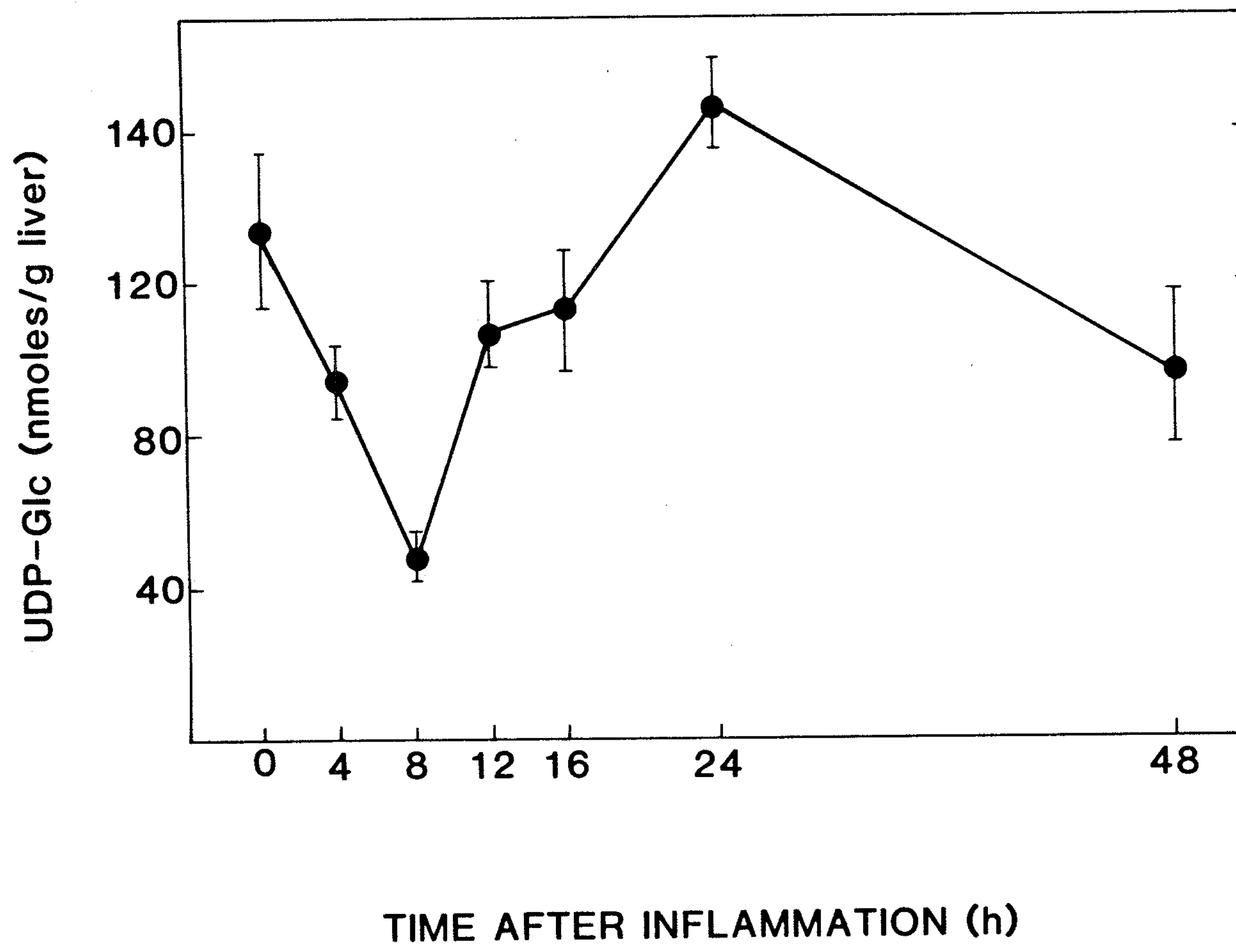

GDP-Man (nmoles/g liver)
8
6
4
2
0
4
8
12
16
24
48
TIME AFTER INFLAMMATION (h)

Fig. 29. Effect of inflammation on hepatic levels of CMP-NeuAc, - ● - , and UDP-Gal, - ▲ - . Results are the means of 4-8 analyses; the bars represent the range of standard deviations of the means.



70
60
50
40
CMP-NeuAc (nmoles/g liver)
50
40
30
20
UDP-Gal (nmoles/g liver)
0
4
8
12
16
24
48
TIME AFTER INFLAMMATION (h)

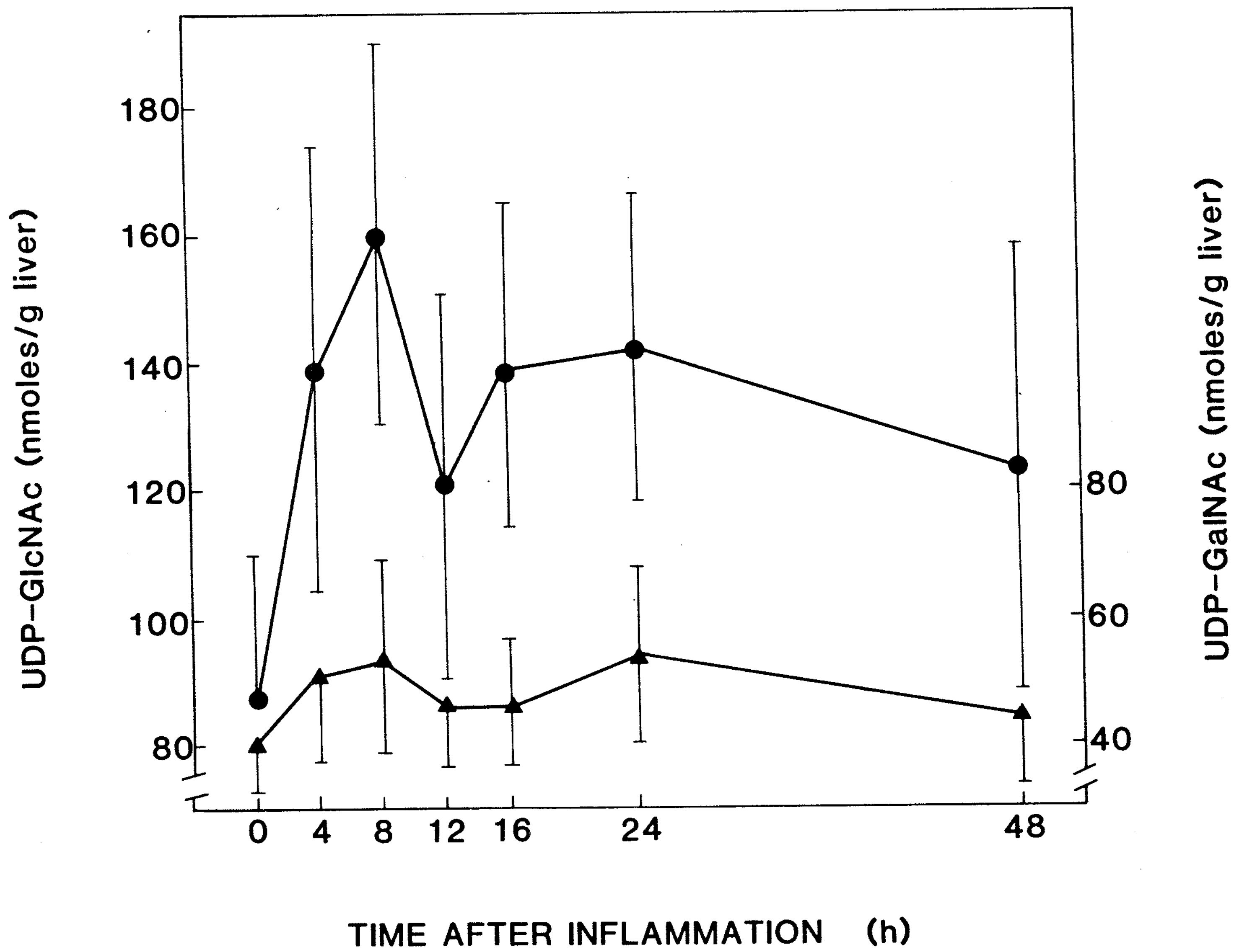
Fig. 30. Effect of inflammation on hepatic levels of UDP-Glc. Results are the means of 4-8 analyses; the bars represent the range of standard deviations of the means.


140
120
80
40
UDP-Glc (nmoles/g liver)
0
4
8
12
16
24
48
TIME AFTER INFLAMMATION (h)

this was reduced by about 60% at 8 h after inflammation; there was then an increase to give a maximum value of about 25% above controls at 24 h before declining to near control values at 48 h after inflammation. The response of UDP-Glc to inflammation resembled that found for CMP-NeuAc and UDP-Gal (Fig. 29). For example, all three nucleotide sugars decreased rapidly to about 30-60% below control values at 8 h after inflammation before rising to a maximum 25-50% above control values at 24 h after inflammation.

In addition to UDP-Gal, UDP-Glc and GDP-Man, the hepatic levels of UDP-N-acetylhexosamines were also analyzed by gas liquid chromatography. Using this method, the effects of inflammation on the hepatic levels of UDP-GlcNAc and UDP-GalNAc were determined and are shown in Fig. 31. Although the pool sizes and alterations of the UDP-N-acetylhexosamines were similar to the results obtained by the method employing the amino acid analyzer (Fig. 26), they were not identical. For example, the changes in UDP-GlcNAc and UDP-GalNAc levels following inflammation did not parallel each other as closely as was found by the method employing the amino acid analyzer (Figs. 26 and 31). In addition, except for control and 4 h values, the pool sizes of UDP-GlcNAc and UDP-GalNAc were found to be somewhat lower when determined by gas liquid chromatography (see Figs. 26 and 31). Analyses of N-acetylhexosamines by gas liquid chromatography are likely to be less reliable than analyses by the amino acid analyzer because of the lower general stability of amino sugars (Gottschalk, 1972) and the fact that the amino sugars are exposed to derivatization. It was noted that the ratio of hexoses to myo-inositol (internal standard) remained constant, whereas with hexosamines considerable variability was observed. Thus, the results

Fig. 31. Effect of inflammation on hepatic levels of UDP-GlcNAc, - ● -, and UDP-GalNAc, - ▲ -, as determined by gas liquid chromatographic analysis. Results are the means of 4-6 analyses; the bars represent the range of standard deviations of the means.



UDP-GlcNAc (nmoles/g liver)
180
160
140
120
100
80
UDP-GalNAc (nmoles/g liver)
80
60
40
0
4
8
12
16
24
48
TIME AFTER INFLAMMATION (h)

obtained from analyses of hexosamines with the amino acid analyzer are more likely to be reliable.

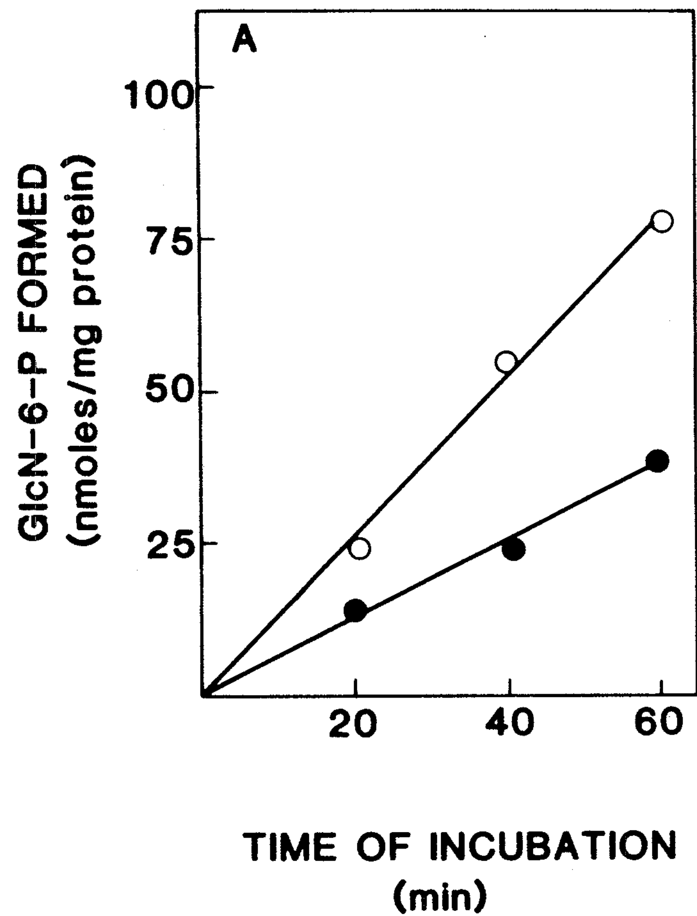
## Enzymes of Nucleotide Sugar Metabolism

The synthesis of UDP-GlcNAc and CMP-NeuAc are known to be regulated by feedback control (Kornfeld *et al*., 1964; Schachter, 1978). The regulatory enzyme for the synthesis of UDP-GlcNAc is glucosamine-6-phosphate synthase which converts fructose-6-phosphate to glucosamine-6-phosphate with glutamine acting as amide donor; the feedback inhibitor of this enzyme is UDP-GlcNAc. For the synthesis of CMP-NeuAc the first reaction is the synthesis of N-acetylmannosamine from UDP-GlcNAc by UDP-GlcNAc 2-epimerase which is subject to feedback control by CMP-NeuAc (Kornfeld *et al*., 1964; Schachter, 1978). In view of the alterations in hepatic levels of UDP-GlcNAc and CMP-NeuAc it was of interest to follow the changes in activities of the regulatory enzymes involved in UDP-GlcNAc and CMP-NeuAc synthesis; in addition, the activity of the nonregulatory enzyme CMP-NeuAc synthase was also measured.

Linear relationships were established for time of incubation and amount of enzyme protein for glucosamine-6-phosphate synthase (Fig. 32), UDP-GlcNAc 2-epimerase (Fig. 33) and CMP-NeuAc synthase (Fig. 34) activities.

The effect of inflammation on glucosamine-6-phosphate synthase is shown in Fig. 35. The specific activity of the enzyme in control livers was 35 units/mg protein/h. Inflammation resulted in a small increase in enzyme activity at 8 h followed by a decline to control values at 12 h after which there was a rapid rise to about twice that of control activities at 24 h after inflammation. Although there was

Fig. 32. Glucosamine-6-phosphate synthase activity as a function of time and amount of enzyme protein. Enzyme activity in control (●) and 24 h experimental (O) liver cell saps. Each plot represents the results from a typical experiment; reproducibilities were within ±10%.


A
100
75
50
25
20
40
60
GlcN-6-P FORMED
(nmoles/mg protein)
TIME OF INCUBATION
(min)

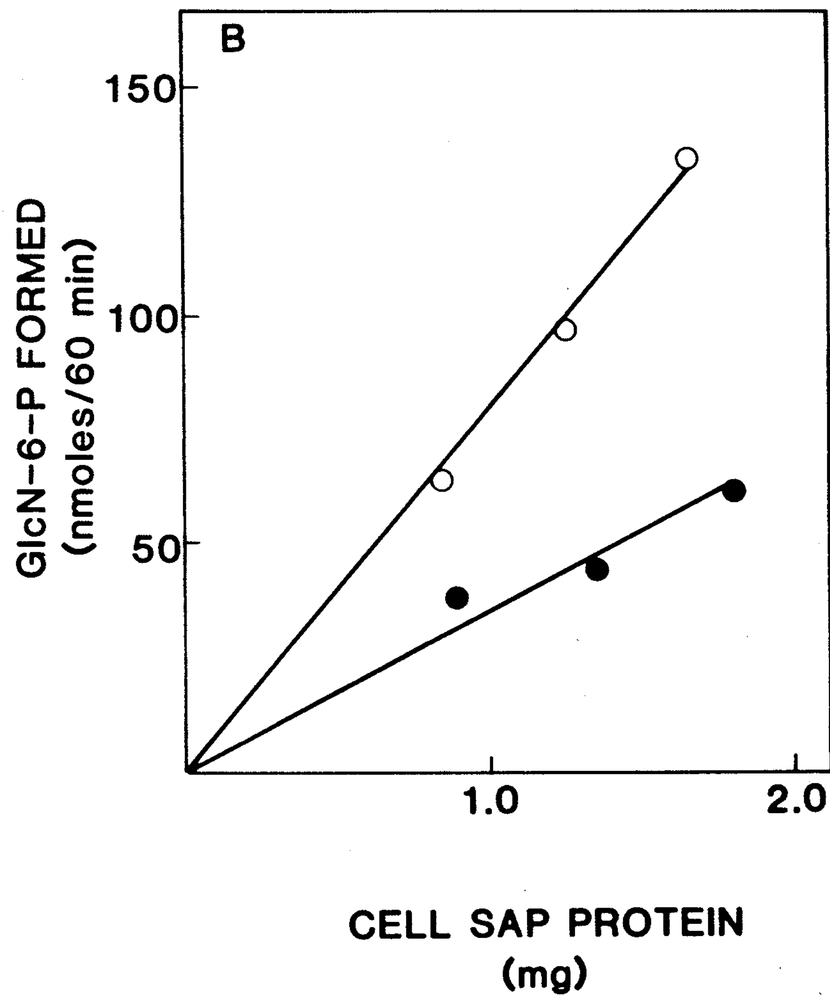
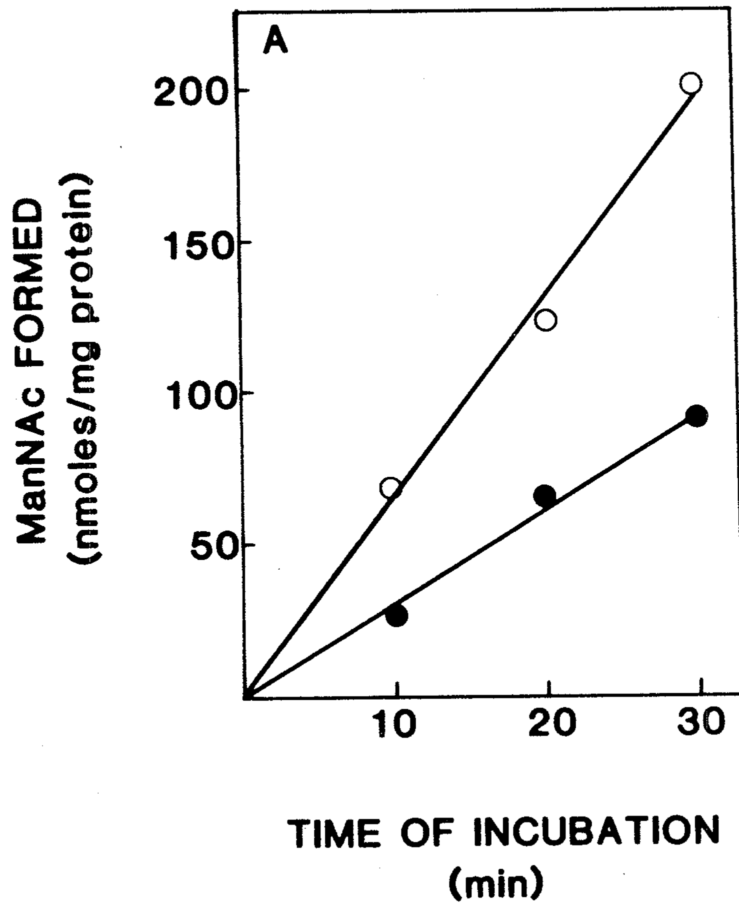

B
150
100
50
1.0
2.0
GlcN-6-P FORMED
(nmoles/60 min)
CELL SAP PROTEIN
(mg)

Fig. 33. UDP-GlcNAc 2-epimerase activity as a function of time and amount of enzyme protein. Enzyme activity in control (●) and 8 h (O) experimental liver cell saps. Each plot represents the results from a typical experiment; reproducibilities were within ±10%.


A
200
150
100
50
ManNAc FORMED
(nmoles/mg protein)
10
20
30
TIME OF INCUBATION
(min)

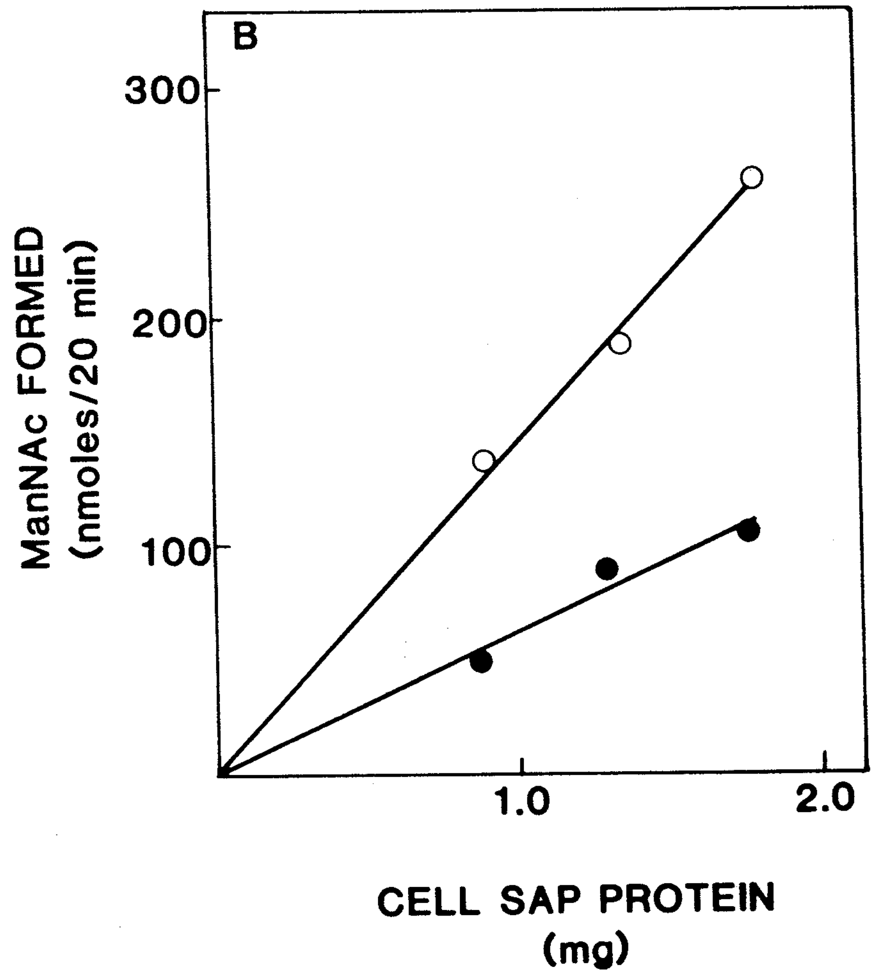
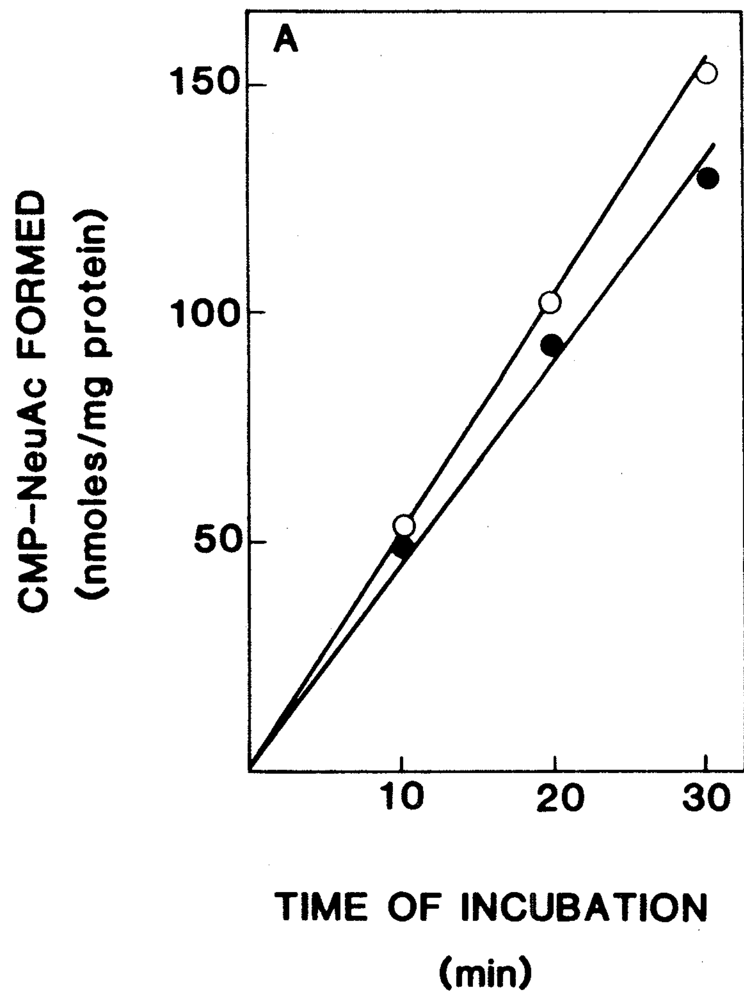

B
300
200
100
ManNAc FORMED
(nmoles/20 min)
1.0
2.0
CELL SAP PROTEIN
(mg)

Fig. 34. CMP-NeuAc synthase activity as a function of time and amount of enzyme protein. Enzyme activity in control (●) and 24 h experimental (O) liver cell saps. Each plot represents the results from a typical experiment; reproducibilities were within ±10%.


A
150
100
50
10
20
30
CMP-NeuAc FORMED
(nmoles/mg protein)
TIME OF INCUBATION
(min)

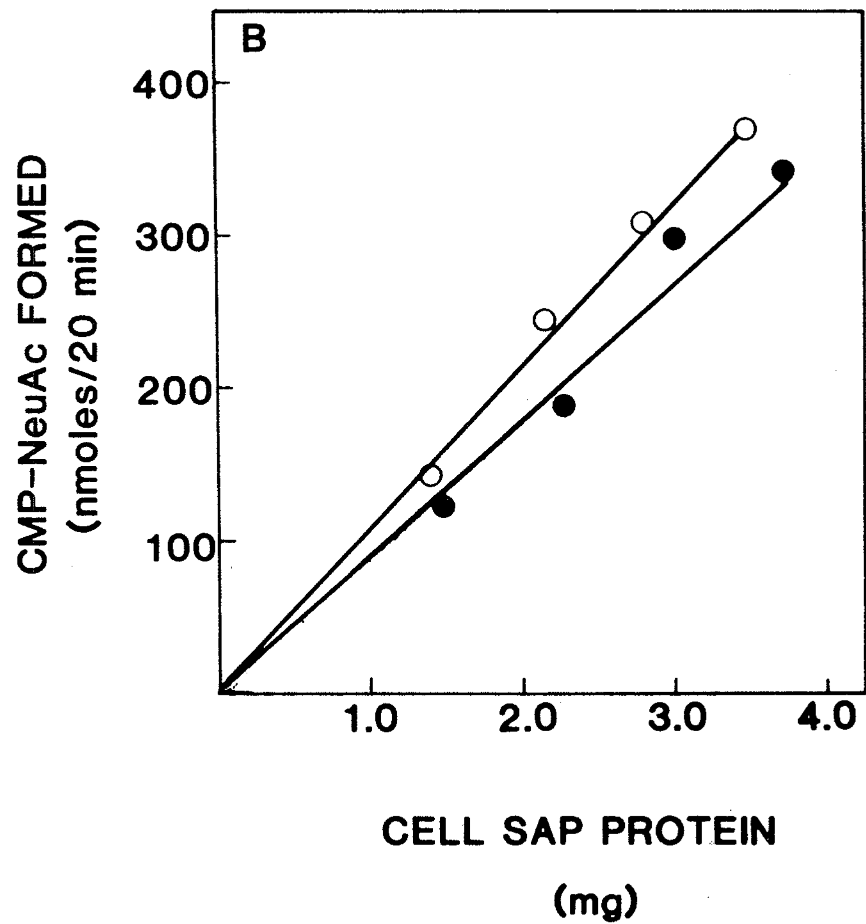
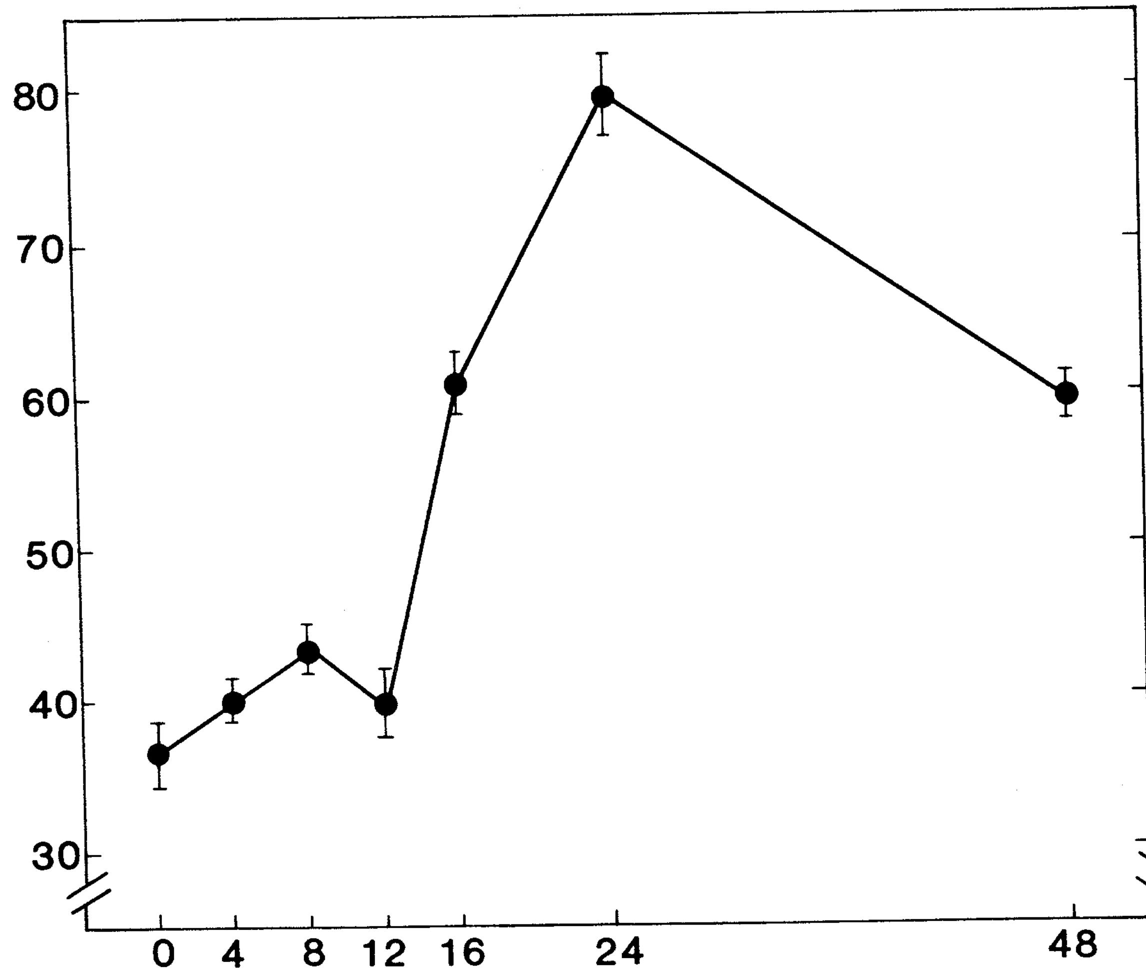

B
400
300
200
100
1.0
2.0
3.0
4.0
CMP-NeuAc FORMED
(nmoles/20 min)
CELL SAP PROTEIN
(mg)

Fig. 35. Effect of inflammation on glucosamine-6-phosphate synthase (or, L-glutamine:D-fructose-6-phosphate aminotransferase) activity in liver. Enzyme activity is expressed as units per mg cell sap protein (see Experimental). Results are the means of 3-6 analyses; the bars represent the range of standard deviations of the means.


GLUCOSAMINE-6-P SYNTHASE ACTIVITY
80
70
60
50
40
30
0
4
8
12
16
24
48
TIME AFTER INFLAMMATION (h)

a decline at 48 h after inflammation, values were still substantially above controls.

The effect of inflammation on UDP-GlcNAc 2-epimerase activities is shown in Fig. 36. The specific acitvity of the enzyme in control livers was 58 units/mg protein/h. In contrast to the response of glucosamine-6-phosphate synthase, the activities of UDP-GlcNAc 2-epimerase dropped to about 60% of control values at 4 h after inflammation which was followed by a rapid increase to just over twice control values before dropping to control values at 24-48 h after inflammation (Fig. 36).

The response of the nonregulatory enzyme, CMP-NeuAc synthase, to inflammation is shown in Fig. 37. Although some oscillation in enzyme activity was observed following inflammation, the changes in the activities of this enzyme were minor compared with the changes in activities found for the two regulatory enzymes studied.

The results of the above studies suggest that, as with elevated glycosyl transferase activities, there was a general increase in nucleotide sugar metabolism leading to increased pool sizes of nucleotide sugars in liver as part of the acute phase response to inflammation.

STUDIES OF THE EFFECT OF INFLAMMATION ON THE FORMATION OF LIPID-LINKED SUGAR INTERMEDIATES INVOLVED IN THE SYNTHESIS OF N-GLYCOSIDICALLY-LINKED GLYCOPROTEINS IN RAT LIVER

As previously stated in the Introduction, the biosynthesis of the oligosaccharide chains of asparagine-linked glycoproteins involves the initial formation of a lipid-linked oligosaccharide, $Glc_3Man_9GlcNAc_2$-P-P-Dol, *via* the dolichol cycle. The assembly of the lipid-linked oligosaccharide involves the ordered step by step transfer of GlcNAc,

Fig. 36. Effect of inflammation on UDP-N-acetylglucosamine 2-epimerase activity in liver. Enzyme activity is expressed as units per mg cell sap protein (see Experimental). Results are the means of 4-6 analyses; the bars represent the range of standard deviations of the means.

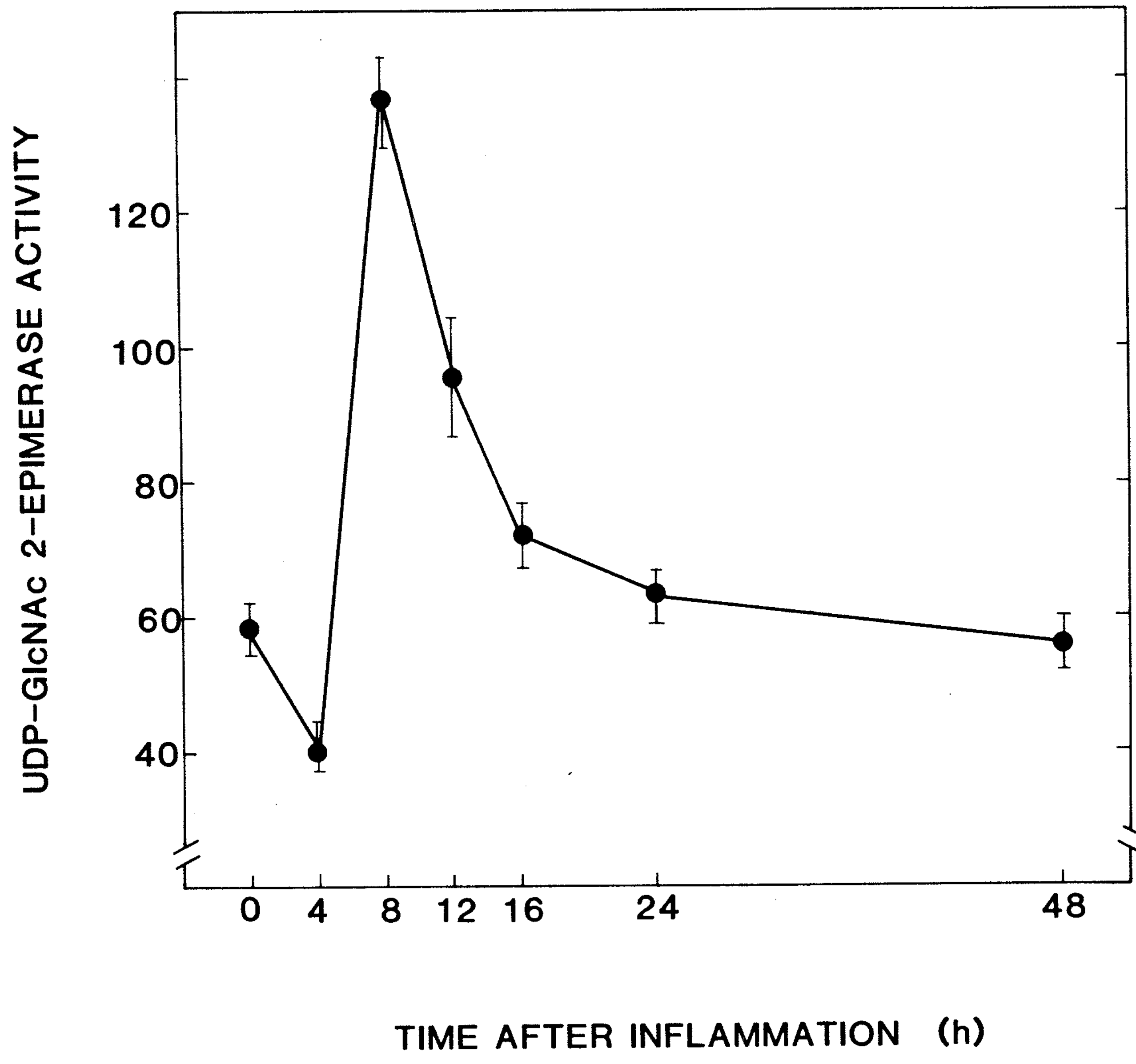
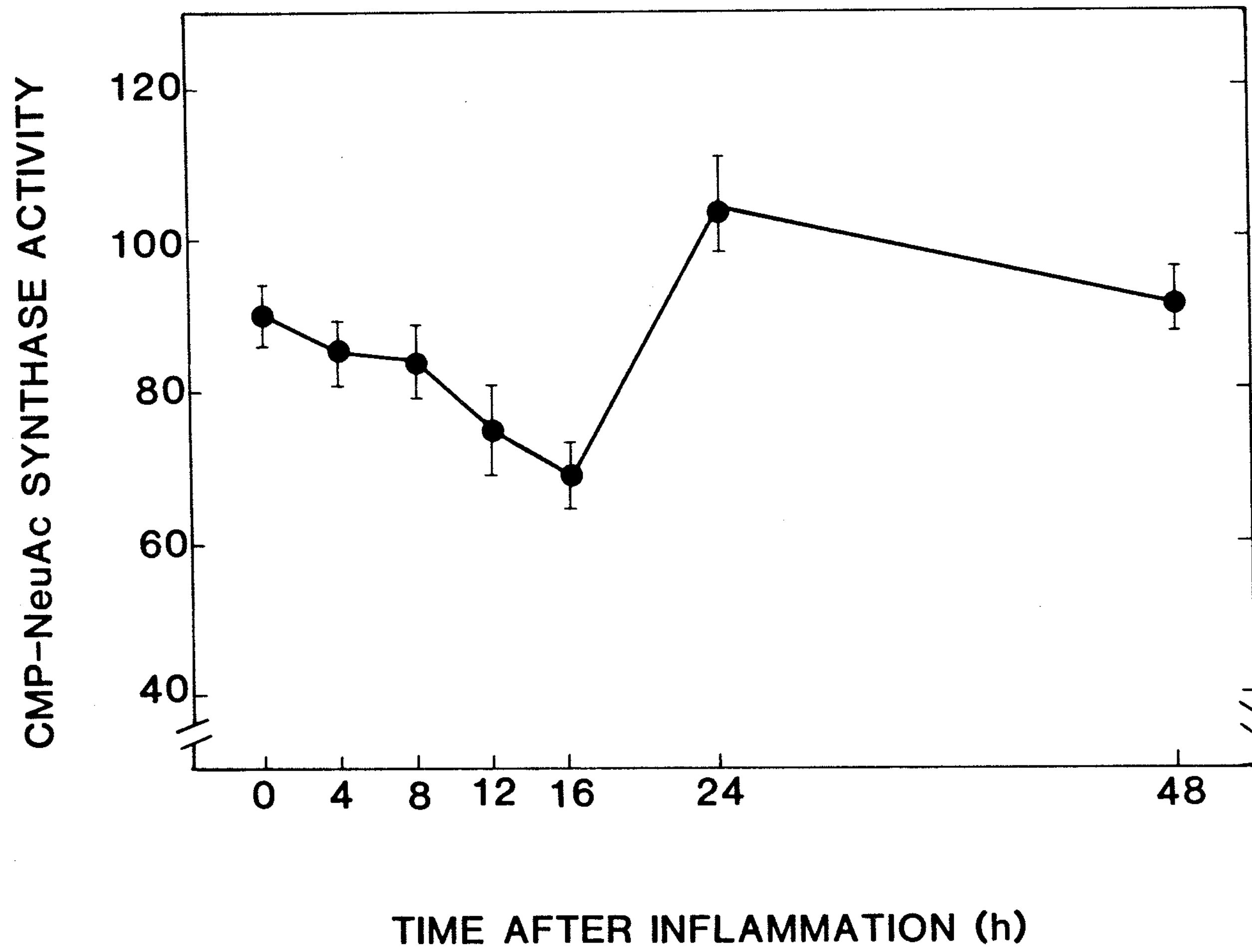

UDP-GlcNAc 2-EPIMERASE ACTIVITY
120
100
80
60
40
0
4
8
12
16
24
48
TIME AFTER INFLAMMATION (h)

Fig. 37. Effect of inflammation on CMP-NeuAc synthase activity in liver. Enzyme activity is expressed as units per mg cell sap protein (see Experimental). Results are the means of 4-6 analyses; the bars represent the range of standard deviations of the means.


CMP-NeuAc SYNTHASE ACTIVITY
120
100
80
60
40
0
4
8
12
16
24
48
TIME AFTER INFLAMMATION (h)

Man and Glc from their nucleotide sugar derivatives to the lipid carrier, either directly or through another lipid intermediate. During inflammation, increased glycoprotein biosynthesis, as well as alterations in the nucleotide sugar pools observed in the preceding studies, might be expected to affect the assembly of the lipid-linked oligosaccharides which in turn could affect protein glycosylation. Thus, studies were performed under in vivo and in vitro conditions to determine the effect of inflammation on the intermediates of the dolichol cycle. The studies involved determination of the effect of inflammation on the incorporation of radiolabeled sugars into lipid-linked sugar intermediates and protein in rat liver, coupled with attempts to characterize the oligosaccharide-lipid intermediates formed in livers from control and inflamed rats.

In Vivo Studies on the Incorporation of Radioactive Sugars into Sugar-Lipid Intermediates and Proteins from Hepatic Rough Membrane Fractions

It is realized in view of the results of pool size measurements on UDP-GlcNAc and GDP-Man that changes in the pool sizes of nucleotide sugars between control and experimental rats will have an effect on incorporation of labeled GlcN and Man into sugar-lipid complexes and protein. However, it was of interest to perform this study to detect any gross changes in incorporation patterns into sugar-lipid intermediates and protein. Control and 12 h inflamed rats were used in an initial study in which the time course of incorporation of [$^{3}$H]GlcN and [$^{14}$C]Man into Lipid I (Man-P-Dol and $(GlcNAc)_{1-2}$-P-P-Dol) and Lipid II (oligosaccharide-P-P-Dol) fractions in liver rough membrane fractions was examined. The results are shown in Figs. 38-40.

Figure 38 shows the time course of incorporation of [$^{3}$H]GlcN and

Fig. 38. *In vivo* incorporation of $[^{3}H]$GlcN and $[^{14}C]$Man into the Lipid I fraction (Man-P-Dol and $(GlcNAc)_{1-2}$-P-P-Dol) of hepatic rough membrane fractions. An intravenous injection of 20 μCi $[^{14}C]$Man (●) and 20 μCi $[^{3}H]$GlcN (▲) was given. Panel A, control; panel B 12 h experimental. Each point represents the mean of 2-4 analyses; reproducibility was with ±10%.

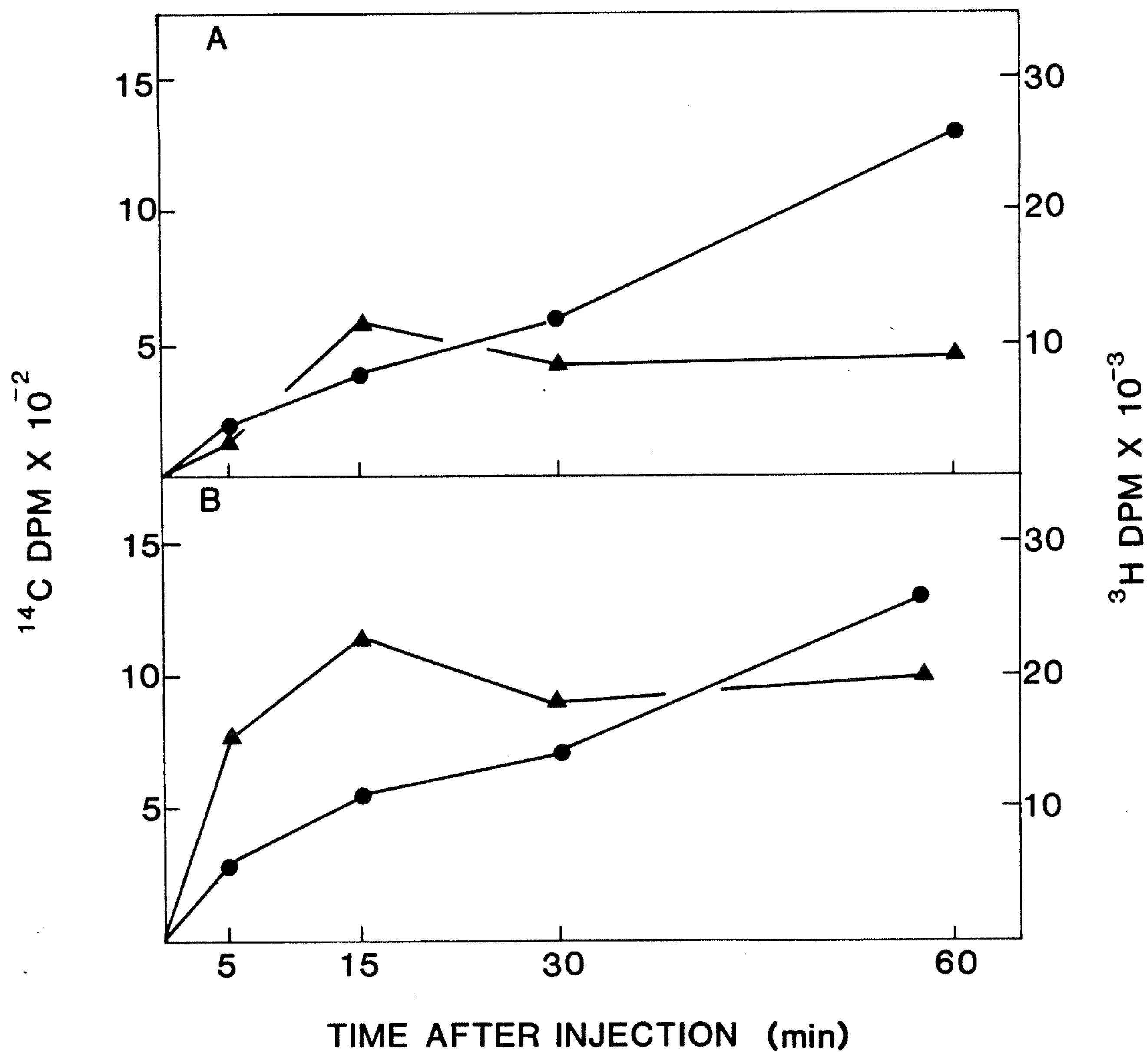

LIPID I
A
B
15
10
5
30
20
10
$^{14}$C DPM X $10^{-2}$
$^{3}$H DPM X $10^{-3}$
5
15
30
60
TIME AFTER INJECTION (min)

Fig. 39. *In vivo* incorporation of $[^3H]$GlcN and $[^{14}C]$Man into lipid-linked oligosaccharides (Lipid II) from hepatic rough membrane fractions. An intravenous injection of 20 μCi each of $[^3H]$GlcN (▲) and $[^{14}C]$Man (●) was given. Panel A, control; panel B, 12 h experimental. Each point represents the mean of 2-4 analyses; reproducibility was within ±12%.

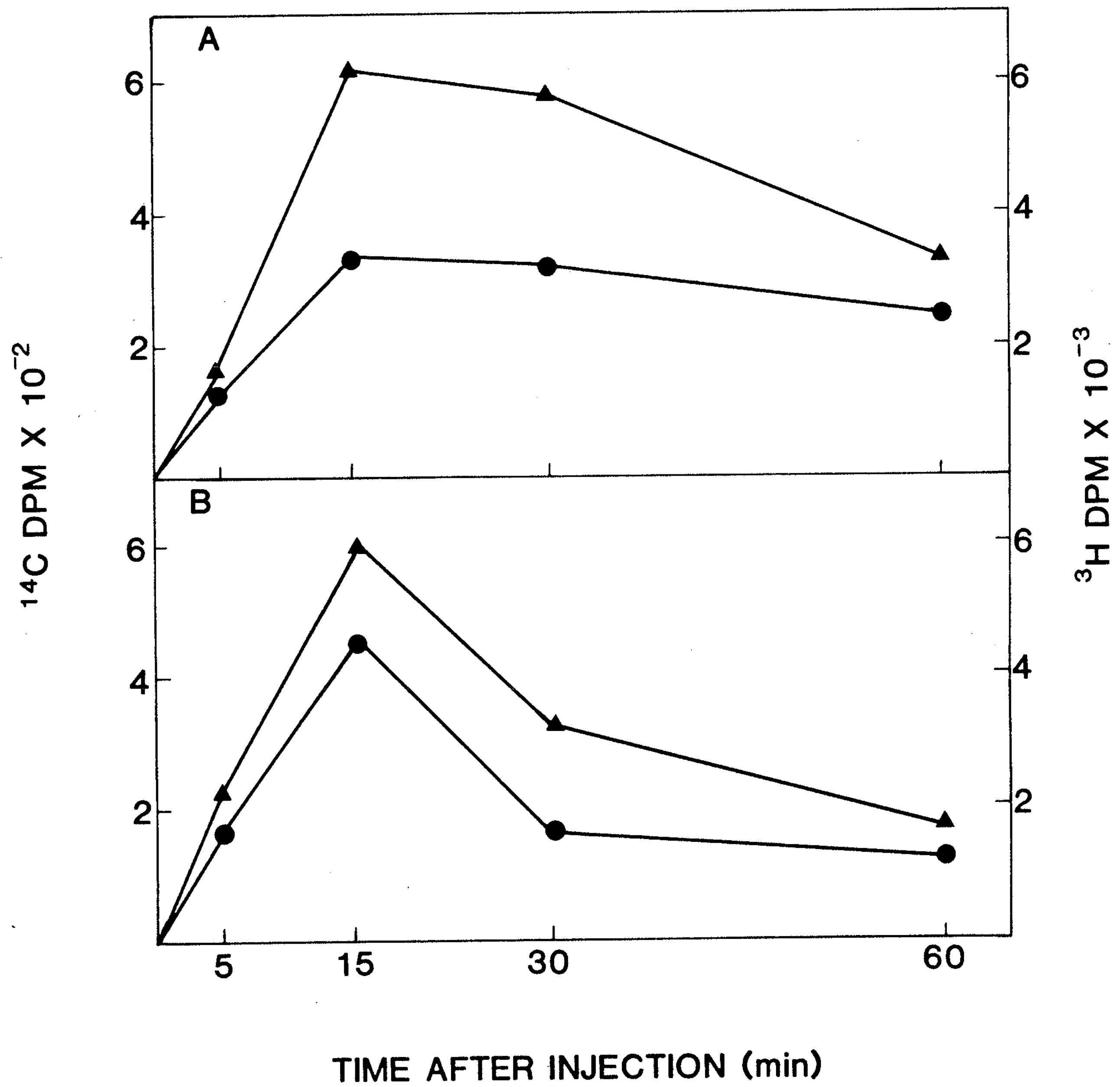
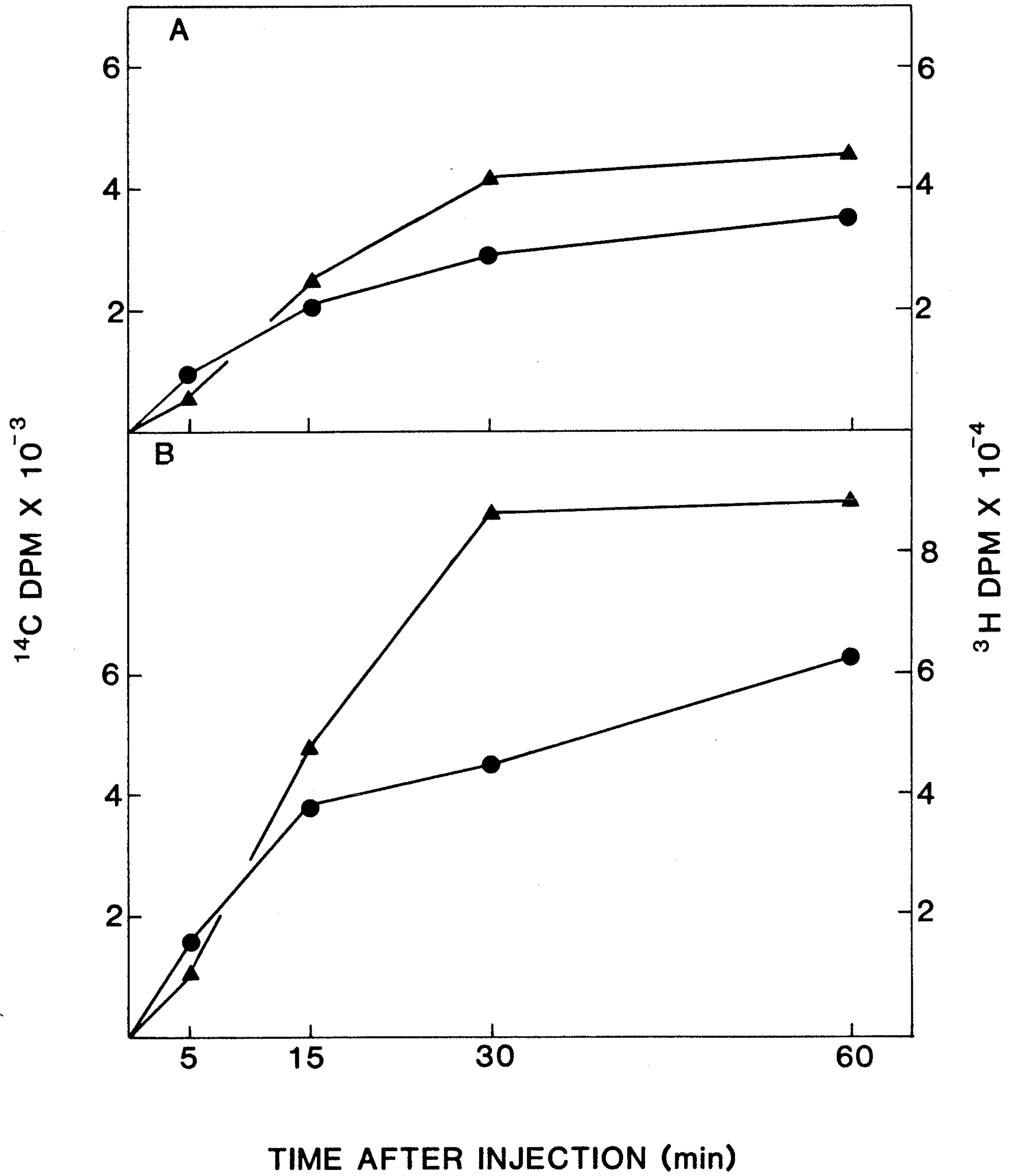

OLIGOSACCHARIDE-P-P-DOL (LIPID II)
A
B
6
4
2
$^{14}C$ DPM X $10^{-2}$
$^{3}H$ DPM X $10^{-3}$
5
15
30
60
TIME AFTER INJECTION (min)

Fig. 40. _In vivo_ incorporation of [$^{3}$H]GlcN and [$^{14}$C]Man into proteins from hepatic rough membrane fractions. An intravenous injection of 20 μCi each of [$^{3}$H]GlcN (▲) and [$^{14}$C]Man (●) was given. Each point represents the mean of 2-4 analyses; reproducibility was within ±12%. Panel A, control; panel B, 12 h experimental.


PROTEIN
A
B
6
4
2
6
4
2
6
4
2
8
6
4
2
$^{14}$C DPM X $10^{-3}$
$^{3}$H DPM X $10^{-4}$
5
15
30
60
TIME AFTER INJECTION (min)

[$^{14}$C]Man into the Lipid I fraction. The maximum level of $^3$H label in in the Lipid I fraction occurred at 15 min after injection of [$^3$H]GlcN for both control and experimental samples, with the experimental sample incorporating about twice as much label. In addition, the incorporation of $^3$H was very rapid with membranes from experimental rats since 70% of the maximum incorporation achieved occurred at 5 min after injection compared with only about 25% for corresponding control samples (Fig. 38). The incorporation of $^{14}$C into Lipid I increased continuously up to 60 min after the injection. The 12 h experimental fractions incorporated greater amounts of $^{14}$C than the controls up to 30 min after injection; however, at 60 min after injection the amount of $^{14}$C in the Lipid I fractions from control and experimental samples was the same (Fig. 38).

The time course of incorporation of [$^3$H]GlcN and [$^{14}$C]Man into oligosaccharide-lipid complexes from liver rough membrane fractions from control and 12 h experimental rats is shown in Fig. 39. The maximum incorporation of $^3$H and $^{14}$C occurred at 15 min after injection for both control and experimental samples. Both control and experimental samples were found to contain about the same amount of $^3$H label at 15 min after injection, whereas $^{14}$C labeling was greater in the experimental sample at this time (Fig. 39). Both $^3$H and $^{14}$C labels declined much more rapidly in the experimental samples compared to the controls after the 15 min peak of incorporation (Fig. 39).

The time course of incorporation of radioactive sugars into proteins from liver rough membrane fractions is shown in Fig. 40. Proteins from experimental samples incorporated about twice as much $^3$H and $^{14}$C compared to controls for up to 60 min after injection.

Taken at face value, these results would suggest that

inflammation caused increased synthesis of sugar-lipid intermediates resulting in increased glycosylation of proteins.

## In Vitro Studies on the Incorporation of [$^{14}$C]Man into Sugar-Lipid Intermediates and Proteins from Hepatic Rough Membrane Fractions

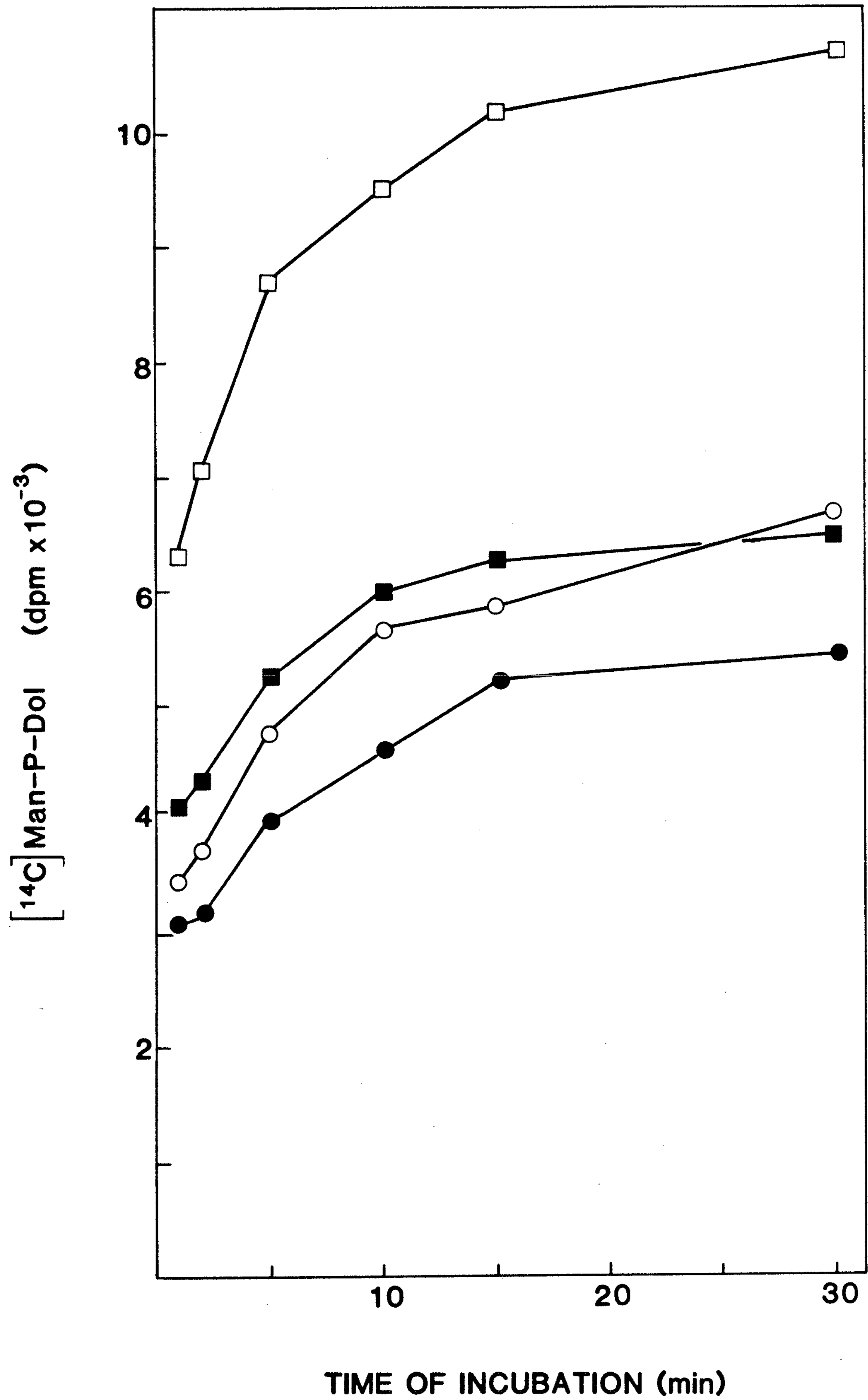
In order to determine the factors responsible for regulating the increased glycosylation of sugar-lipid intermediates and proteins in liver rough membrane fractions during inflammation, a series of experiments was performed in which incorporation of [$^{14}$C]Man from GDP-[$^{14}$C]Man was followed into sugar-lipid complexes and proteins in two types of cell-free systems. In the first system a regenerating source of ATP was omitted, thus depriving the system of an energy source to drive protein synthesis. In the second system energy was provided in the form of ATP, phosphenolpyruvate and pyruvate kinase. Thus, incorporation of [$^{14}$C]Man from GDP-[$^{14}$C]Man into sugar-lipid complexes and proteins was followed in absence and presence of concurrent protein synthesis. The object of these experiments was to determine if the formation of sugar-lipid complexes was dependent on protein synthesis, and how the results would be influenced by a system in which there was enhanced glycoprotein biosynthesis.

### In vitro mannosylation in absence of a cell-free protein synthesizing system.

Hepatic rough membrane fractions from control and 24 h experimental rats were incubated with GDP-[$^{14}$C]Man with and without Triton X-100. The incorporation of [$^{14}$C]Man into sugar-lipid complexes and proteins under these conditions is shown in Fig. 41-43.

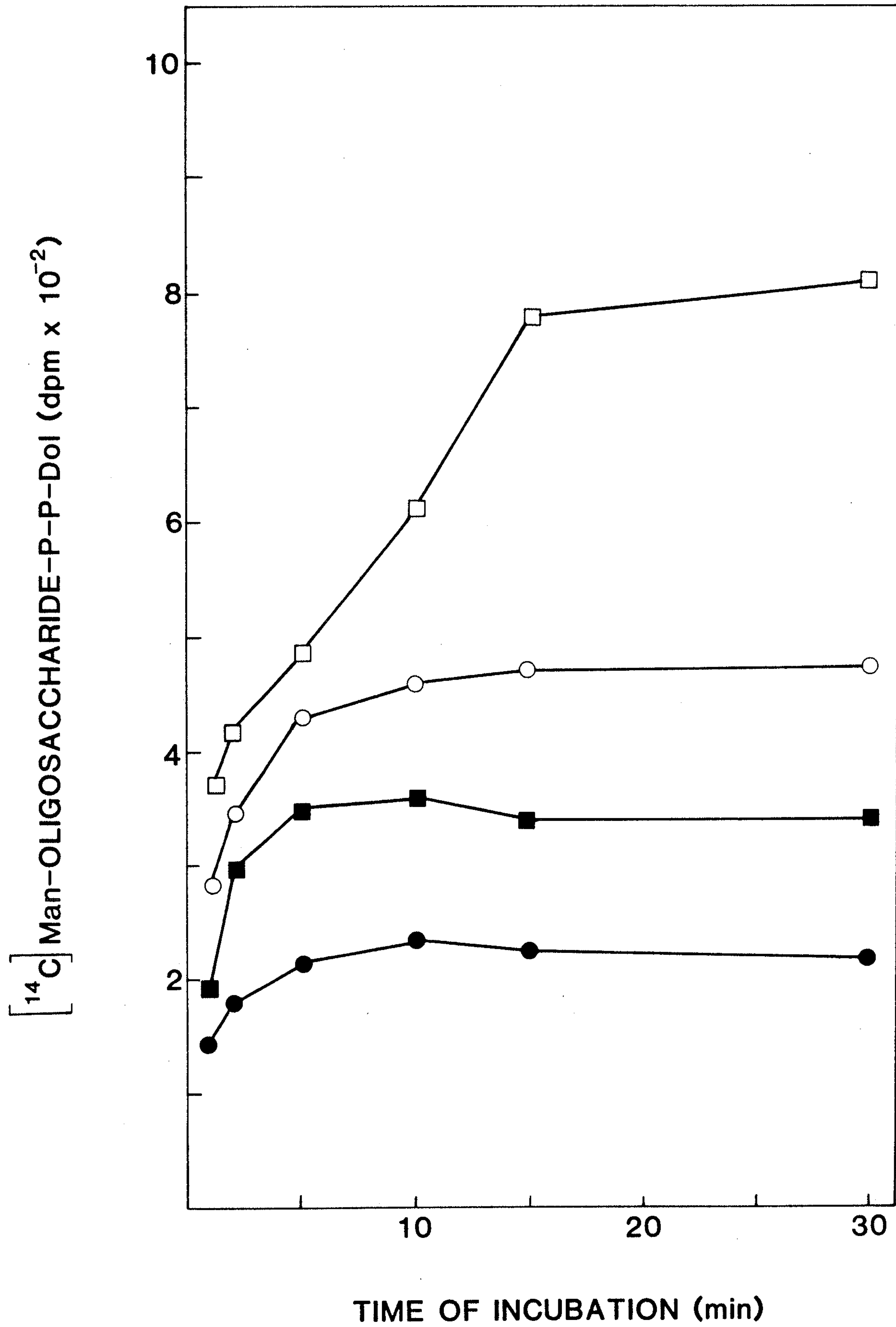
Figure 41 shows that the formation of [$^{14}$C]Man-P-Dol in absence of detergent was about 20% greater in the experimental rough membrane fractions over the time course of incubation. The addition of Triton X-100 to the assay (0.1% final concentration increased the formation of [$^{14}$C]Man-P-Dol by about 20% and 70% in the control and experimental rough membrane

Fig. 41. *In vitro* incorporation of [$^{14}$C]Man from GDP-[$^{14}$C]Man into Dol-P from hepatic rough membrane fractions in absence of a cell-free protein synthesizing system. Incorporation is expressed as dpm per mg rough membrane fraction protein. Control (●, O) and 24 h experimental (■, □) rough membrane fractions; open symbols represent the presence of 0.1% Triton X-100. Each point represents the mean of 3-4 analyses; reproducibility was within ±10%.



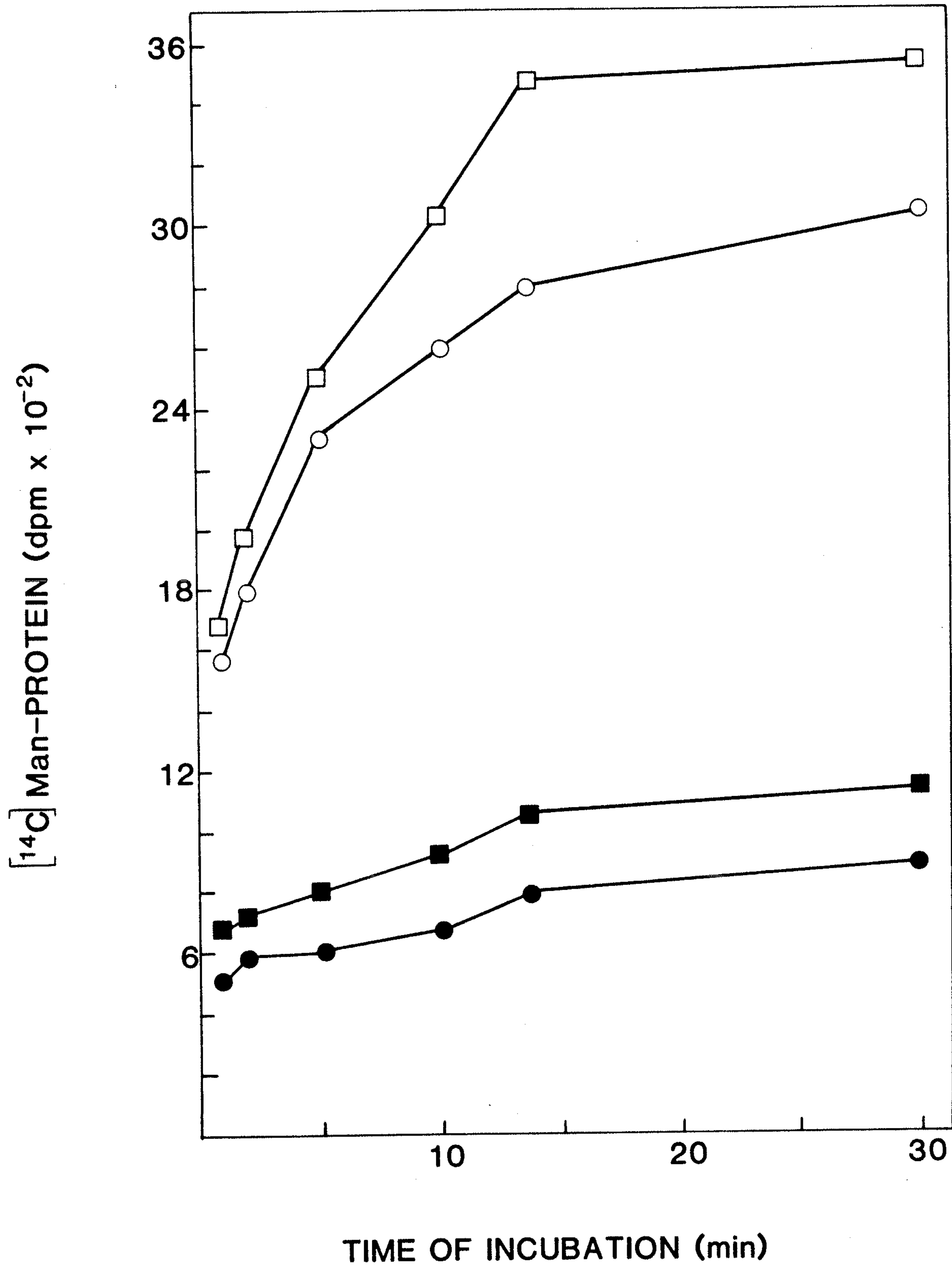
10
8
6
4
2
[14C] Man-P-Dol (dpm x10-3)
10
20
30
TIME OF INCUBATION (min)

Fig. 42. *In vitro* incorporation of $[^{14}C]$Man from GDP-$[^{14}C]$Man into oligosaccharide-P-P-Dol from hepatic rough membrane fractions in absence of a cell-free protein synthesizing system. Incorporation is expressed as dpm per mg rough membrane fraction protein. Control (●, O) and 24 h experimental (■, □) rough membrane fractions; open symbols represent the presence of 0.1% Triton X-100. Each point represents the mean of 3-4 analyses; reproducibility was within ±10%.



10
8
6
4
2
[14C] Man-OLIGOSACCHARIDE-P-P-Dol (dpm x 10⁻²)
10
20
30
TIME OF INCUBATION (min)

Fig. 43. *In vitro* incorporation of [$^{14}$C]Man from GDP-[$^{14}$C]Man into proteins from hepatic rough membrane fractions in absence of a cell-free protein synthesizing system. Incorporation is expressed as dpm per mg rough membrane fraction protein. Control (●, O) and 24 h experimental (■, □) rough membrane fractions; open symbols represent the presence of 0.1% Triton X-100. Each point represents the mean of 3-4 analyses; reproducibility was within ±10%.


36
30
24
18
12
6
10
20
30
[14C] Man-PROTEIN (dpm x 10-2)
TIME OF INCUBATION (min)

fractions, respectively (Fig. 41).

Fig. 42 shows that the formation of [$^{14}$C]Man-oligosaccharide-P-P-Dol in absence of detergent was about 50% greater in experimental rough membrane fractions compared to controls over the incubation period. Incubations in presence of 0.1% Triton X-100 increased the incorporation of [$^{14}$C]Man into oligosaccharide-P-P-Dol by about 100% in both control and experimental rough membrane fractions (Fig. 42).

Figure 43 shows that the incorporation of [$^{14}$C]Man into endogenous proteins from experimental rough membrane fractions was about 25% greater than in controls, in absence of detergent, over the incubation period. In the presence of 0.1% Triton X-100, the incorporation of [$^{14}$C]Man into endogenous proteins from both control and experimental rough membrane fractions increased by 300-500% (Fig. 43).

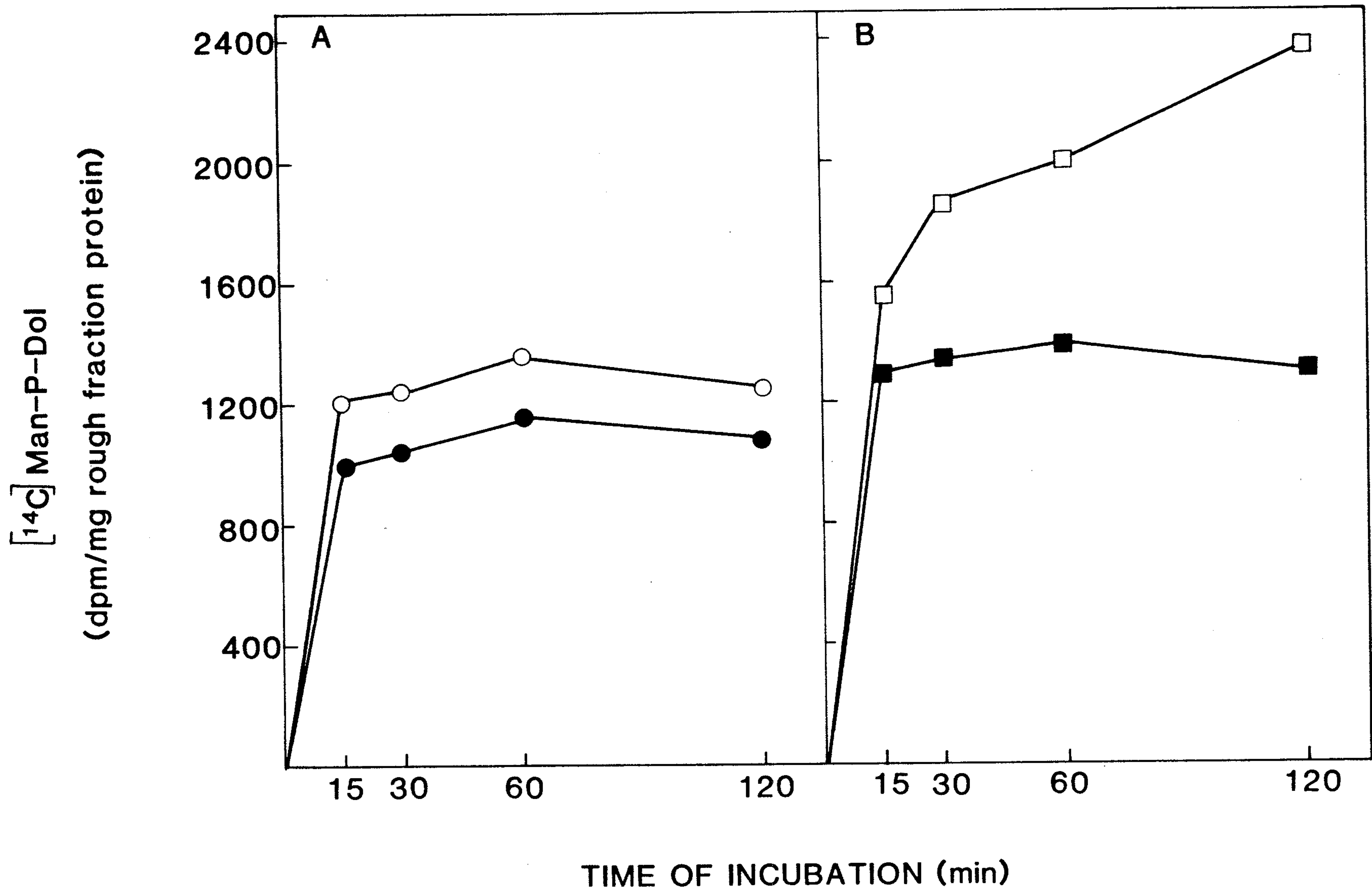
These results suggest that hepatic rough membrane fractions from experimental rats have a higher capacity for glycosylation of sugar-lipid intermediates and proteins.

In vitro mannosylation in presence of a cell-free protein synthesizing system

The incorporation of [$^{14}$C]Man from GDP-[$^{14}$C]Man into sugar-lipid complexes and proteins from control and 24 h experimental liver rough membrane fractions was studied under conditions of concurrent cell-free protein synthesis as previously described. Preparations of liver cell sap were included in the incubations to supply such factors as amino acids, tRNAs and amino-acyl-tRNA synthetases which are required for protein synthesis.

Figures 44, 45, and 46 show the results of time course experiments on the formation of [$^{14}$C]Man-P-Dol, [$^{14}$C]Man-oligosaccharide-P-P-Dol and [$^{14}$C]Man-protein, respectively, for both control and 24 h experimental rough membrane fractions. Both control and experimental rough membrane fractions incorporated a greater amount of [$^{14}$C]Man into the lipid-linked sugar

Fig. 44. *In vitro* incorporation of [$^{14}$C]Man from GDP-[$^{14}$C]Man into Man-P-Dol from hepatic rough membrane fractions in presence of concurrent cell-free protein synthesis. Panel A, control rough membrane fractions with control (●) and 24 h experimental (O) cell saps; panel B, 24 h experimental rough membrane fractions with control (■) and 24 h experimental (□) cell saps. Each point represents the mean of 3-4 analyses; reproducibility was within ±10%.



A
B
2400
2000
1600
1200
800
400
15 30 60 120
15 30 60 120
[14C] Man-P-Dol
(dpm/mg rough fraction protein)
TIME OF INCUBATION (min)

Fig. 45. *In vitro* incorporation of [$^{14}$C]Man from GDP-[$^{14}$C]Man into oligosaccharide-P-P-Dol from hepatic rough membrane fractions in presence of concurrent cell-free protein synthesis. Panel A, control rough membrane fractions with control (●) and 24 h experimental (O) cell saps; panel B, 24 h experimental rough membrane fractions with control (■) and 24 h experimental (□) cell saps. Each point represents the mean of 3-4 analyses; reproducibility was within ±10%.

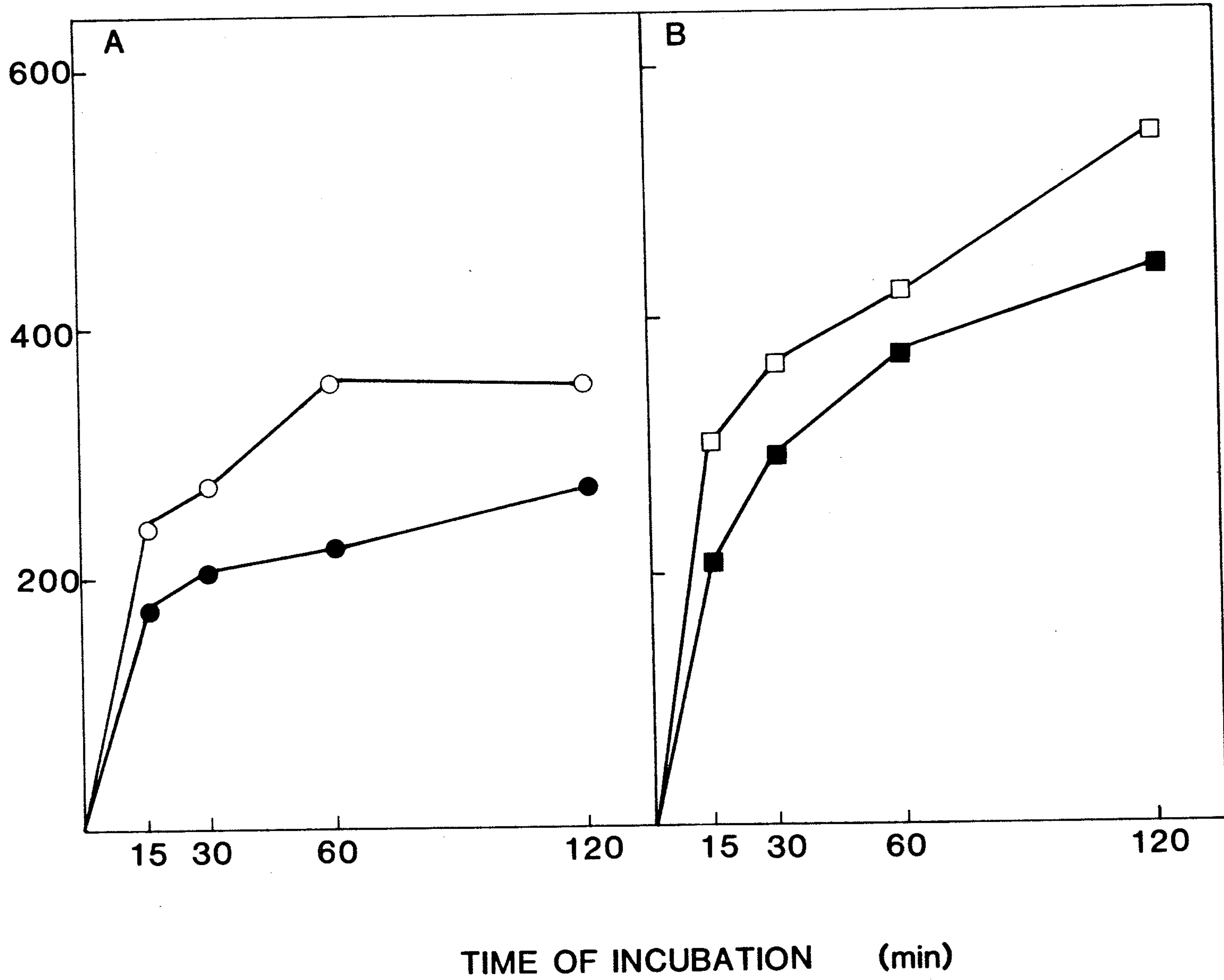
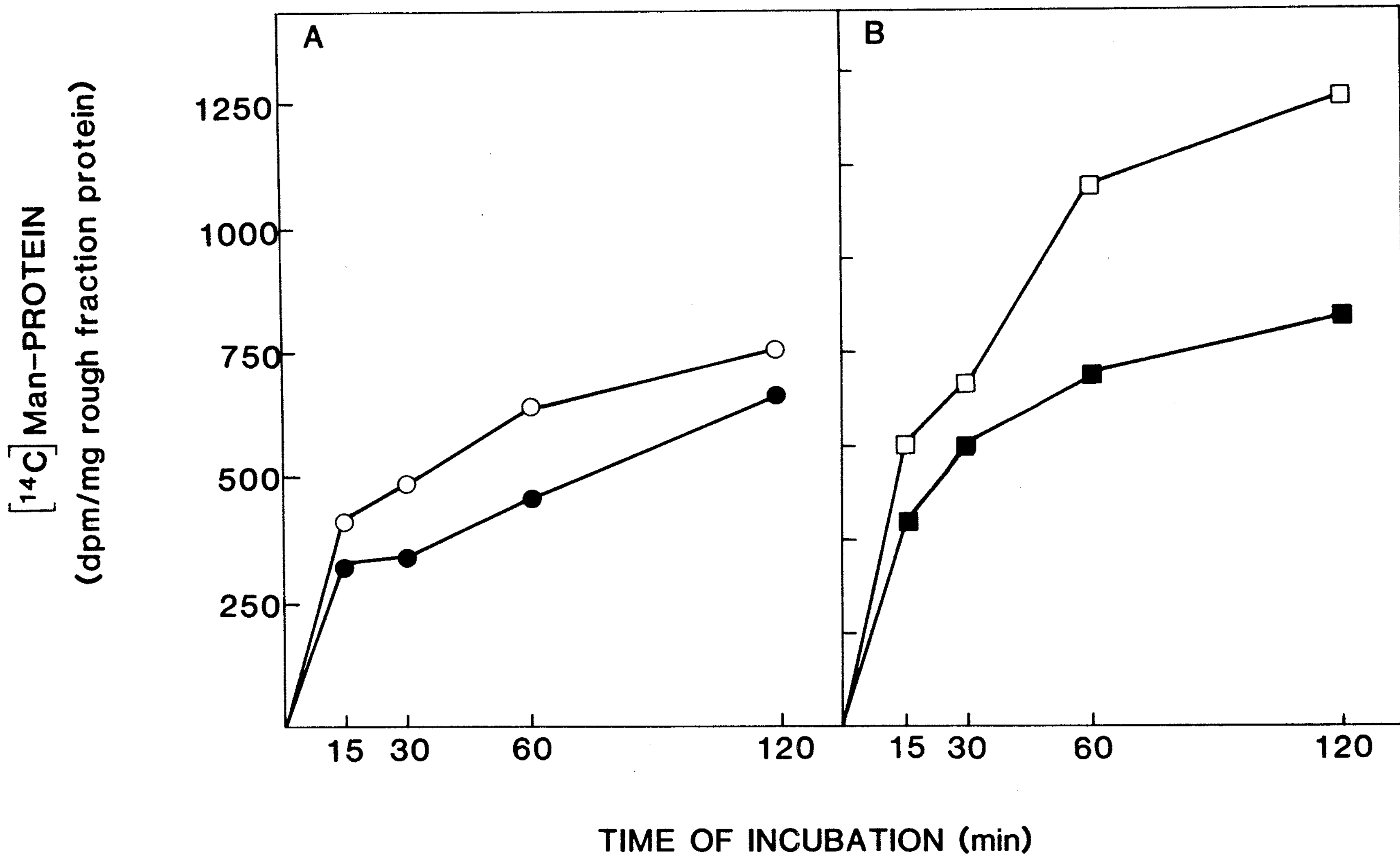

A
B
600
400
200
[14C] Man-OLIGOSACCHARIDE-P-P-Dol
(dpm/mg rough fraction protein)
15 30 60 120
15 30 60 120
TIME OF INCUBATION (min)

Fig. 46. *In vitro* incorporation of [$^{14}$C]Man from GDP-[$^{14}$C]Man into proteins from hepatic rough membrane fractions in presence of concurrent cell-free protein synthesis. Panel A, control rough membrane fractions with control (●) and 24 h experimental (O) cell saps; panel B, 24 h experimental rough membrane fractions with control (■) and 24 h experimental (□) cell saps. Each point represents the mean of 3-4 analyses; reproducibility was within ±10%.


A
B
[14C] Man-PROTEIN
(dpm/mg rough fraction protein)
1250
1000
750
500
250
15 30 60 120
15 30 60 120
TIME OF INCUBATION (min)

complexes and proteins when incubated with 24 h experimental cell sap than with control cell sap; however, in the presence of 24 h experimental cell sap the experimental rough membrane fractions incorporated more [$^{14}$C]Man into the sugar lipid complexes and proteins that control rough membrane fractions (Figs. 44-46).

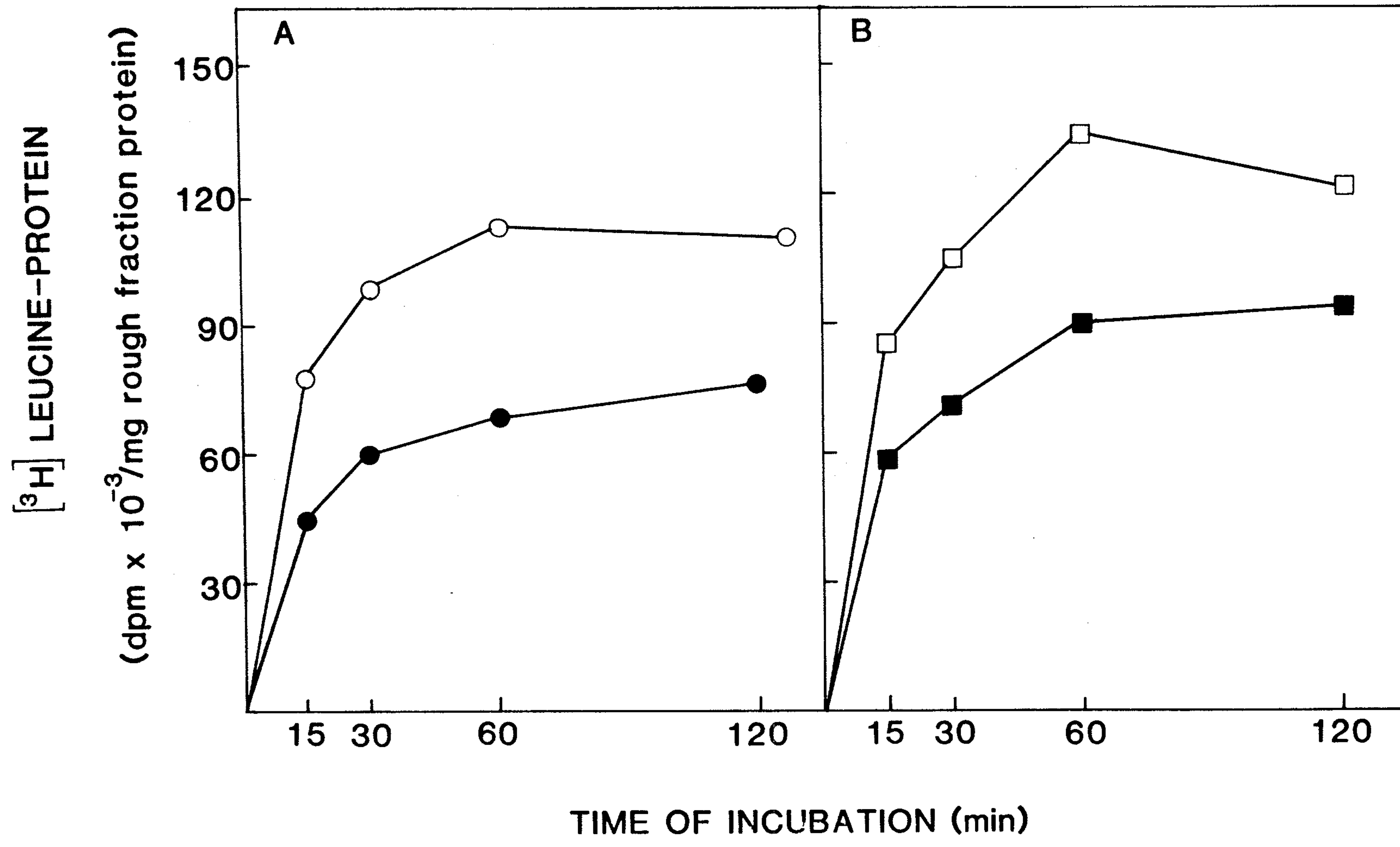
The incorporation of [$^{3}$H]leucine into proteins from control and 24 h experimental rough membrane fractions was also monitored. Figure 47 shows that, as was the case with [$^{14}$C]Man, [$^{3}$H]leucine incorporation into proteins was greater in the presence of 24 h experimental cell sap for both control and experimental rough membrane fractions.

These results suggest that, under conditions of concurrent cell-free protein synthesis, 24 h experimental rough membrane fractions have a higher capacity for mannosylation of endogenous sugar-lipid intermediates and proteins than control rough membrane fractions. Increased [$^{3}$H]leucine incorporation into proteins also suggests a greater capacity for protein synthesis by experimental rough membrane fractions. In addition, cell sap prepared from 24 h experimental livers has been shown to be able to stimulate [$^{14}$C]Man incorporation into sugar-lipid intermediates and proteins from control rough membrane fractions, as well as, [$^{3}$H]leucine incorporation into the same proteins.

Effect of cell sap on mannosylation

In order to further examine the effect of cell sap on the mannosylation of the sugar-lipid intermediates and proteins from liver rough membrane fractions, a series of experiments was performed. These involved examining the incorporation of [$^{14}$C]Man from GDP-[$^{14}$C]Man into sugar-lipid intermediates and proteins from control and 12 h experimental rough membrane fractions in the presence of control and various experimental liver cell sap preparations.

Fig. 47. *In vitro* incorporation of [$^{3}$H]leucine into proteins from hepatic rough membrane fractions in presence of concurrent cell-free protein synthesis. Panel A, control rough membrane fractions with control (●) and 24 h experimental (O) cell saps; panel B, 24 h experimental rough membrane fractions with control (■) and 24 h experimental (□) cell saps. Each point represents the mean of 3-4 analyses; reproducibility was within ±10%.


A
B
150
120
90
60
30
15 30 60 120
15 30 60 120
[3H] LEUCINE-PROTEIN
(dpm x 10-3/mg rough fraction protein)
TIME OF INCUBATION (min)

Tables 13 and 14 show the effects of cell saps prepared from control and several different experimental livers on the mannosylation of sugar-lipid intermediates and proteins from control and 12 h experimental rough membrane fractions, respectively; the effect on $[^{3}H]$leucine incorporation into proteins is also shown. Cell saps from different times after onset of inflammation had either slightly inhibitory or stimulatory effects on the formation of $[^{14}C]$Man-P-Dol in both control and 12 h experimental rough membrane fractions. The incorporation of $[^{3}H]$leucine into proteins from both control and 12 h experimental rough membrane fractions was also stimulated in the presence of experimental cell sap preparations.

Results from the preceding study in this thesis have shown that GDP-Man levels in liver change during inflammation (see Fig. 28). Thus, the cell saps from control and experimental livers used in the above mannosylation assays contained different amounts of GDP-Man. Due to this fact, the incorporation of $[^{14}C]$Man from GDP-$[^{14}C]$Man into sugar-lipid complexes and proteins from both control and 12 h experimental rough membrane fractions was calculated on the basis of the specific radioactivity of GDP-$[^{14}C]$Man in each assay (see footnotes of Table 13 for further details). Correcting for changes in specific radioactivity for assays with both control and 12 h experimental rough fractions showed that the formation of $[^{14}C]$Man-P-Dol was not significantly affected by the various experimental cells saps, whereas $[^{14}C]$Man incorporation into oligosaccharide-lipid complexes and proteins was significantly increased (Tables 13 and 14). Hepatic leucine pools have also been found to change during inflammation (Woloski _et al_., 1983b). Correcting for changes in the specific

Table 13. Effect of experimental liver cell saps on the incorporation of [$^{14}$C]mannose and [$^{3}$H]leucine into sugar-lipid intermediates and proteins from control liver rough membrane fractions.

| | Control[a] | | Cell sap[b] | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 4h | 8h | 12h | 16h | 24h | 48h |
| Fraction | dpm/mg | [pmoles/mg] | Ratio inflamed/control | | | | | |
| [$^{14}$C]Man-P-Dol | 1080 | | 0.72 | 1.1 | 1.2 | 0.80 | 1.1 | 0.96 |
| | | [5.9] | (0.82) | (1.0) | (1.1) | (0.81) | (1.0) | (1.0) |
| [$^{14}$C]Man-oligosaccharide-P-P-Dol | 225 | | 1.0 | 1.6 | 1.8 | 1.9 | 1.8 | 1.4 |
| | | [1.2] | (1.1) | (1.6) | (1.6) | (1.8) | (2.0) | (1.4) |
| [$^{14}$C]Man-protein | 490 | | 1.2 | 1.4 | 1.8 | 1.4 | 1.4 | 1.3 |
| | | [2.7] | (1.3) | (1.4) | (1.6) | (1.4) | (1.5) | (1.4) |
| [$^{3}$H]leucine-protein | 65,600 | | 1.1 | 1.1 | 1.5 | 1.5 | 2.0 | 1.8 |
| | | [50] | (0.87) | (1.4) | (1.7) | (2.2) | (1.1) | (2.0) |

[a]Incorporation is expressed as dpm/mg rough membrane fraction protein/60 min; values in square brackets represent results expressed as pmoles Man or leucine incorporated/mg rough membrane fraction protein/60 min; values in parentheses are ratios of values in square brackets.

[b]Values in brackets and parentheses represent corrections for changes in specific radioactivities of GDP-[$^{14}$C]Man and [$^{3}$H]leucine due to altered hepatic pools of GDP-Man (see Fig. 28) and leucine (see Woloski et al., 1983b) in the cell sap preparations. Uncorrected value for GDP-[$^{14}$C]Man is 108 mCi/mmole; corrected values with control, 4h, 8h, 12h, 16h, 24h, and 48h cell saps are 83, 73, 84, 92, 84, 73, and 79 mCi/mmole, respectively. Uncorrected value for $^{3}$H leucine is 5 Ci/mmole; corrected values with control, 4h, 8h, 12h, 16h, 24h, and 48h cell saps are 0.59, 0.73, 0.44, 0.50, 0.39, 1.0, and 0.53 Ci/mmole, respectively. The above values were calculated on the basis of 1 mg cell sap protein (per assay) obtained from 0.03 g liver by the subcellular fraction procedure.
Values represent the means of 3 analyses; standard deviations of the means were within ±10%.

Table 14. Effect of experimental cell saps on the incorporation of [$^{14}$C]mannose and [$^{3}$H]leucine into sugar-lipid intermediates and proteins from 12 h experimental rough membrane fractions.

| | Control | | Cell sap | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 4h | 8h | 12h | 16h | 24h | 48h |
| Fraction | dpm/mg | [pmoles/mg] | Ratio inflamed/control | | | | | |
| [$^{14}$C]Man-P-Dol | 1266 | | 0.84 | 1.2 | 1.2 | 0.81 | 1.1 | 0.97 |
| | | [6.9] | (0.95) | (1.2) | (1.1) | (0.80) | (1.1) | (1.0) |
| [$^{14}$C]Man-oligosaccharide-P-P-Dol | 220 | | 1.1 | 1.8 | 1.6 | 1.4 | 1.4 | 1.2 |
| | | [1.2] | (1.2) | (1.8) | (1.5) | (1.4) | (1.6) | (1.3) |
| [$^{14}$C]Man-protein | 760 | | 1.0 | 1.3 | 1.4 | 1.2 | 1.2 | 0.97 |
| | | [4.1] | (1.2) | (1.3) | (1.3) | (1.2) | (1.4) | (1.0) |
| [$^{3}$H]leucine-protein | 87,410 | | 0.97 | 1.0 | 1.3 | 1.3 | 1.8 | 1.5 |
| | | [67] | (0.80) | (1.3) | (1.5) | (1.9) | (0.90) | (1.7) |

Values represent the means of 3 analyses; standard deviations of the means were within ±10%. Values were calculated as described in footnotes of Table 13.

radioactivity of [$^{3}$H]leucine in the assays, it was found that, with the possible exception of the 24 h experimental cell sap, experimental cell saps stimulated [$^{3}$H]leucine incorporation into proteins from both control and 12 h experimental rough membrane fractions (Tables 13 and 14). These results suggest that, under the assay conditions, increased [$^{14}$C]Man and [$^{3}$H]leucine incorporation into rough membrane fraction oligosaccharide-lipid complexes and proteins was independent of GDP-Man and leucine levels in the experimental cell saps.

To determine if the elevated [$^{14}$C]Man incorporation into oligosaccharide-lipid complexes and proteins was due to a high molecular weight component of cell sap, the following study was performed. Control rough membrane fractions were incubated under conditions of concurrent cell-free protein synthesis with control and 12 h experimental cell saps and with a 1:1 mixture (by protein content) of control cell sap and a high molecular weight fraction of 12 h experimental cell sap (see Experimental for preparation). The incorporation of [$^{14}$C]Man from GDP-[$^{14}$C]Man into sugar-lipid intermediates and proteins under these assay conditions is shown in Table 15. The results show that both the experimental cell sap and the high molecular weight fraction of the experimental cell sap were able to cause increased [$^{14}$C]Man incorporation into oligosaccharide-lipid complexes and proteins from control rough membrane fractions. [$^{3}$H]Leucine incorporation into proteins was also stimulated by 12 h experimental cell sap, but not by a high molecular weight fraction prepared from 12 h experimental cell sap (Table 15). The formation of [$^{14}$C]Man-P-Dol was not significantly affected by either 12 h experimental cell sap or a high molecular weight fraction of 12 h experimental cell sap (Table 15).

Table 15. Effect of a high molecular weight fraction of liver cell sap on the incorporation of [$^{14}$C]mannose and [$^{3}$H]leucine into sugar-lipid complexes and proteins from control liver rough membrane fractions.

| | Cell sap | | |
|---|---|---|---|
| | Control | 12 h Experimental | HMF[a] |
| Fraction | pmoles/h/mg rough membrane fraction protein | | |
| [$^{14}$C]Man-P-Dol | 6.8 | 7.2 | 6.9 |
| [$^{14}$C]Man-oligosaccharide-P-P-Dol | 1.4 | 2.6 | 2.5 |
| [$^{14}$C]Man-protein | 2.7 | 3.8 | 3.6 |
| [$^{3}$H]leucine-protein | 45 | 74 | 44 |

Values represent the means of 3-4 analyses; standard deviations of the means were within ±10%. Specific radioactivities of GDP-[$^{14}$C]Man and [$^{3}$H]leucine were calculated as described in the footnotes of Table 13.

[a]HMF consists of a 1:1 mixture (by protein content) of control cell sap and a high molecular weight-enriched fraction of 12 h experimental cell sap (see Experimental).

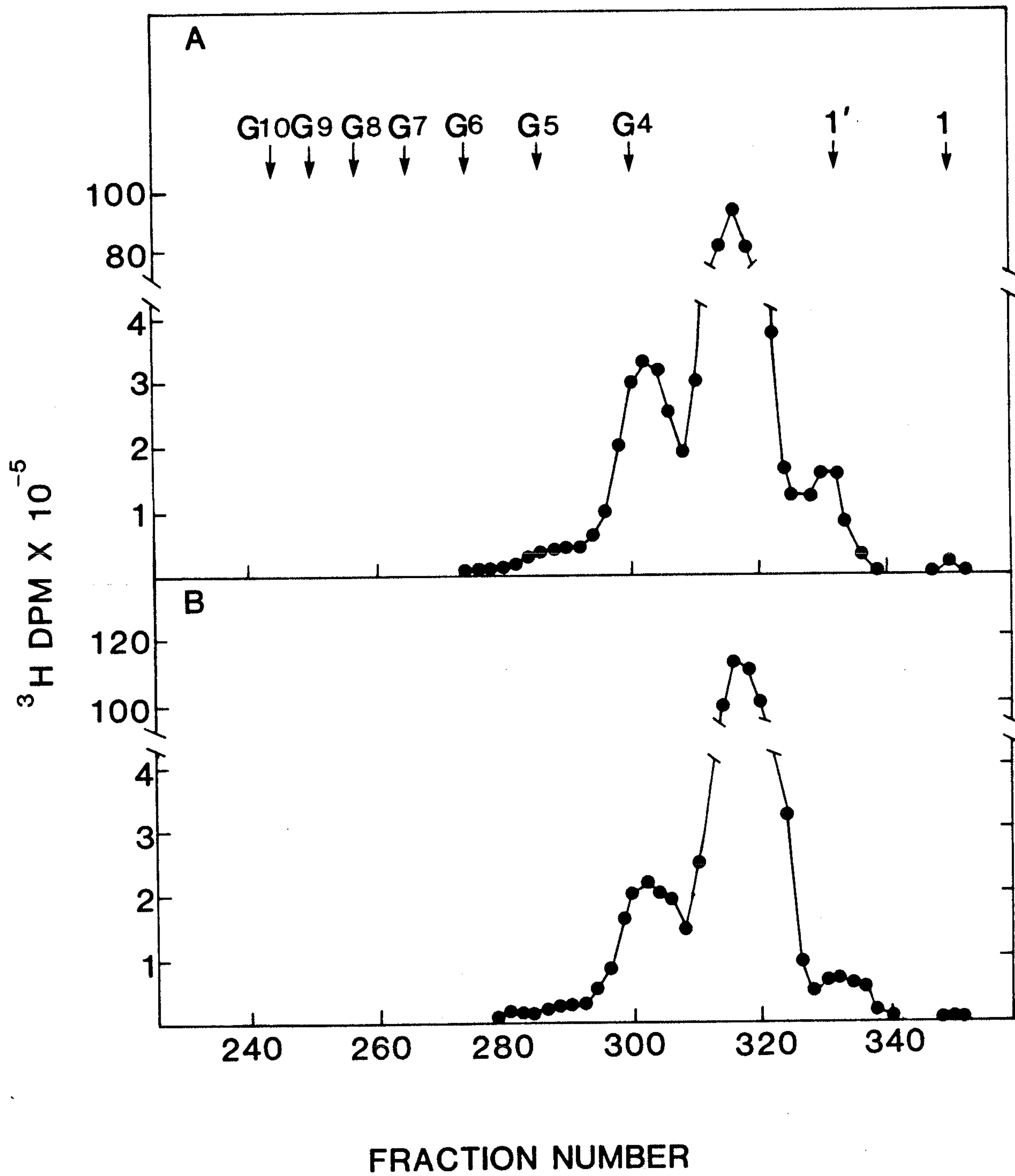
These results, coupled with those from previous *in vitro* studies presented above, suggest that experimental cell sap preparations contain one or more high molecular weight components which have the ability to increase glycosylation, in rough membrane fractions, of oligosaccharide-lipid complexes and proteins.

## Analysis of Oligosaccharide-Lipid Complexes

The above *in vitro* cell-free studies suggest that there are differences in the incorporation of [$^{14}$C]Man from GDP-[$^{14}$C]Man into lipid-linked oligosaccharides in control and experimental rough membrane fractions. However, the oligosaccharide-lipid preparation contains all the oligosaccharide intermediates of the dolichol cycle (Fig. 9). In order to determine if inflammation caused a change in the distribution of the various intermediates in rough membrane fractions, the oligosaccharide-lipids were separated on the basis of size. This was accomplished by mild acid hydrolysis of the oligosaccharide-lipid complexes to remove the lipid, followed by fractionation of the oligosaccharides on columns of Bio-Gel P4 resin.

Oligosaccharides derived from oligosaccharide-lipid complexes from control and 12 h experimental livers were labeled at the reducing end by treatment with NaB[$^{3}$H]$_4$ (see Experimental) prior to fractionation by gel filtration chromatography. This method allows for a quantitative labeling of the oligosaccharide complexes such that the amount of label in the different oligosaccharide complexes is proportional to their relative distribution within the total oligosaccharide population. Gel filtration chromatography profiles of the oligosaccharides are shown in Fig. 48. The control and experimental oligosaccharide profiles were very similar, consisting of one major

Fig. 48. Chromatography of oligosaccharides derived from oligosaccharide-lipid complexes from hepatic microsome fractions. After mild acid hydrolysis of the lipid-linked oligosaccharides, the free oligosaccharides were labeled by treatment with $NaB[^3H]_4$ and were chromatographed on a 1 x 208 cm column of Bio-Gel P-4 in 0.5 M acetic acid. Aliquots of 0.2 ml containing $7 \times 10^8$ dpm were chromatographed. The fraction size was 0.4 ml, and the inclusion and exclusion volumes were at fractions 350 and 138, respectively. Panel A, control liver; panel B, 12 h experimental liver. Numbered arrows indicate the elution positions of standards: 1, $[^{14}C]$mannose; 1', $[^3H]$GlcNAc; G4-G10, maltosaccharides $Glc_4$, $Glc_5$...$Glc_{10}$ labeled by treatment with $NaB[^3H]_4$.


A
G10 G9 G8 G7 G6 G5 G4 1′ 1
100
80
4
3
2
1
B
120
100
4
3
2
1
240 260 280 300 320 340
$^3$H DPM X $10^{-5}$
FRACTION NUMBER

peak flanked on each side by a small peak; the major peak in each profile contained over 90% of the total radioactivity in the profile (Fig. 48). The largest oligosaccharide species resolved eluted in the region of the standard maltooligosaccharide $Glc_4$. Comparison of these results with those of Kobata's group (Mizouchi et al., 1980), who performed the same type of chromatographic analysis on similar $NaB[^3H]_4$-treated oligosaccharides, suggests that the composition of the oligosaccharide migrating in the $Glc_4$ region is GlcNAc-N-acetylglucosaminitol. Thus, no mannose-containing oligosaccharides were apparently resolved using this approach. The reason for the inability to resolve mannose-containing oligosaccharides is not clear. Possible explanations are that the peaks obtained were mainly artifacts produced during the reduction or acid hydrolysis procedures.

The above procedure used for the labeling of lipid-derived oligosaccharides was abandoned, and a carbohydrate labeling method based on that described by Hubbard and Robbins (1980) was used. Hubbard and Robbins (1980) studied the assembly of lipid-derived oligosaccharide complexes after the incorporation of radioactive sugars into oligosaccharide-lipid complexes in Chinese hamster ovary (CHO) cells. This procedure allows for the labeling of the lipid-linked oligosaccharides without using $NaB[^3H]_4$.

Liver slices from control and 12 h experimental rats were incubated with $[^3H]$GlcN and $[^{14}C]$Man and the oligosaccharide-lipid complexes isolated as previously described in the Experimental. High-resolution gel filtration chromatography elution profiles of the $[^3H]$GlcN-labeled and $[^{14}C]$Man-labeled lipid derived oligosaccharides are shown in Figs. 49 and 50, respectively. The profiles

Fig. 49. Chromatography of [$^3$H]GlcN-labeled oligosaccharides derived from oligosaccharide-lipid complexes from liver microsome fractions. Oligosaccharide-lipids were extracted from microsome fractions prepared from 5 g of liver slices which were incubated with [$^3$H]GlcN (see Experimental). See legend of Fig. 48 for further details. Resolved peaks are indicated by lettered arrows. Panel A, control liver; panel B, 12 h experimental liver.

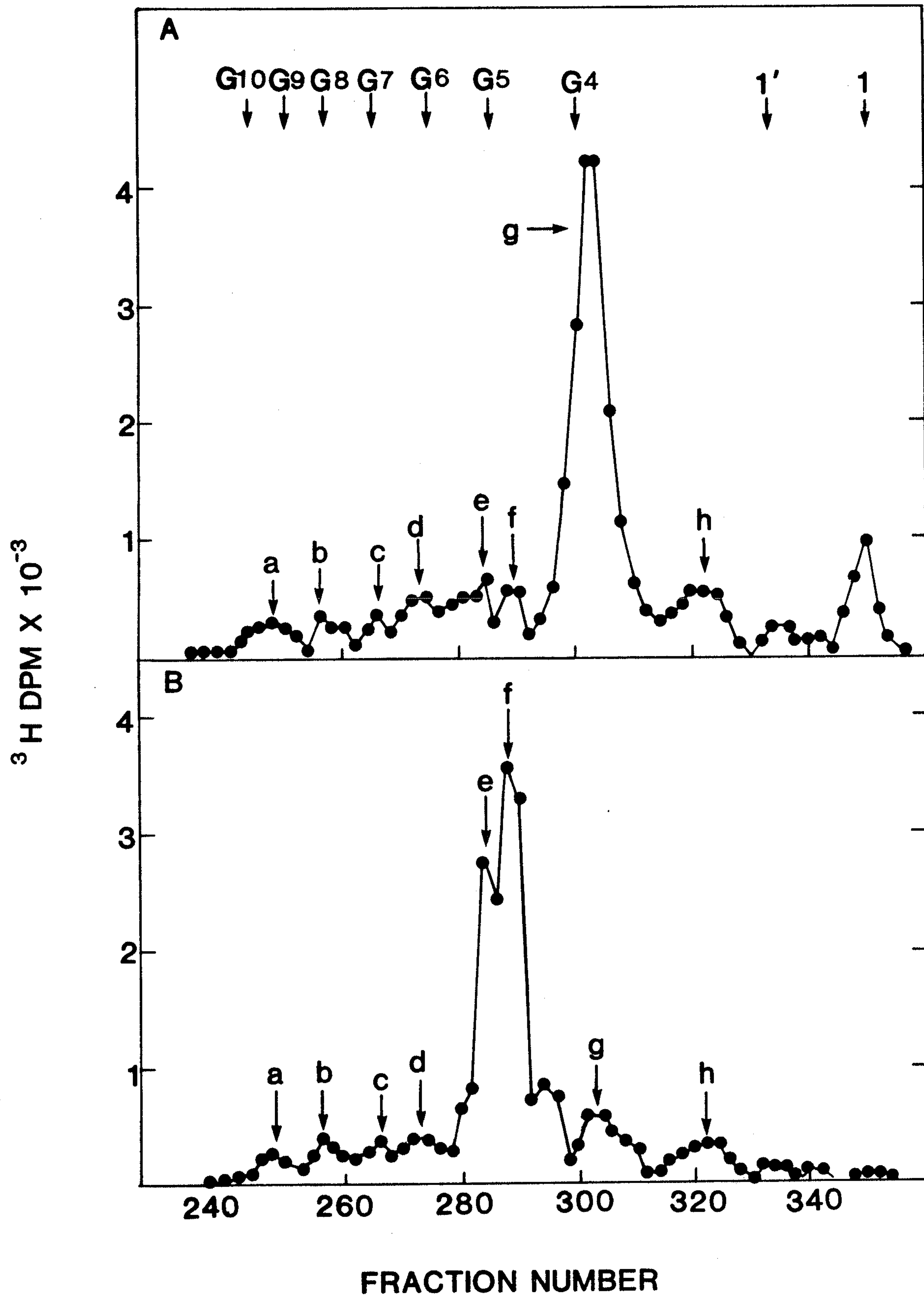
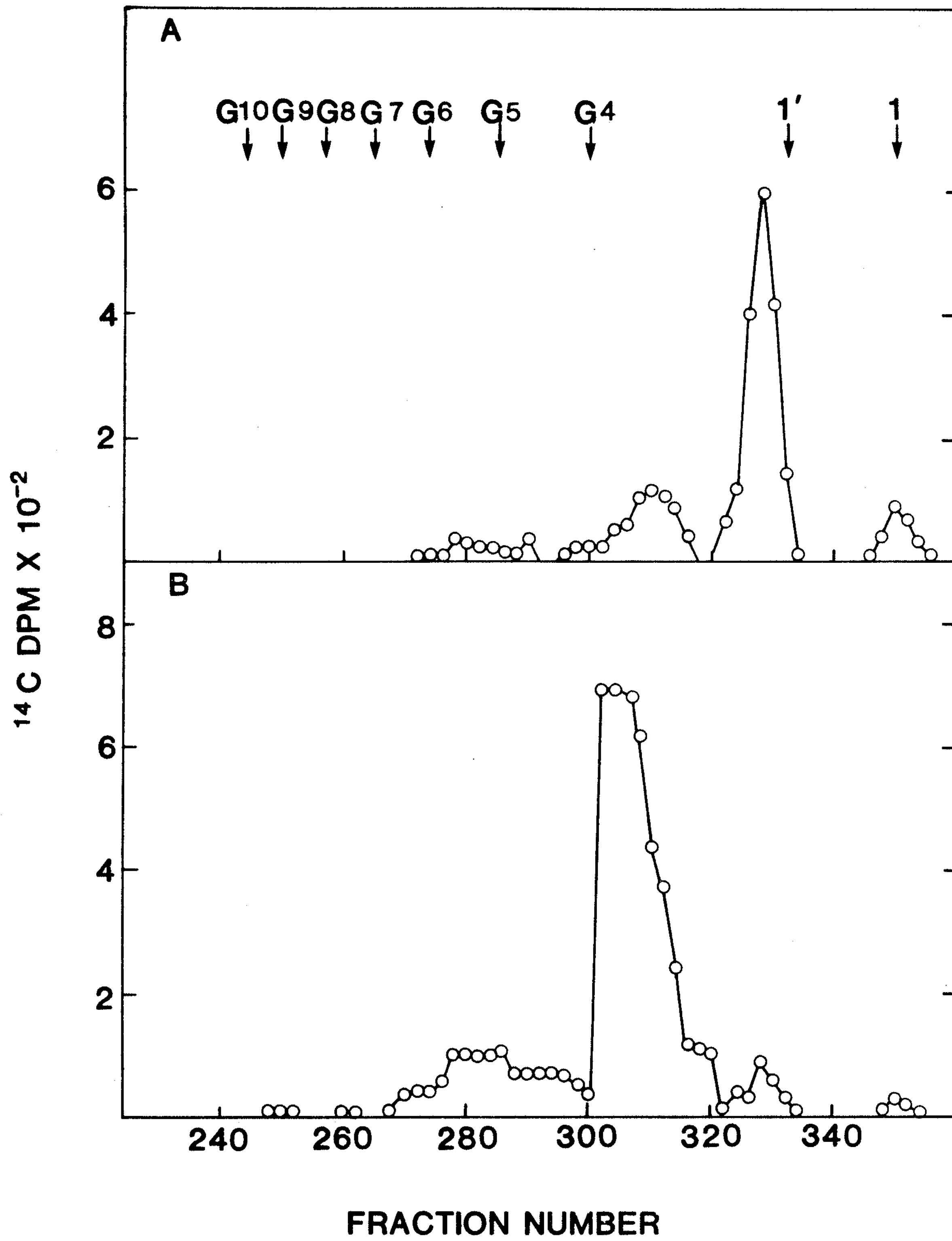

A
G10 G9 G8 G7 G6 G5 G4 1′ 1
g
a b c d e f h
B
a b c d e f g h
3H DPM X 10-3
4
3
2
1
240 260 280 300 320 340
FRACTION NUMBER

Fig. 50. Chromatography of [$^{14}$C]Man-labeled oligosaccharides derived from oligosaccharide-lipid complexes from liver microsome fractions. Oligosaccharide-lipids were extracted from microsome fractions prepared from 5 g of liver slices incubated with [$^{14}$C]Man (see Experimental). See legend of Fig. 48 for further details. Resolved peaks are indicated by lettered arrows. Panel A, control livers; panel B, 12 h experimental livers.


A
G10
G9
G8
G7
G6
G5
G4
1′
1
6
4
2
B
8
6
4
2
$^{14}C$ DPM X $10^{-2}$
240
260
280
300
320
340
FRACTION NUMBER

of the [$^3$H]GlcN-labeled oligosaccharides from control and experimental liver slices were very similar (Fig. 49). The main differences in the [$^3$H]GlcN-labeling in these profiles occured in the common peaks identified as e, f and g (Fig. 49). The largest peak in the control profile was peak g, while peaks e and f contained considerably less radioactivity; in contrast, peak g was minor and peaks e and f were the major labeled peaks in the experimental profile (Fig. 49). Little or no significant [$^{14}$C]Man-labeled oligosaccharides appeared in the region where the maltooligosaccharide standards $Glc_5$-$Glc_{10}$ eluted (Fig. 50). Thus, no attempt was made to compare the elution profiles of the [$^{14}$C]Man-labeled lipid-derived oligosaccharides from control and 12 h experimental livers.

Inflammation results in increased hepatic protein biosynthesis (Jamieson and Ashton, 1973a; Kushner, 1982; Jamieson _et al_., 1983). Thus the increased glycosylation of the lipid-linked oligosaccharides observed in the preceding _in vitro_ cell-free studies could be associated with increased protein biosynthesis and, thus, increased acceptor availability. Some differences were observed in the elution profiles of the lipid-derived [$^3$H]GlcN-labeled oligosaccharides from control and experimental livers (Fig. 49). Thus, it was of interest to examine the effect of puromycin, an inhibitor of protein synthesis, on the incorporation of radioactive sugars into oligosaccharide-lipid complexes in liver slices from control and experimental rats.

The elution profiles of [$^{14}$C]GlcN-labeled and [$^3$H]Man-labeled lipid-derived oligosaccharides formed in liver slices in the presence of puromycin, are shown in Figs. 51 and 52, respectively. While the addition of puromycin resulted in a loss of [$^{14}$C]GlcN-labeled material

Fig. 51. Chromatography of [$^{14}$C]GlcN-labeled oligosaccharides derived from oligosaccharide-lipid complexes from microsome fractions prepared from liver slices incubated in the presence of [$^{14}$C]GlcN and puromycin. See Experimental and legends of Figs. 48-49 for further details. Resolved peaks are indicated by lettered arrows. Panel A, control livers; panel B, 12 h experimental livers.

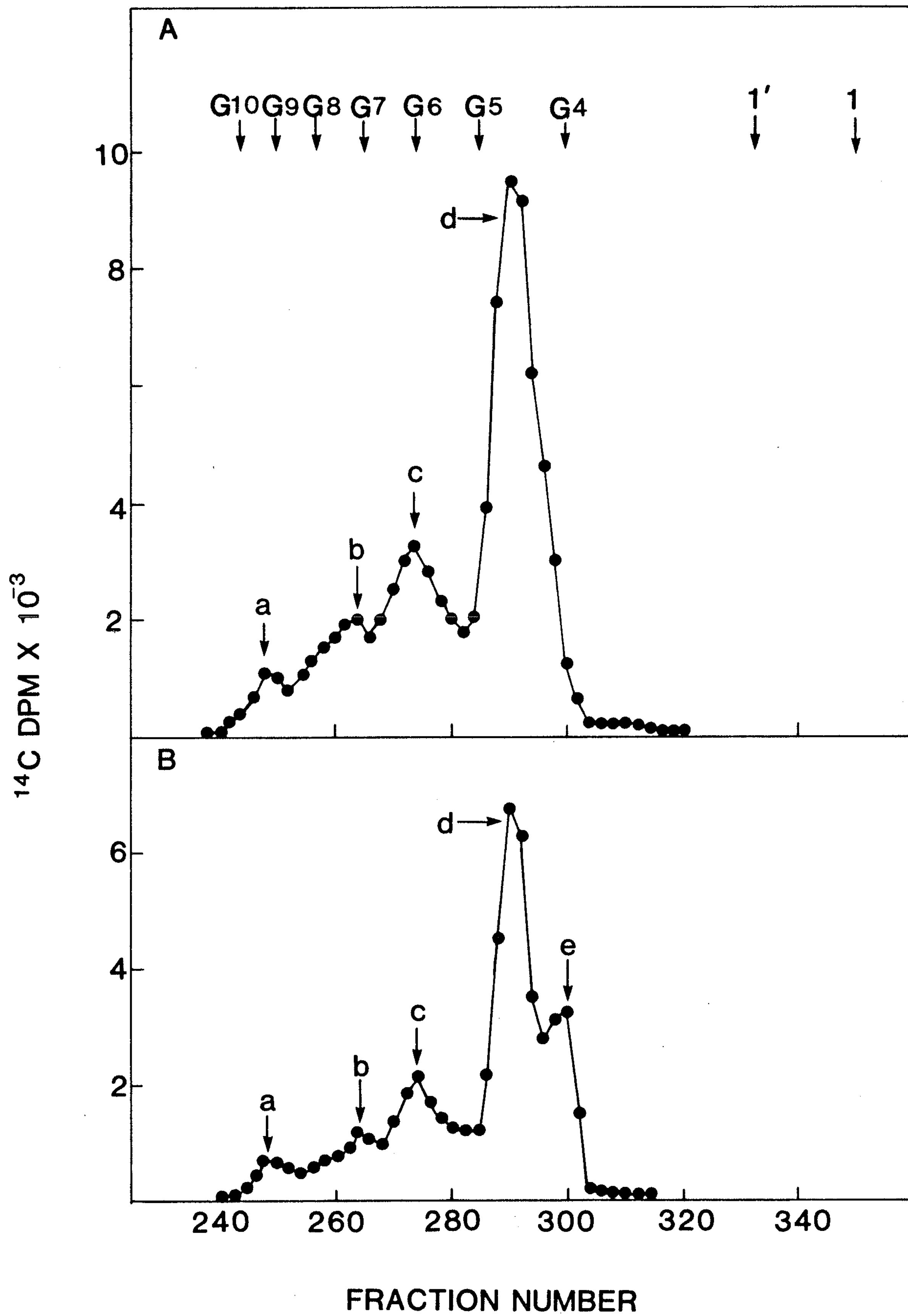
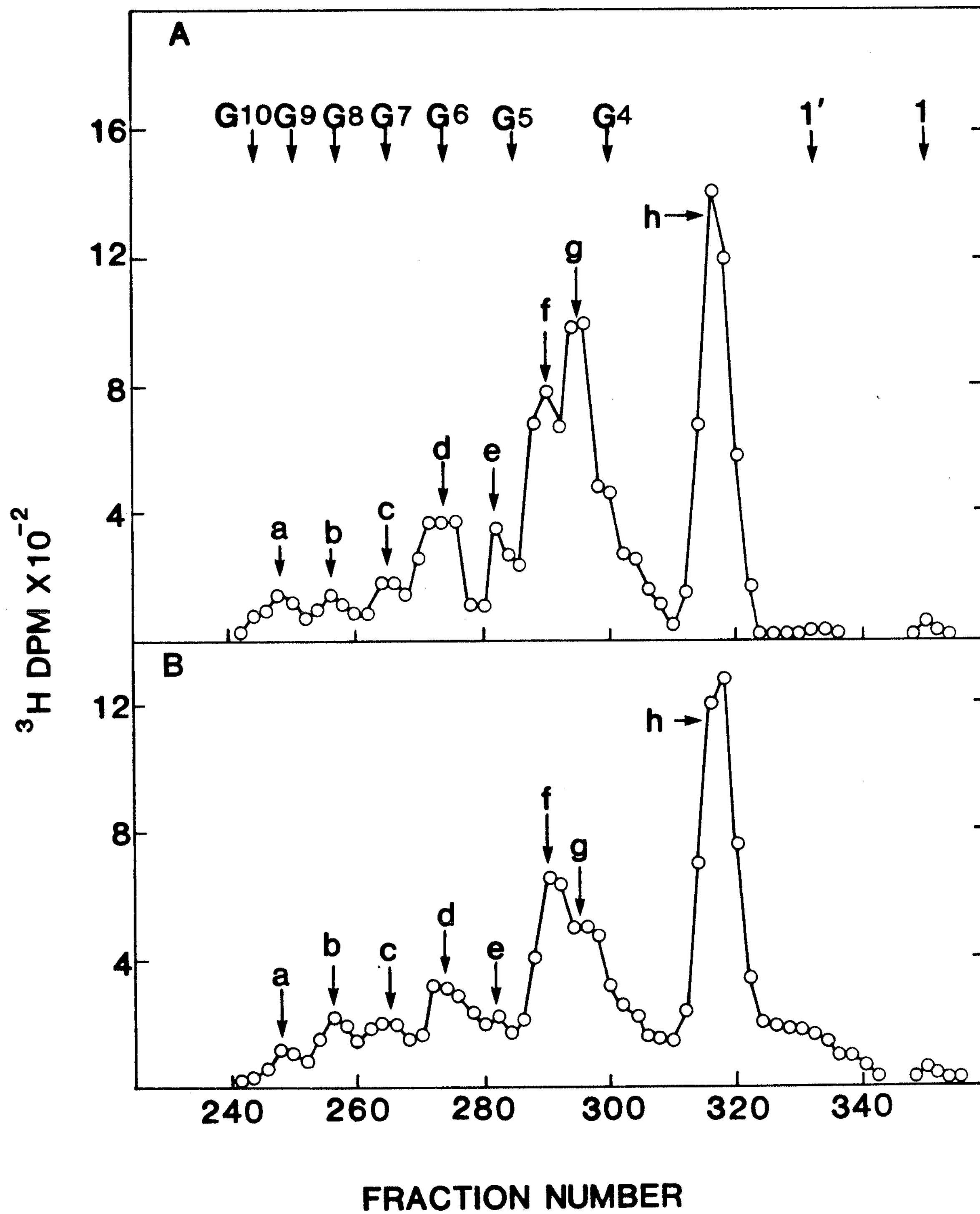

A
G10
G9
G8
G7
G6
G5
G4
1'
1
10
8
4
2
a
b
c
d
B
6
4
2
e
240
260
280
300
320
340
$^{14}C$ DPM X $10^{-3}$
FRACTION NUMBER

Fig. 52. Chromatography of [$^{3}$H]Man-labeled oligosaccharides derived from oligosaccharide-lipid complexes from microsome fractions prepared from liver slices incubated in the presence of [$^{3}$H]Man and puromycin. See Experimental and Figs. 48-49 for further details. Resolved peaks are indicated by lettered arrows. Panel A, control liver; panel B, 12 h experimental liver.


A
B
G10 G9 G8 G7 G6 G5 G4 1′ 1
a
b
c
d
e
f
g
h
16
12
8
4
$^3$H DPM X10$^{-2}$
240
260
280
300
320
340
FRACTION NUMBER

eluting after the $Glc_4$ standard, there were no differences in the profiles obtained from the control and experimental livers (Fig. 51). Profiles of [$^3$H]Man-labeled oligosaccharides from control and experimental livers were also very similar (Fig. 52). Apparently, the inhibition of protein synthesis does not result in differences in the assembly of lipid-linked oligosaccharide precursors of N-linked glycoproteins.

Identification of lipid-derived oligosaccharides

As shown in Figs. 49, 51 and 52, the elution profiles of the lipid-derived labeled oligosaccharides in livers from control and 12 h experimental rats were very similar. An attempt was made to identify the oligosaccharide species associated with the observed peaks on the basis of comparison with reported relative elution constant ($K_d$) values for a homologous series of high-mannose oligosaccharides given in Appendix I. The $K_d$ values and possible compositions of the oligosaccharide peaks from Figs 49, 51 and 52 are given in Tables 16, 17, 18, respectively. As previously stated, the main difference in the profiles from control and 12 h experimental livers, in absence of puromycin, appeared to reside in the amount of radioactivity in peaks e, f and g in Fig. 49. However, as shown in Table 16 and Appendix I, these peaks correspond to species of a lower molecular weight than $Man_1GlcNAc_2$ and were thus, not identified. In addition, the labeled species in Figs. 49 and 52 which eluted after fraction number 280 could not be identified as they were also of a molecular weight lower than that of $Man_1GlcNAc_2$ (see Tables 16 and 18; see also Appendix I). It is possible that these low molecular weight species were artifacts which could have resulted from the degradation

Table 16. Identification of chromatographed lipid-derived [$^{3}H$]glucosamine-labeled oligosaccharides from control and 12 h experimental liver slices.

| Peak | $K_d$ value | | Corresponding oligosaccharide |
|---|---|---|---|
| | Control | Experimental | |
| a | 0.52 | 0.52 | $Man_4GlcNAc_2$ |
| b | 0.56 | 0.56 | $Man_3GlcNAc_2$ |
| c | 0.60 | 0.60 | $Man_2GlcNAc_2$ |
| d | 0.64 | 0.64 | $Man_1GlcNAc_2$ |
| e | 0.69 | 0.69 | n.d. |
| f | 0.71 | 0.71 | n.d. |
| g | 0.78 | 0.78 | n.d. |
| h | 0.87 | 0.87 | GlcNAc |

Peaks designated a-h are from the gel chromatography profile shown in Fig. 49. Relative elution constant ($K_d$) values were calculated and oligosaccharides assigned as shown in Appendix I. Abbreviation: n.d., not determined.

Table 17. Identification of chromatographed [$^{14}$C]glucosamine-labeled lipid-derived oligosaccharides from control and 12 h experimental liver slices incubated with puromycin.

| Peak | $K_d$ value | | Corresponding oligosaccharide |
|---|---|---|---|
| | Control | Experimental | |
| a | 0.52 | 0.52 | $Man_4GlcNAc_2$ |
| b | 0.59 | 0.59 | $Man_2GlcNAc_2$ |
| c | 0.64 | 0.64 | $Man_1GlcNAc_2$ |
| d | 0.72 | 0.72 | n.d. |
| e | - | 0.76 | n.d. |

Peaks designated a-e are from the gel chromatography profile shown in Fig. 51. See Table 16 for further details.

Table 18. Identification of chromatographed [$^{3}$H]mannose-labeled lipid-derived oligosaccharides from control and 12 h experimental liver slices incubated with puromycin.

| | $K_d$ value | | |
|---|---|---|---|
| Peak | Control | Experimental | Corresponding oligosaccharide |
| a | 0.52 | 0.52 | $Man_4GlcNAc_2$ |
| b | 0.56 | 0.56 | $Man_3GlcNAc_2$ |
| c | 0.60 | 0.60 | $Man_2GlcNAc_2$ |
| d | 0.64 | 0.64 | $Man_1GlcNAc_1$ |
| e | 0.68 | 0.68 | n.d. |
| f | 0.72 | 0.72 | n.d. |
| g | 0.75 | 0.75 | n.d. |
| h | 0.84 | 0.84 | n.d. |

Peaks designated a-h are from the gel chromatography profile shown in Fig. 52. See Table 16 for further details.

of the lipid-linked oligosaccharides during the extraction or hydrolysis procedures. The largest lipid-derived oligosaccharide identified in both control and 12 h experimental livers was $Man_4GlcNAc_2$ (Tables 16-18).

From the above studies on the analysis of lipid-linked oligosaccharides, it was evident that there were no significant qualitative differences in the assembly of hepatic oligosaccharide-lipid complexes between control and experimental rats.

## Effect of Inflammation on the Activities of the Oligosaccharide Processing Glycosidases

As previously stated in the Introduction, after transfer to acceptor proteins, the precursor lipid-derived oligosaccharides undergo a series of modifications that eventually produce the complex-type of oligosaccharide chains found in mature N-linked glycoproteins. The initial modifications, termed oligosaccharide processing, involve the removal of the glucose residues and several of the mannoses from the $Glc_3Man_9GlcNAc_2$ structure by specific α-glucosidases and α-mannosidases located in the microsome fraction (Hubbard and Ivatt, 1981; Kornfeld, 1982). These glycosidase activities differ from the corresponding lysosomal enzyme activities in their subcellular localization, substrate specificities, pH optima and other kinetic and physical parameters (Lejeune *et al.*, 1963; Brown *et al.*, 1972; Tulsiani *et al.*, 1977; Opheim and Touster, 1978; Grinna and Robbins, 1979; Tabas and Kornfeld, 1979; Burns and Touster, 1982; Tulsiani *et al.*, 1982; Bischoff and Kornfeld, 1983). The hepatic lysosomal glycosidases are involved in the catabolism of serum glycoproteins internalized by the liver (Aronson, 1972; Gregoriadis, 1975; Pricer and Ashwell, 1976).

Decreased activities of the hepatic lysosomal glycosidases, β-N-acetylglucosaminidase and β-galactosidase, have been reported during inflammation (Kaplan and Jamieson, 1977). This suggests a decreased lysosomal degradation of the carbohydrate moieties of glycoproteins during inflammation. Previous studies have shown that inflammation resulted in increased hepatic synthesis of rat $\alpha_1$-acid glycoprotein, a typical acute phase protein, which led to an increased content of this protein in the microsome fraction (Jamieson et al., 1972b; Jamieson and Ashton, 1973a). A high-mannose containing precursor species of rat $\alpha_1$-acid glycoprotein has been isolated from liver rough fractions (Friesen and Jamieson, 1980), suggesting that initial glycosylation of this protein occurs via the lipid-linked pathway. These results concerning $\alpha_1$-acid glycoprotein, coupled with those previously presented in this thesis showing increased glycosylation of sugar-lipid intermediates and proteins in rough membrane fractions, suggest an elevated level of substrates for the processing glycosidases during inflammation. Thus, a study was performed to determine the effect of inflammation on the hepatic activities of processing α-glucosidases and α-mannosidases in rough and smooth fractions and Golgi, in addition to the acidic glycosidases in the lysosomes.

The pH optima determinations of microsome fraction α-glucosidase and α-mannosidase activities are shown in Figs. 53 and 54, respectively. The pH optima of α-glucosidase and α-mannosidase activities in control microsome fractions were 6.5 and 5.5, respectively; no changes in the pH optima of the enzyme activities were observed in 24 h experimental microsome fractions (Figs. 53-54). Linear relationships were established for incubation time and amount of enzyme protein for both

Fig. 53. The effect of pH on hepatic microsome fraction α-glucosidase activities. Assays were performed in citrate-phosphate buffer adjusted to the appropriate pH; the substrate used was p-nitrophenyl-α-D-glucopyranoside (see Experimental for further details). Panels A and B, control and 24 h experimental hepatic microsome fractions, respectively. Control and 24 h experimental values for 100% activity are 2.9 and 2.4 units/mg microsome fraction protein, respectively. Results are the means of 3-4 analyses; reproducibility was within ±10%.

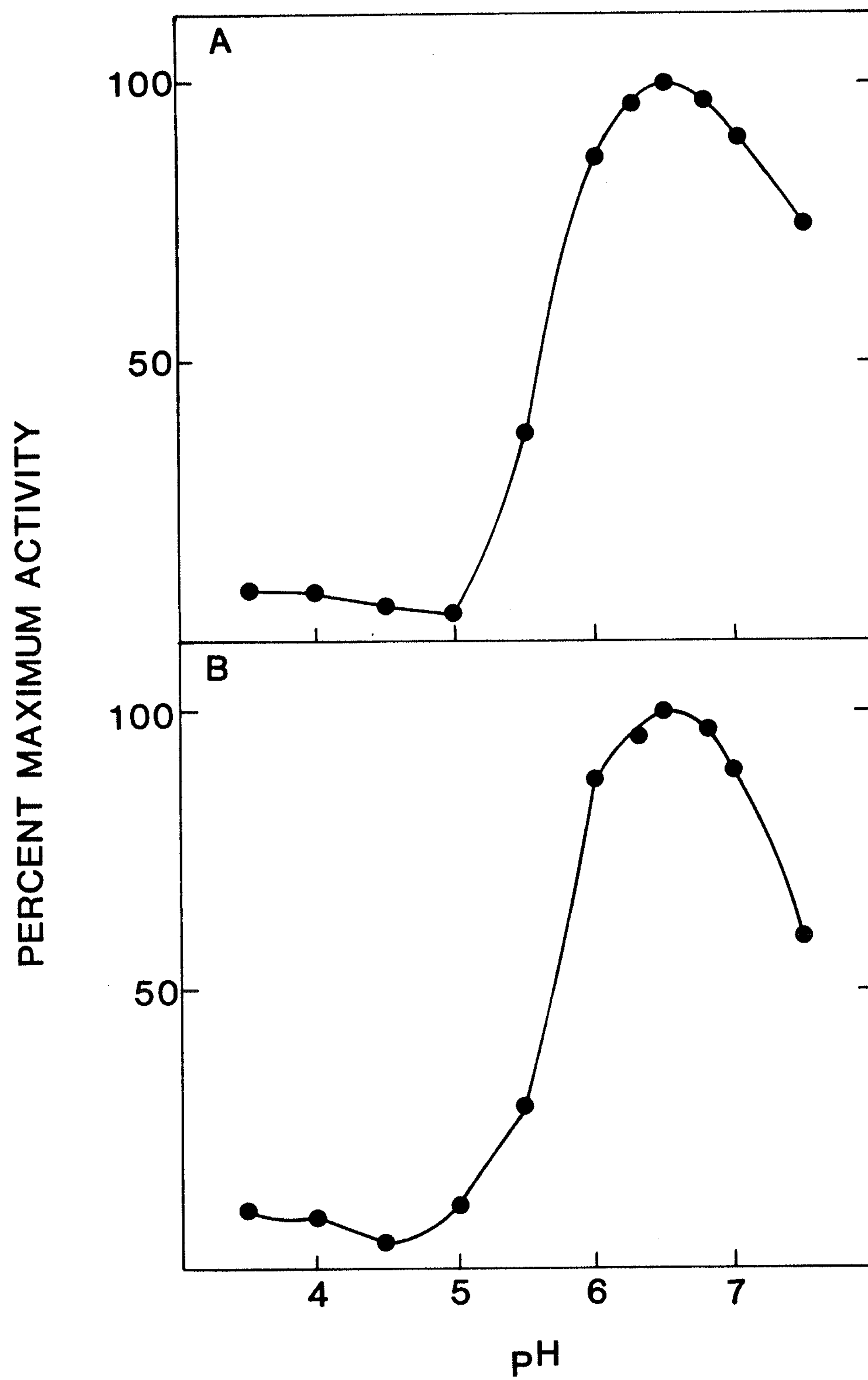
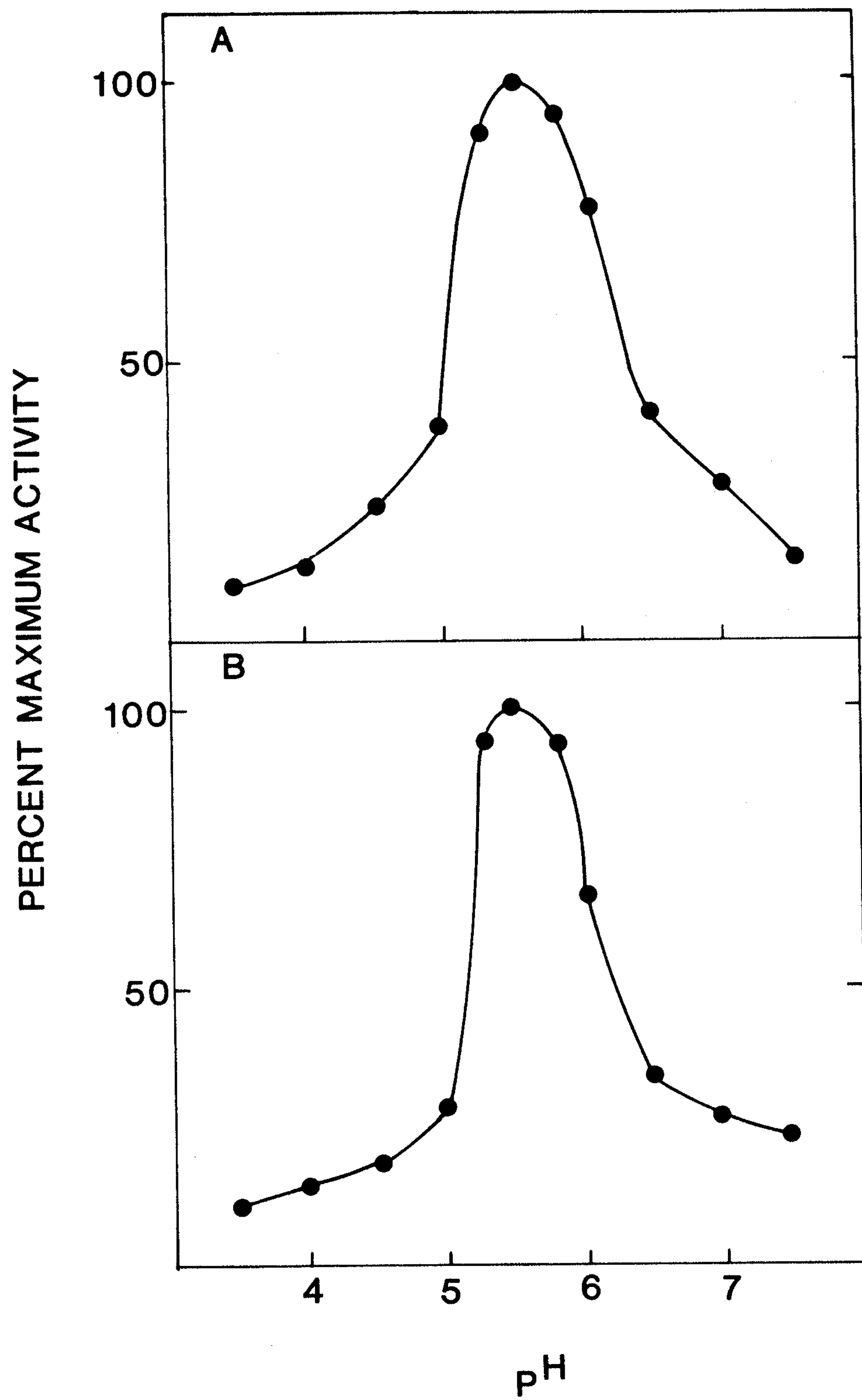
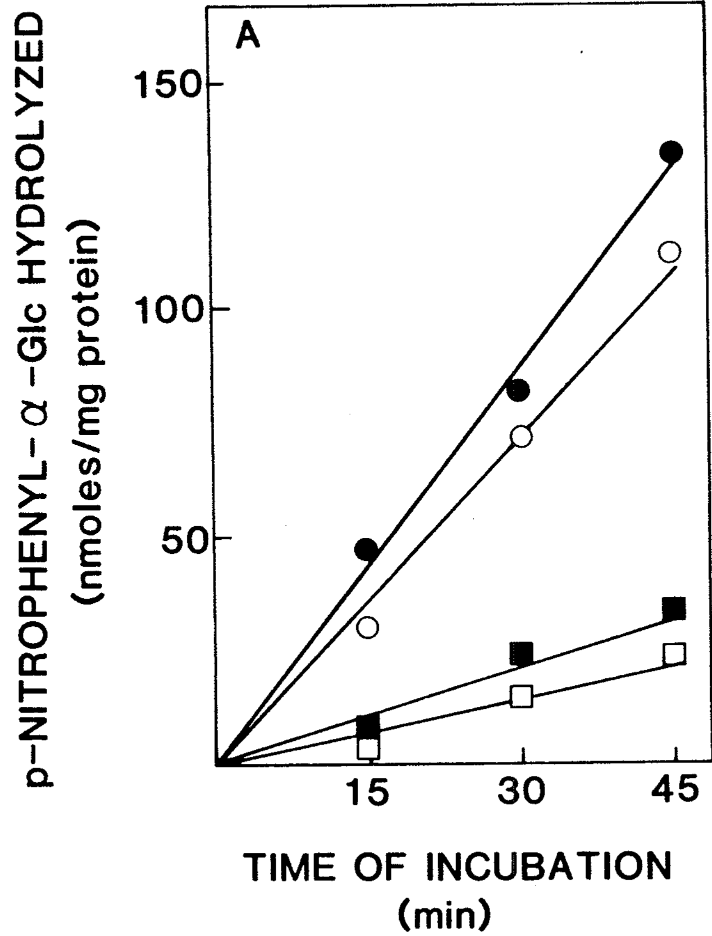

A
100
50
B
100
50
PERCENT MAXIMUM ACTIVITY
4
5
6
7
pH

Fig. 54. The effect of pH on hepatic microsome fraction α-mannosidase activities. Assays were performed in citrate-phosphate buffer adjusted to the appropriate pH; the substrate was p-nitrophenyl-α-D-mannopyranoside (see Experimental for further details). Panels A and B, control and 24 h experimental hepatic microsome fractions, respectively. Control and 24 h experimental values for 100% activity are 2.2 and 1.6 units/mg microsome fraction protein, respectively. Results are the means of 3-4 analyses; reproducibility was within ±10%.


A
B
100
50
100
50
PERCENT MAXIMUM ACTIVITY
4
5
6
7
pH

microsome fraction α-glucosidase (Fig. 55) and α-mannosidase (Fig. 56) activities. The effect of time after inflammation on the specific activities of α-glucosidase and α-mannosidase in rough and smooth membrane fractions, Golgi and lysosomes is shown in Tables 19 and 20. Following inflammation changes were observed in the activities of the neutral processing glycosidases. Activities of α-glucosidase in 12-24 h experimental rough membrane fractions, 48 h experimental smooth membrane fractions and 48 h experimental Golgi preparations decreased by about 20%, 50% and 50%, respectively. Activities of α-mannosidase in 24-48 h experimental rough membrane fractions increased by about 65%; activities in Golgi declined to about 40% control levels by 48 h after inflammation, while remaining unchanged in smooth membrane fractions. Comparison of control microsome subfractions indicates that the Golgi preparations had the highest specific activities of both α-glucosidase and α-mannosidase (Tables 19-20). Both lysosomal α-glucosidase and α-mannosidase activities decreased, reaching a minimum of about 40% below control levels at 24 h after inflammation (Tables 19-20). In contrast to the behavior of the lysosomal enzyme activities, the Golgi processing enzyme activities continued to decrease throughout the time course studied (Tables 19-20).

Fig. 55. Effect of incubation time and amount of enzyme protein on microsome fraction and lysosome α-glucosidase activities. Control (●) and 24 h experimental (O) microsome fractions; control (■) and 24 h experimental (□) lysosomes. Results represented the means of 2-3 analyses; reproducibility was within ±10%.


A
p-NITROPHENYL-α-Glc HYDROLYZED (nmoles/mg protein)
150
100
50
15
30
45
TIME OF INCUBATION (min)

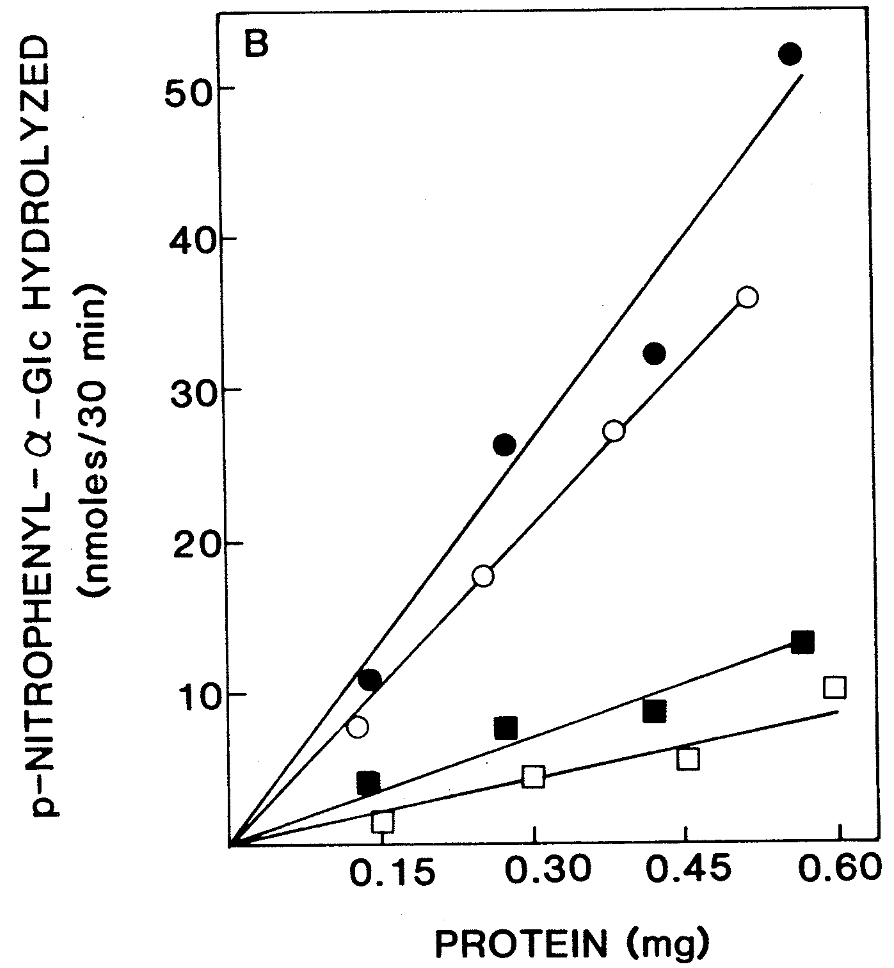
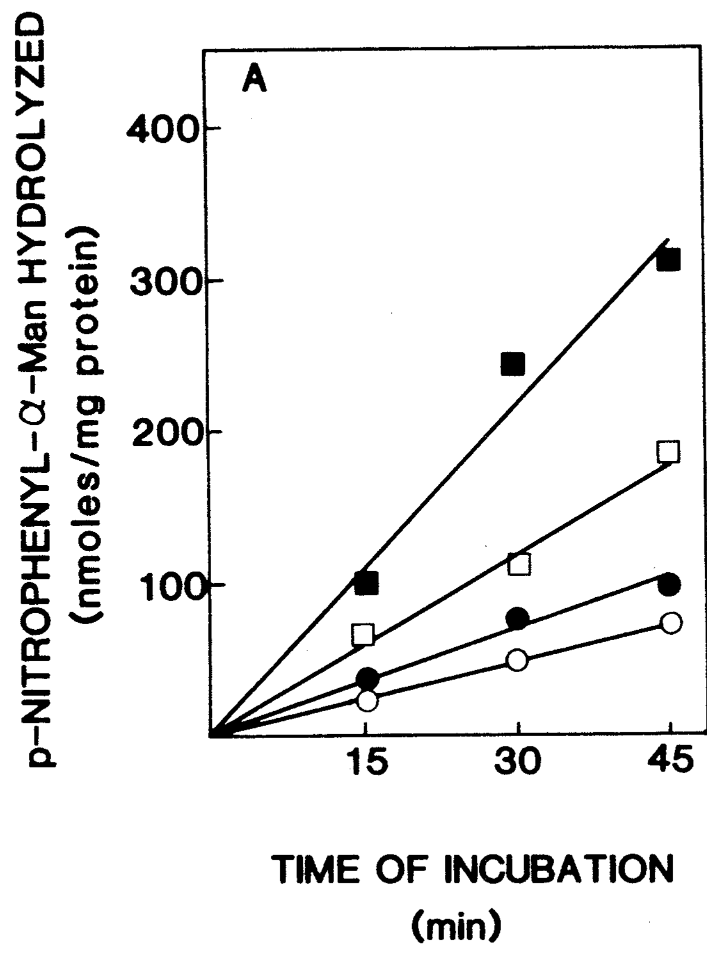
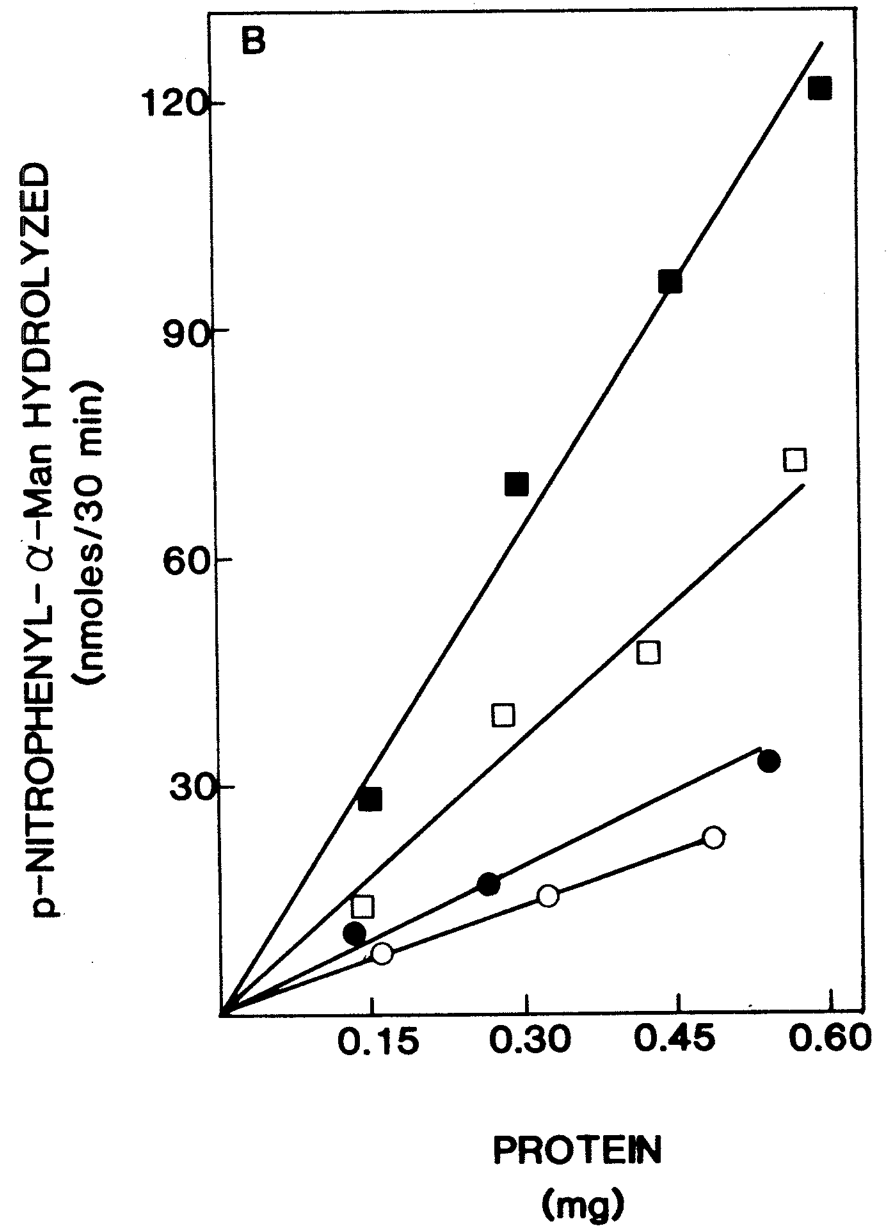

B
p-NITROPHENYL-α-Glc HYDROLYZED (nmoles/30 min)
50
40
30
20
10
0.15
0.30
0.45
0.60
PROTEIN (mg)

Fig. 56. Effect of incubation time and amount of enzyme protein on microsome fraction and lysosome α-mannosidase activities. Control (●) and 24 h experimental (O) microsome fractions; control (■) and 24 h experimental (□) lysosomes. Results represent the means of 2-3 analyses; reproducibility was within ±10%.



A
p-NITROPHENYL-α-Man HYDROLYZED
(nmoles/mg protein)
400
300
200
100
15
30
45
TIME OF INCUBATION
(min)

B
p-NITROPHENYL-α-Man HYDROLYZED
(nmoles/30 min)
120
90
60
30
0.15
0.30
0.45
0.60
PROTEIN
(mg)

Table 19. Effect on inflammation on α-glucosidase activities in liver microsome subfractions and lysosomes.

| Time after inflammation (h) | α-Glucosidase activity | | | |
|---|---|---|---|---|
| | Rough | Smooth | Golgi | Lysosomes |
| 0 | 2.87 ± 0.18 | 3.86 ± 0.16 | 4.07 ± 0.21 | 0.72 ± 0.04 |
| 12 | 2.10 ± 0.17 | 3.86 ± 0.23 | 3.40 ± 0.28 | 0.59 ± 0.04 |
| 24 | 2.48 ± 0.21 | 3.22 ± 0.22 | 3.31 ± 0.16 | 0.45 ± 0.03 |
| 48 | 2.74 ± 0.13 | 1.80 ± 0.12 | 2.26 ± 0.21 | 0.56 ± 0.04 |

Values represent the means and standard deviations of 4-6 analyses. Enzyme activities of rough and smooth membrane fractions and Golgi were assayed at pH 6.5, the optimum pH for microsome fraction α-glucosidase activity (see Fig. 53). Lysosomal enzyme activity was assayed at pH 4.2 (Brown _et al_., 1972). Enzyme activities are expressed as units per mg protein (see Experimental).

Table 20. Effect of inflammation on α-mannosidase activities in liver microsome subfractions and lysosomes.

| Time after inflammation (h) | α-Mannosidase activity | | | |
|---|---|---|---|---|
| | Rough | Smooth | Golgi | Lysosomes |
| 0 | 0.62 ± 0.06 | 2.87 ± 0.21 | 5.40 ± 0.49 | 7.20 ± 0.41 |
| 12 | 0.74 ± 0.05 | 2.83 ± 0.17 | 5.32 ± 0.41 | 6.11 ± 0.31 |
| 24 | 1.02 ± 0.06 | 2.73 ± 0.16 | 2.77 ± 0.26 | 3.90 ± 0.21 |
| 48 | 1.05 ± 0.10 | 2.77 ± 0.22 | 2.01 ± 0.20 | 5.12 ± 0.28 |

Values represent the means and standard deviations of 4-6 analyses. Enzyme activities of rough and smooth membrane fractions and Golgi were assayed at pH 5.5, the optimum pH for microsome fraction α-mannosidase activity (see Fig. 54). Lysosomal enzyme activity was assayed at pH 4.6 (Opheim and Touster, 1978). Enzyme activities are expressed as units per mg protein (see Experimental).

## STUDIES ON THE EFFECT OF INFLAMMATION ON RAT LIVER AND SERUM SIALYL AND GALACTOSYL TRANSFERASE ACTIVITIES

Elevated levels of sialyl and galactosyl transferase activities were observed in the serum of rats following onset of inflammation. Elevated glycosyl transferase activities have also been observed in animals and humans with a variety of inflammatory and pathological conditions; these include various liver diseases (Kim *et al.*, 1972; Mookerjea *et al.*, 1972), partial hepatectomy (Ip, 1979), and various neoplastic diseases (Kessel and Allen, 1975; Podolsky and Weiser, 1975; Kessel *et al.*, 1976, 1977; Podolsky *et al.*, 1978). As mentioned previously, proteins that increase in concentration in serum following inflammation are termed acute phase reactants; thus, on this basis sialyl and galactosyl transferase are behaving as if they were acute phase reactants. Since liver is the site of synthesis of acute phase reactants, hepatic sialyl and galactosyl transferase activities were examined. It was found that, as in serum, both sialyl and galactosyl transferase activities increased in liver following inflammation. Similar elevations in rat hepatic glycosyl transferase activities following inflammation were observed by Lombart *et al.* (1980) using human asialo- and asialoagalacto-$\alpha_1$-acid glycoproteins as acceptor substrates for sialyl and galactosyl transferases, respectively.

These results resemble those found with acute phase proteins, such as $\alpha_1$-acid glycoprotein (Kushner, 1982; Jamieson *et al.*, 1983). Using a liver slice system to study the synthesis and secretion of $\alpha_1$-acid glycoprotein, Jamieson *et al.* (1975) demonstrated elevated rates of synthesis and increased secretion of this acute phase

protein by liver slices prepared from inflamed rats; it is believed that elevated hepatic biosynthesis accounts for the increased serum content of $\alpha_1$-acid glycoprotein following inflammation (Jamieson et al., 1972b; Jamieson et al., 1983). In this thesis, direct evidence for a hepatic origin of the elevated serum sialyl transferase activity was provided by similar studies using liver slices; elevated serum galactosyl transferase could not be accounted for by hepatic release.

The results from the liver slice experiments clearly show that liver slice medium and serum enzymes have similar properties. For example, the pH optima and apparent $K_m$ values, with respect to CMP-NeuAc, and rat and human asialo-$\alpha_1$-acid glycoprotein, were observed to be very similar for the serum and liver slice medium sialyl transferase activities. Both medium and serum sialyl transferase activities were potently inhibited by nucleoside triphosphates, as well as cytidine mono- and di-phosphates. As mentioned previously, liver is known to contain at least six sialyl transferases, two of which have been purified by Paulson's group (Weinstein et al., 1982a, b). These enzymes have been shown to be responsible for the formation of NeuAc$\alpha$2→3Gal$\beta$1→4(3)GlcNAc and NeuAc$\alpha$2→6Gal$\beta$1→4GlcNAc linkages in the oligosaccharides of N-linked glycoproteins. However, the studies described in this thesis show that only the $\alpha$2→6 sialyl transferase appeared to be released by liver. These results are all consistent with the hypothesis of a hepatic origin for the serum sialyl transferase activity following inflammation. These results are in agreement with those of Hudgin and Schachter (1971) who have compared the properties of porcine liver and serum sialyl transferases and have

shown that the serum enzyme resembles the liver enzyme. In addition studies presented here suggest that the elevated levels of serum sialyl transferase activity in humans suffering from neoplastic diseases also are due to increases in the $\alpha2\rightarrow6$ sialyl transferase activity. This would suggest that, as with the rat enzyme, the human enzyme is likely to be of hepatic origin and may also be behaving as an acute phase reactant.

A study by Shah and Raghupathy (1977) showed differential effects of nucleotides on rat liver and serum sialyl transferase activities, suggesting that the serum and liver enzymes may be different. In that study, all nucleotides inhibited the serum sialyl transferase activity with nucleoside triphosphates and all cytosine nucleotides having the greatest inhibitory effect. Uracil nucleotides, UDP and UTP, caused a twofold activation of the liver sialyl transferase activity, whereas GDP, GTP and ATP were without significant activating or inhibitory effects. Thus, the results obtained from the study in this thesis agree with the above results with regard to the serum enzyme activity, but not the liver enzyme activity. The reason for this discrepancy is not clear. However, in the present work, rat asialo-$\alpha_1$-acid glycoprotein was used as acceptor substrate, whereas, in the study by Shah and Raghupathy (1977), asialo-fetuin was used as acceptor substrate. Fetuin contains three N-glycosidically linked and three O-glycosidically linked carbohydrate chains (Spiro, 1973; Spiro and Bhoyroo, 1974; Nilsson et al., 1979), whereas rat $\alpha_1$-acid glycoprotein has five N-glycosidically linked carbohydrate chains (Yoshima et al., 1981). Therefore, the increase in hepatic sialyl transferase activity observed in the presence of

uridine nucleotides in the study of Shah and Raghupathy (1977) could be explained by the effects on the sialyl transferase(s) responsible for attaching NeuAc to the O-linked oligosaccharide chains. Purified rat liver sialyl transferases responsible for attaching NeuAc to N-linked oligosaccharide chains do not react with O-linked oligosaccharide substrates (Weinstein et al., 1982b).

As previously mentioned in the Introduction, other studies in our laboratory have led to the isolation of a protein fraction derived from peritoneal monocytes which is believed to be a central mediator of the acute phase response. In these studies, it was found that liver and serum sialyl transferase activities were elevated suggesting that this monokine acts on the liver, not only to stimulate $\alpha_1$-acid glycoprotein biosynthesis and depress albumin biosynthesis (Woloski et al, 1983a), but possibly to cause release of sialyl transferase into serum. Thus, it would appear that the alterations in liver and serum sialyl transferase activities after inflammation could be controlled by the same factors responsible for elevated hepatic synthesis of acute phase reactants, lending further support for a possible hepatic origin for the serum sialyl transferase activity.

Since sialyl and galactosyl transferases are located mainly in the Golgi complex (Schachter et al., 1970; Bretz et al., 1980; Beyer and Hill, 1982) and both enzymes are closely associated in function (Beyer and Hill, 1982), the lack of appearance of large amounts of galactosyl transferase activity in liver slice medium supports the idea of a preferential release of sialyl transferase from livers from both control and experimentally inflamed rats. In addition, the results obtained using galactosyl transferase as a control enzyme

probably exclude cell rupture as an explanation for the appearance of sialyl transferase in medium; the degradation of galactosyl transferase in the liver slice system was discounted by suitable control experiments which showed that both sialyl and galactosyl transferase activities were stable in liver slices and medium over the incubation times used. Thus, it was of interest to examine the nature of the release of sialyl transferase from liver.

As previously mentioned in the Introduction, glycosyl transferases which have been purified and characterized thus far have been found to be glycoproteins. The pathway of secretion that has been characterized for many liver synthesized glycoproteins such as $\alpha_1$-acid glycoprotein (Jamieson _et al_., 1983), and other secretory proteins such as albumin (Peters, 1977) involves synthesis on bound polyribosomes in the rough endoplasmic reticulum and secretion into blood _via_ the channels of rough and smooth endoplasmic reticulum and Golgi complex. Along this pathway, the proteins undergo covalent modifications such as the addition of carbohydrate moieties to the polypeptide chain, processing and elongation of the oligosaccharide chains, as well as proteolytic removal of the propeptide (see Introduction). In this thesis, tunicamycin, puromycin, cycloheximide, colchicine and proteases were all used in a liver slice system to obtain some information to allow us to understand how sialyl transferase is released from liver.

Glycosyl transferases are believed to be glycoproteins containing N-linked oligosaccharide chains (Beyer _et al_., 1981; Fujita-Yamaguchi and Yoshida, 1981). In the studies in this thesis, tunicamycin, an inhibitor of N-linked glycosylation, was found to have little or no effect on the release of sialyl transferase activity into medium from

slice experiments using livers prepared from control and inflamed rats. This suggests that glycosylation of the enzyme is not required for its release from the liver.

Studies on the effects of inhibition of glycosylation by tunicamycin on cell surface and secretable glycoproteins indicate that while for some glycoproteins carbohydrate is not obligatory for export or secretion, the opposite is true for other glycoproteins. For example, glycosylation is apparently not required for export or secretion of transferrin and $\alpha_1$-acid glycoprotein from rat liver (Edwards et al., 1979), ovalbumin from hen oviduct (Struck et al., 1978), immunoglobulin G (IgG) from mouse plasmacytoma cells (Hickman and Kornfeld, 1978), or fibronectin from chick embryo fibroblasts (Olden et al., 1978), nor for the insertion of rhodopsin into retinal disk membranes (Poncz and Kean, 1980). In other instances, glycosylation has been found to be required for proper export and secretion of invertase and acid phosphatase from yeast (Kuo and Lampen, 1976), IgA, IgE, and IgM of mouse plasmacytoma cells (Hickman et al., 1977), secretory and surface proteins of Baby Hamster Kidney fibroblasts (Damsky et al., 1979), as well as vesicular stomatitis virus (VSV) envelope glycoprotein (Leavitt et al., 1977a,b). Thus, when glycosylation is blocked, the effects on the export and secretion of specific proteins are variable. This variability also extends to the fate of nonglycosylated, nonsecreted proteins. For example, it has been found that nonglycosylated fibroblast fibronectin is hypersusceptible to intracellular proteolytic degradation (Olden et al., 1978), whereas the VSV nonglycosylated envelope glycoprotein accumulates intracellularly, but is not appreciably degraded (Leavitt et al.,

1977a,b). In the case of VSV envelope glycoprotein, the aggregation of the nonglycosylated virus protein was explained as an alteration in the tertiary structure (Gibson _et al._, 1979). Taken together, the results presented above suggest that the carbohydrate moieties exert specific effects on the functional conformation of glycoproteins, but that some glycoproteins are more dependent on their carbohydrate moieties for maintaining functional conformation than are others. In other words, it seems that post-translational modifications of proteins, such as glycosylation, required for the formation of proper tertiary structure may be necessary to ensure proper transport and secretion of some proteins. In the case of sialyl transferase release from liver in the studies in this thesis, glycosylation does not appear important to ensure its release from liver.

Liver contains an intracellular pool of sialyl transferase in the Golgi complex as indicated by the sialyl transferase activity of Golgi membrane (Schachter _et al._, 1970; Schachter and Roseman, 1980). Thus, it is possible that the release of sialyl transferase activity from liver occurred from the Golgi pool rather than by release of newly synthesized enzyme. This possibility was supported by the experiments with the protein synthesis inhibitors, puromycin and cycloheximide, which had no effect on the release of sialyl transferase activity into the medium. Studies on the synthesis and secretion of albumin, $\alpha_1$-acid glycoprotein and transferrin have determined that the transit time from liver for these proteins is 20-30 minutes after initiation of translation (Schreiber _et al._, 1979). This suggest that, since liver slices were incubated with puromycin and cycloheximide for 3 h, release of sialyl transferase

from liver was not dependent on _de novo_ synthesis of the enzyme. Also, since initial glycosylation of N-linked glycoproteins occurs on polypeptide chains in the rough endoplasmic reticulum, the lack of effect of tunicamycin on sialyl transferase release from liver was most likely due to the fact that released enzyme activity originated from the pre-existing enzyme pool in the Golgi complex.

The final steps in the secretion of plasma proteins from liver involve the migration of Golgi-derived secretory vesicles to the sinusoidal cell surface, where the vesicles fuse with the plasma membrane and empty their contents into the extracellular space (Glaumann, 1970; see also Introduction). The migration of secretory vesicles to the plasma membrane is believed to be mediated by microtubules. Microtubules have been implicated in the release of a number of hormones and proteins, including insulin (Lacy _et al._, 1972), thyroid, pituitary and parathyroid hormones (Williams and Wolf, 1972; Labrie _et al._, 1973; Reaven and Reaven, 1975), collagen (Ehrlich and Bornstein, 1972), low density lipoproteins (Stein and Stein, 1973), and plasma proteins (Redman _et al._, 1975). Most of this work is based on the use of antimitotic drugs, such as colchicine, which are known to exert an effect on microtubules. Colchicine interacts with the primary microtubular protein, tubulin (Wilson _et al._, 1974), and causes the dissolution of formed microtubules. In the studies in this thesis, colchicine, added to liver slice incubations containing livers from control and 24 h experimental rats, caused decreases in release of sialyl transferase activity into the medium; no significant change was observed in hepatic sialyl transferase activity. Release of sialyl transferase activity from control liver slices was inhibited

by about 25% at all concentrations of colchicine used; inhibition of release of sialyl transferase activity from 24 h experimental liver slices increased from 23% to 45% as the concentration of colchicine was increased from 1 x $10^{-5}$ M to 5 x $10^{-5}$ M. Although there may be differences in the release of sialyl transferase between livers from control and inflamed rats, the results from the studies with colchicine would suggest that disruption of microtubules correlates with an inhibition of release of sialyl transferase. The differential effects of colchicine found with control and experimental livers could be explained by the increased levels of microtubules in experimental livers as suggested by Feldmann (1982). Feldmann (1982) suggests that the increase in the number of microtubules is required for the secretion of increased intracellular levels of acute phase proteins which result from elevated biosynthesis following inflammation. Thus, it is possible that a greater concentration of colchicine may be required to interact with the increased number of microtubules in experimental livers to achieve a maximum level of inhibition of sialyl transferase release as apparently is obtained with control livers in the present study.

It is interesting to note that a maximum inhibition of only about 25% was achieved in the release of sialyl transferase activity from control liver slices under the assay conditions used. Studies by Wilson *et al*. (1974) on the interaction of colchicine with microtubules showed that colchicine does not bind to certain microtubules, termed stable microtubules, but does bind to other microtubules, termed labile microtubules, resulting in their dissolution. Based on the results of these studies, Wilson *et al*. (1974) proposed that

the colchicine binding site is one of the protein-protein interaction sites between tubulin molecules, and that the binding of colchicine at that site prevents normal microtubule assembly. Thus, the ability of colchicine to disrupt assembled microtubules would be dependent on the stability of the microtubule; the more stable the microtubule, the more resistant it is to the action of colchicine. Thus, one explanation for the results obtained in the present work would be that control livers contain a large number of stable microtubules, thus limiting the inhibition of sialyl transferase activity release into medium to 25% over the range of colchicine used. Furthermore, the increased number of microtubules in experimental livers may be largely of the labile type since nearly double the percentage of inhibition of release of sialyl transferase activity into medium was observed for experimental livers compared with controls at the highest concentration of colchicine used.

Studies by others on the effect of colchicine on the secretion of plasma proteins from liver have yielded similar results to those obtained in this thesis regarding sialyl transferase release. Using liver slices, Redman _et al_. (1975) observed about a 60% inhibition of albumin secretion with $10^{-5}$ M colchicine. Redman _et al_. (1975) also showed that colchicine did not interfere with the synthesis and transport of albumin to the Golgi-derived secretory vesicles where the protein accumulated. _In vivo_ studies on the effect of colchicine on the secretion of hepatic serum glycoproteins produced similar results to those obtained above for albumin (Banerjee _et al_., 1976). Although colchicine inhibited the secretion of the serum glycoproteins and caused their intracellular accumulation in the secretory vesicles, it

did not interfere with the Golgi-localized addition of galactose and sialic acid (Schachter, 1978; Schachter and Roseman, 1980) to these glycoproteins. The results from the above studies suggest that the colchicine-inhibited release of sialyl transferase activity observed in the studies in this thesis was a post-Golgi event involving the microtubular system. These results also suggest that the release of sialyl transferase from liver occurs in the same manner or in a similar manner to that of other plasma proteins, that is, via secretory vesicles.

Assuming that the membrane-bound and soluble sialyl transferases are identical, as is strongly suggested in this thesis, then some type of post-translational modification must be responsible for the release of the liver enzyme from the membrane. The most obvious suggestion is that there is proteolytic cleavage of the membrane-bound enzyme releasing the catalytically active portion of protein from the membrane. Once released from the membrane, the enzyme may be secreted via Golgi-derived secretory vesicles. The selective release of the $\alpha 2 \rightarrow 6$ and not the $\alpha 2 \rightarrow 3$ sialyl transferase from the liver suggests that the membrane-bound $\alpha 2 \rightarrow 6$ sialyl transferase may be more exposed or more suitable for attack by proteolytic enzymes. As mentioned in the Introduction, both sialyl transferase and Golgi-derived secretory vesicles are associated with the trans Golgi. Thus, it is possible that the portion of the Golgi membrane destined to form secretory vesicles contains membrane-bound sialyl transferase enzymes, and that the dissociation of the sialyl transferases from the membrane occurs after formation of the secretory vesicles. The proteolytic conversion of proalbumin to albumin occurs predominantly

in secretory vesicles (Ikehara et al., 1976; Redman et al., 1978). Thus, a similar type of proteolytic activity could possibly be responsible for releasing the sialyl transferase into the cisternal space of the secretory vesicle which contains other secretory proteins. The dissociation of α2→6 sialyl transferase from the Golgi or secretory vesicle membrane, as suggested above, represents an intracellular dissociation mechanism. However, although glycosyl transferases are located primarily in the endoplasmic reticulum and Golgi complex, there is ample evidence that these enzymes also exist on the cell surface membranes of many cell types (Shur and Roth, 1975; Pierce et al., 1980). Studies on the hepatic subcellular localization of glycosyl transferases demonstrated that while the Golgi complex is definitely enriched in sialyl, galactosyl, N-acetylglucosaminyl, and fucosyl transferase activities, low levels of these enzyme activities are present on the plasma membrane (Munro et al., 1975). The origin of the hepatic plasma membrane glycosyl transferases, such as sialyl transferase, could possibly be the Golgi complex. Membrane-bound glycoproteins, such as glycosyl transferases, may be transported to the plasma membrane via Golgi-derived secretory vesicles, as are other surface membrane and secretory proteins. The secretory vesicle migrates to the plasma membrane and fuses with it, releasing its contents and generating a new patch of plasma membrane complete with integral glycoproteins (Morre et al., 1979), such as the glycosyl transferases. There is substantial evidence that the plasma membrane undergoes turnover as part of normal membrane function (Baumann and Doyle, 1982). During this process certain substances may be naturally shed or released from the cell surface (Baumann and Doyle, 1982;

Doljanski, 1982). Thus, the release of $\alpha 2 \rightarrow 6$ sialyl transferase from the liver could possibly occur at the cell surface.

At this time, the weight of evidence would suggest that sialyl transferase is most likely to have been released from the Golgi complex. Paulson et al. (1977a) purified a soluble sialyl transferase from bovine colostrum that exhibited two major forms with molecular weights of about 54000 and 42000. Such heterogeneity has been found in other purified glycosyl transferases (Beyer et al., 1981) and has been suggested to be the result of proteolytic degradation which releases portions of the polypeptide nonessential for catalytic activity (Magee et al., 1973, 1976; Powell and Brew, 1974), such as those involved in anchoring the polypeptide to the Golgi membrane (Sadler et al., 1979b; Westcott and Hill, 1982). Thus, the soluble forms of the bovine colostrum sialyl transferase are believed to represent degraded species of the membrane-bound enzyme in the Golgi complex of lactating mammary glands. Studies in this thesis show that liver, medium and serum sialyl transferases have remarkably similar kinetic properties. Thus, it is likely that the soluble forms of sialyl transferase in serum and medium also arise as a result of proteolytic cleavage of the liver membrane-bound enzyme which releases it from the membrane, resulting in the enzyme being secreted from the liver.

Recently, Paulson's group (Weinstein et al., 1982a) has prepared antiserum to their purified rat liver $\alpha 2 \rightarrow 6$ sialyl transferase. Clearly, the use of antiserum would provide the means to answer some of the questions posed in the present work. It would allow for the determination of immunological identity of the liver, medium and serum $\alpha 2 \rightarrow 6$ sialyl transferases. It would also provide the means for studying the

enzyme's biosynthetic pathway, as well as its turnover rates in liver and serum by use of pulse-chase experiments with radioactive amino acids. These experiments would allow for a direct comparison of the amount of enzyme protein translated, transported and secreted in control and experimental livers, as was performed on $\alpha_1$-acid glycoprotein in our laboratory (Jamieson and Ashton, 1973b; Jamieson et al., 1975). In the studies determining the effects of colchicine, tunicamycin, puromycin and cycloheximide on the release of sialyl transferase from liver slices, antiserum could be used to determine the effect on the polypeptide itself and not just on the activities. Clearly, the results which would be provided by the above studies would help to more fully delineate the nature of the release of the $\alpha 2 \rightarrow 6$ sialyl transferase from both control and experimental livers.

The metabolic role of the Gal$\beta$1$\rightarrow$4GlcNAc$\alpha$2$\rightarrow$6 sialyl transferase released from the liver into serum under normal or inflammatory conditions is not known. While the liver enzyme is responsible for catalyzing the addition of sialic acid to the terminal positions of N-linked complex type oligosaccharides, the involvement of the serum enzyme in the glycosylation of serum glycoproteins seems unlikely because of the absence of nucleotide sugars in the blood (Weiser et al., 1982). The $\alpha 2 \rightarrow 6$ sialyl transferase appears to be behaving as an acute phase reactant. As previously mentioned in the Introduction, acute phase reactants play important roles in the non-specific immune responses of the host, thus, the $\alpha 2 \rightarrow 6$ sialyl transferase may also have a similar function. Another possibility is that the presence of the sialyl transferase in serum may be the result of normal hepatic function in which intracellular material in the

endoplasmic reticulum or Golgi complex is translocated to the cell surface membrane, where some is released or shed into the extracellular space. Thus, the sialyl transferase would then be a byproduct of membrane turnover rather than having a specific extracellular metabolic role. The function, if any, of the serum sialyl transferase remains to be elucidated.

The results from the present work strongly suggest that $\alpha 2 \rightarrow 6$ sialyl transferase is behaving like an acute phase reactant. In view of the elevation of the human $\alpha 2 \rightarrow 6$ sialyl transferase activity in pathological conditions, future studies have the potential of leading to the use of this serum enzyme as a clinical diagnostic indicator for the presence of acute inflammatory conditions.

STUDIES ON THE EFFECT OF INFLAMMATION ON NUCLEOTIDE SUGAR METABOLISM

The acute phase response in mammals is accompanied by elevated hepatic biosynthesis of acute phase reactants, such as $\alpha_1$-acid glycoprotein, fibrinogen, haptoglobin and C-reactive protein (Kushner, 1982; Jamieson et al, 1983). Almost all acute phase reactants are glycoproteins containing N-linked complex type carbohydrate chains composed of GlcNAc, Man, Gal and NeuAc. Elevated biosynthesis of acute phase reactants during inflammation must therefore be accompanied by increases in the biosynthesis of the carbohydrate moieties of these glycoproteins. As previously stated in the Introduction, there are two pathways in which sugars are incorporated into the oligosaccharide chains of N-linked glycoproteins; one involves direct transfer of sugars to N-linked oligosaccharide chains, while the other involves transfer of sugars to lipid-linked intermediates. In both cases, the individual sugars utilized for the synthesis of the oligosaccharide

chains must be in the form of nucleotide sugars. Therefore, studies on the alterations in metabolism of nucleotide sugars in liver during the acute phase response are important because they can provide us with an insight into aspects of control of acute phase glycoprotein biosynthesis.

As previously stated in the Introduction, the starting point for the synthesis of sugars required for glycoprotein biosynthesis is glucose, which is converted by established pathways to the various nucleotide sugars. The nucleotide sugar UDP-GlcNAc occupies a key position in this scheme since it is the donor of the GlcNAc residues found in the inner and terminal regions of complex type carbohydrate chains found in glycoproteins. However, UDP-GlcNAc is also the starting point for the synthesis of CMP-NeuAc, the donor of NeuAc residues found in terminal positions of complex type carbohydrate chains. The regulatory enzyme for the synthesis of UDP-GlcNAc is glucosamine-6-phosphate synthase, which converts fructose-6-phosphate to glucosamine-6-phosphate with glutamine acting as amide donor; this enzyme is subject to feedback inhibition by UDP-GlcNAc (Kornfeld et al., 1964). For the synthesis of CMP-NeuAc, the two key enzymes are UDP-GlcNAc 2-epimerase (which forms UDP-GlcNAc to N-acetylmannosamine, the first reaction leading to CMP-NeuAc formation) and CMP-NeuAc synthase (which forms the nucleotide sugar from CTP and NeuAc). UDP-GlcNAc 2-epimerase is the regulatory enzyme in this pathway and it is subject to feedback inhibition by CMP-NeuAc (Kornfeld et al., 1964).

Clearly, during the acute phase response to inflammation, where there is enhanced demand for GlcNAc and NeuAc for glycoprotein biosynthesis, changes might be expected in UDP-N-acetylhexosamine and

CMP-NeuAc pools and the activities of the enzymes leading to their formation. The control value of 141 nmoles/g liver of UDP-N-acetylhexosamines, composed of 95 nmoles UDP-GlcNAc and 46 nmoles UDP-GalNAc, compares favorably with some literature values, but is slightly lower than others. For example, Okubo and Chandler (1976) reported values ranging from 127-534 nmoles/g liver from fed rats; with starved rats, Bley et al. (1973) reported 207 nmoles/g liver and Bates et al. (1966) reported 270 nmoles/g liver. The variability in UDP-N-acetylhexosamine levels could reflect the use of different strains of rats, or result from variations in the nutritional status of the animals; UDP-GlcNAc pools have been shown to be affected by diet (Tepperman et al., 1981). Because of the variations in UDP-N-acetylhexosamine pools reported in the literature, care was taken in the present studies to use animals of the same sex and weight; animals were also maintained under constant light conditions and were always sacrificed at the same time of day. Increases in UDP-N-acetylhexosamine pools following trauma have been reported by others, but the earliest time examined was at 18 h (Bley et al., 1973; Okubo and Chandler, 1976). At times after trauma of 18 h or longer, it was observed that UDP-N-acetylhexosamine pools increased by 20-30%, which compares favorably with the 50% increase found in the present work at longer times after inflammation (Fig. 26). The earlier workers, however, would not have detected the sudden rise in UDP-N-acetylhexosamine pools to about twice control values that occurred at 8 h after inflammation, or the subsequent dip in pool levels found at 12 h after inflammation (Fig. 26). These results show that the response of UDP-N-acetylhexosamine pools to inflammation is much more rapid than was

previously believed.

As indicated above, glucosamine-6-phosphate synthase is the rate limiting enzyme in the pathway leading to the formation of UDP-GlcNAc. Therefore, this enzyme activity might be expected to increase in parallel with UDP-N-acetylhexosamine pools during inflammation. As mentioned previously in the Introduction, glucosamine-6-phosphate synthase activity is regulated in a complicated way. Although it is subject to feedback inhibition by UDP-GlcNAc, the degree of inhibition is dependent on secondary factors, such as AMP, UTP and glucose-6-phosphate, which alter the binding constant of UDP-GlcNAc to the enzyme (Kornfeld et al., 1964; Bates et al., 1966; Kornfeld, 1967; Miyagi and Tsiuki, 1971). Also, it appears that the enzyme is normally about 90% inhibited in vivo (Hardingham and Phelps, 1968; Winterburn and Phelps, 1971) which leaves substantial latitude for regulatory control. The specific activity of the enzyme in control livers was 35 units/ mg/ protein/h which is slightly lower than the 40 units reported by Bley et al. (1973), and the 35.5-46 units reported by Okubo and Chandler (1976). In the present studies, glucose-6-phosphate was added to the assay system to prevent the conversion of fructose-6-phosphate to glucose-6-phosphate by phosphoglucose isomerase which is present in high levels in liver (Miyagi and Tsuiki, 1971). However, as mentioned previously, glucose-6-phosphate can enhance the feedback effect of UDP-GlcNAc on the enzyme, and this probably explains the slightly lower activity of glucosamine-6-phosphate synthase found in the present work compared with the data presented by Bley et al. (1973) and Okubo and Chandler (1976). Inflammation resulted in a small increase in enzyme activity at 8 h followed by a decline to control values at

12 h; after which there was a rapid rise to about twice the control activities at 24 h after inflammation (Fig. 35). Although there was a decline at 48 h after inflammation, the enzyme activity was still substantially above controls. These results are consistent with the findings of Bley _et al_. (1973) and Okubo and Chandler (1976) who reported large increases in glucosamine-6-phosphate synthase activities following laparotomy. The times studied by these workers were 18 h to 8 days after injury when it was found that the increases in glucosamine-6-phosphate synthase could be blocked by actinomycin D or cycloheximide suggesting that the elevated activities of the enzyme found 18 h after the sham operation were mainly due to _de novo_ synthesis of the enzyme, rather than by regulatory control. However, it is difficult to reconcile the behavior of the enzyme at early times after inflammation, that is, up to 12 h after turpentine injection, with the two-fold increase in the pool size of UDP-GlcNAc found at 8 h after inflammation (Fig. 26). Although it is not clear why UDP-N-acetylhexosamine pools increase rapidly in liver, increased glucosamine-6-phosphate synthase activity does not appear to be responsible for the early response. It must therefore be assumed that control over UDP-GlcNAc formation at early times after onset of inflammation is exerted at a step beyond the formation of glucosamine-6-phosphate, possibly by increased utilization of the pools of the intermediates glucosamine-6-phosphate, N-acetylglucosamine-6-phosphate and N-acetylglucosamine-1-phosphate (see Fig. 11). There may also be an increase in the channelling of hepatic free GlcNAc and glucosamine, derived mainly from catabolism of hexosamine-containing glycoconjugates (Allen and Walker, 1980; Voisey and Winterburn, 1982), into N-acetylglucosamine-6-phosphate

(Schachter and Roden, 1973) and eventually into UDP-GlcNAc. Thus, it would be of interest to measure the pool sizes of the hexosamines leading to UDP-GlcNAc formation together with the enzymes responsible for their interconversions at early times after inflammation.

At later times after inflammation the elevated UDP-N-acetylhexosamine pool is best explained by increased glucosamine-6-phosphate synthase activities caused mainly by the presence of increased enzyme protein as suggested by Bley et al. (1973). The above explanations for the behavior of the UDP-N-acetylhexosamine pool are complicated by fact that increased glucosamine-6-phosphate synthase activities and changes in UDP-N-acetylhexosamine pools cannot be considered in isolation. As mentioned previously, other factors can influence glucosamine-6-phosphate synthase activities, and UDP-GlcNAc also acts as the substrate for UDP-GlcNAc 2-epimerase, the first enzyme in the pathway leading to CMP-NeuAc formation. This reaction is not detectably reversible (Sommar and Ellis, 1972) and it provides a means to reduce the UDP-N-acetylhexosamine pool and channel the UDP-GlcNAc into the biosynthetic pathway leading to CMP-NeuAc formation. The control activity of UDP-GlcNAc 2-epimerase was 58 units/mg protein which is comparable to the value of 44.7 units/mg protein calculated from the data reported by Kikuchi et al. (1971). (These workers reported 134 units of activity defining a unit as 1 nmole product formed/h; in the present work, a unit is 1 nmole product formed/20 min.) Inflammation resulted in a rapid reduction in the activity of UDP-GlcNAc 2-epimerase to about 60% of the control value at 4 h after inflammation. Although the explanation for this response is not clear, one outcome would be a decreased utilization of UDP-GlcNAc which could partially explain the rapid rise in the UDP-GlcNAc pool after

inflammation (Fig. 26). However, at 8 h after inflammation, the epimerase activity increased dramatically to twice control values. This change could be explained by the behavior of CMP-NeuAc, the feedback inhibitor for this enzyme. It is noteworthy that the CMP-NeuAc levels in liver in the present work are in the range of values determined by Kornfeld _et al_. (1964), in a study on the allosteric inhibition of UDP-GlcNAc 2-epimerase, that can affect the epimerase activity. The control level of CMP-NeuAc was 49 nmoles/g liver, which is slightly higher than the 40.8 nmoles/g reported by Harms _et al_. (1973). However, the pool of CMP-NeuAc was reduced by about 30% at 8-12 h after inflammation, the times at which the UDP-GlcNAc 2-epimerase activities were the highest (Figs. 29 and 36). Thus, increased epimerase activities could have resulted from lower levels of CMP-NeuAc, the feedback inhibitor for the enzyme. The CMP-NeuAc pool in turn was replenished at 16-48 h following inflammation as a result of the elevated epimerase activity at 8-12 h after inflammation, which in turn channels more UDP-GlcNAc into the CMP-NeuAc pathway. Consequently, this could explain the dip in the UDP-GlcNAc pool observed at 12 h following inflammation (Fig. 26). The activity of CMP-NeuAc synthase, which is not a regulatory enzyme, did show some oscillation in activity following inflammation (Fig. 37), but the changes in activity were minor compared to those found for the two regulatory enzymes studied. The reduced CMP-NeuAc pools found at 8-12 h after inflammation are probably best explained by an increased demand for the synthesis of liver synthesized glycoproteins like the $\alpha_1$-glycoprotein studied in our laboratory (Jamieson _et al_., 1975). Increased synthesis of $\alpha_1$-acid glycoprotein is well underway

at 8-12 h following inflammation, so that the synthesis of this and other acute phase glycoproteins would tend to deplete the hepatic pool of CMP-NeuAc.

The other sugars that are essential for glycoprotein biosynthesis are galactose and mannose which are derived from UDP-Gal and GDP-Man, respectively. The control level of UDP-Gal was 31 nmoles/g liver which is lower than the value of 92 nmoles reported by Bauer et al. (1976) using an enzyme assay system, rather than analysis by gas chromatography, to measure the nucleotide sugar. Although the reason for this discrepancy is not clear, Bauer et al. (1976) did report that UDP-Gal pools fell by 20-30% at short times after trauma induced by laparotomy or partial hepatectomy, but this was followed by increases at 24-48 h after the operation. The behavior of UDP-Gal pools in response to trauma by Bauer et al. (1976) is thus consistent with the data presented in the present work. It is of interest to note that the responses of UDP-Gal and CMP-NeuAc pools to inflammation were very similar (Fig. 29). Residues of NeuAc and Gal are known to occupy terminal and penultimate positions, respectively, on oligosaccharide chains of liver synthesized N-linked glycoproteins, and it is well known that these two sugars are transferred directly from their nucleotide sugars by the appropriate glycosyl transferases to growing oligosaccharide chains (Schachter, 1978; Schachter and Roseman, 1980; see also Introduction). Thus, although CMP-NeuAc synthesis is under regulatory control by UDP-GlcNAc 2-epimerase (Kornfeld et al., 1964; see Introduction), the pools of CMP-NeuAc and UDP-Gal might be expected to change in a similar way in response to inflammation; moreover, the changes are likely to be related to changes in glycoprotein biosynthesis found after inflammation.

Therefore, it is interesting to speculate that the decline in CMP-NeuAc and UDP-Gal pools at 4-16 h after inflammation might reflect increased demands for NeuAc and Gal for liver glycoprotein biosynthesis which is substantially increased at 8-24 h after inflammation (Jamieson et al., 1975); indeed the hepatic activities of both sialyl and galactosyl transferases are considerably elevated by 12 h following inflammation (this Thesis).

The control level of GDP-Man was 4.8 nmoles/g liver which is considerably lower than the pool sizes of the other nucleotide sugars studied in the present work. Although the literature does not appear to contain information on hepatic GDP-Man levels, low levels of this nucleotide sugar have been found in other tissues. For example, Mendicino and Rao (1975) reported a value of 12 nmoles/g for lymph node, a tissue which happens to contain a UDP-GlcNAc pool of 95 nmoles/g which is identical to that found in liver in the present work. The rapid rise of GDP-Man following inflammation followed by a decline at 12 h, a second peak at 24 h, and a gradual decline to control values at 48 h after inflammation, is similar to the response of the UDP-N-acetylhexosamine pools to inflammation (Figs. 26 and 28). The incorporation of GlcNAc and Man into oligosaccharide chains is a complex process involving the initial synthesis of an oligosaccharide-lipid intermediate containing two GlcNAc and nine Man residues; the first five Man residues come directly from GDP-Man and the other four come from Man-P-Dol which is formed from GDP-Man and Dol-P (Hubbard and Ivatt, 1981; Jamieson, 1983; see Introduction). After the completed high-mannose containing complex is transferred to protein, and after removal of the excess Man residues, additional GlcNAc, as well as

Gal and NeuAc, are added directly from their nucleotide sugars to produce a typical complex oligosaccharide chain (Hubbard and Ivatt, 1981; Jamieson, 1983; see Introduction). Thus, because of the complex manner in which Man and GlcNAc are incorporated into glycoproteins, it is difficult to relate changes in GDP-Man and UDP-GlcNAc pools in inflammation directly to changes in glycoprotein biosynthesis. A further complication in this relationship is that, as previously stated, the synthesis of UDP-GlcNAc is under regulatory control by glucosamine-6-phosphate synthase. However, since transfer of Man and GlcNAc to glycoproteins involves formation of oligosaccharide-lipid complexes, it is not surprising that pools of GDP-Man and UDP-GlcNAc show similar changes following inflammation.

Although glucose is not a constituent of liver synthesized serum glycoproteins, it is an essential component in the synthesis of the high-mannose containing dolichol-linked precursor oligosaccharide (Hubbard and Ivatt, 1981; Jamieson, 1983). The sequential addition of three Glc residues to the lipid-linked high-mannose oligosaccharide appears to be required for the transfer of the oligosaccharide from the lipid carrier to protein acceptor (Hubbard and Ivatt, 1981; Jamieson, 1983). The Glc residues come from Glc-P-Dol which is formed from UDP-Glc and Dol-P, and they are removed shortly after the oligosaccharide is transferred to the protein (Hubbard and Ivatt, 1981; Jamieson, 1983). The control level of UDP-Glc was 127 nmoles/g liver which is identical to that reported by Nordlie _et al_. (1980) but lower than the 331 nmoles reported by Bauer _et al_. (1976). However, Bauer _et al_. (1976) did report that UDP-Glc levels fell at short times after trauma induced by laparotomy or partial hepatectomy, and then

by a decline to control values at longer times. The behaviour of UDP-Glc pools was similar in the present studies. Indeed, although it is involved in the synthesis of lipid-linked oligosaccharides, the response of the UDP-Glc pool to inflammation more closely resembled that of UDP-Gal rather than GDP-Man or UDP-GlcNAc. Since UDP-Glc is also an intermediate in the pathway leading to the synthesis of UDP-Gal from glucose (Fig. 11), it might be expected that UDP-Glc and UDP-Gal pools would change in a similar way in response to inflammation. As suggested previously, changes in the UDP-Gal pool are likely related to altered glycoprotein biosynthesis found after inflammation. This may also be true for changes in the UDP-Glc pool which may reflect an increase in the synthesis and utilization of UDP-Gal for elevated glycoprotein bioynthesis following inflammation. However, the role of glucose in oligosaccharide-lipid synthesis may also be contributing to the observed changes in the UDP-Glc pool after inflammation. Thus, as with UDP-GlcNAc and GDP-Man, it is difficult to relate changes in UDP-Glc pools in inflammation directly to changes in glycoprotein biosynthesis.

As mentioned previously, glucose serves as the precursor for the synthesis of all the nucleotide sugars. Glucose metabolism is tightly regulated and is under many different controls (Hers, 1976). Three major alterations in hepatic glucose metabolism occur in inflammation which can alter liver glucose levels. These are: (1) increased synthesis of glucose from a number of sources, such as gluconeogenesis, glycogenolysis and lipolysis (Shutler et al., 1977; Beisel, 1980; Kushner, 1982); (2) increased release of glucose from liver to fuel energy demands elsewhere in the body (Beisel, 1975,

1980; Langstaff et al., 1980); and (3) increased utilization of glucose, as for the synthesis of the carbohydrate moieties of glycoproteins. Work by Shutler et al. (1977) showed that the hepatic level of glycogen decreased almost immediately after induction of inflammation. Results from the present studies indicate that changes in hepatic nucleotide sugar pools also occur at very short times after induction of inflammation. Thus, changes in hepatic glucose levels, resulting from altered glucose metabolism during inflammation, may be responsible for some of the observed changes in nucleotide sugar pools in inflammation.

As mentioned previously, alternate sources of sugars for nucleotide sugar biosynthesis are the free sugars, such as Man, Gal and GlcNAc, which are derived from glycoconjugate catabolism in lysosomes. Sialic acid free serum glycoproteins have been shown to bind to liver and subsequently appear associated with liver lysosomes (Pricer and Ashwell, 1976). It is believed that this mechanism represents the main pathway for catabolism of serum glycoproteins. Lysosomes are rich in glycosidases which are involved in the catabolism of the carbohydrate chains of serum glycoproteins. Work by Kaplan and Jamieson (1977) showed that experimentally induced inflammation resulted in substantial reductions in the activities of the lysosomal glycosidases, β-galactosidase and β-N-acetylglucosaminidase. These results implied that there was a diminished catabolism of serum glycoproteins following inflammation. A diminished glycoprotein catabolism could result in a reduced supply of free sugars, such as Man, Gal and GlcNAc, thus further suggesting that glucose is the major source of sugar for nucleotide sugar biosynthesis following inflammation, and that altered hepatic glucose metabolism may play an important role in nucleotide sugar biosynthesis.

Nucleotides may also play a role in the changes in nucleotide sugar pools observed following inflammation. Following inflammation there is an increase in hepatic RNA synthesis which is related to the elevated synthesis of acute phase proteins (Thompson and Wannemacher, 1973; Princen et al, 1981; Ricca et al., 1982; Kushner, 1982). Elevated RNA synthesis coupled with enhanced nucleotide sugar synthesis following inflammation would result in an increased utilization of nucleotides, such as UTP, GTP, CTP and ATP, which could alter their intracellular levels and metabolism. In nucleotide sugar synthesis, feedback inhibition of glucosamine-6-phosphate synthase by UDP-GlcNAc is decreased by UTP which competes for the UDP-GlcNAc binding site of the enzyme (Phelps et al., 1970). Elevated levels of UDP-GlcNAc were found in livers of rats fed on a diet rich in orotate, a uridine precursor (Bates et al., 1966). The increased hepatic pool of UDP-GlcNAc was suggested to be due to elevated levels of UTP available for nucleotide sugar synthesis, as well as for the circumvention of the feedback inhibition of glucosamine-6-phosphate synthase by UDP-GlcNAc. Also, work by Gorski and Mueller (1963) suggested that there may be a relationship between the increased hepatic pool sizes of UTP and UDP-GlcNAc found in estrogen-treated rats. Thus, altered nucleotide metabolism following inflammation might be expected to play a role in the regulation of nucleotide sugar biosynthesis.

It is evident from the above discussion that, in addition to enhanced glycoprotein biosynthesis, alterations in hepatic glucose and nucleotide levels following inflammation could possibly affect the synthesis of nucleotide sugars. Therefore, studies on hepatic carbohydrate and nucleotide metabolism following inflammation would be

important to gain a greater insight into nucleotide sugar metabolism during the acute phase response to inflammation.

This study has examined the behavior of nucleotide sugar pools during inflammation, concentrating on those nucleotide sugars involved in the synthesis of the oligosaccharide chains of acute phase glycoproteins. In addition, the effect of inflammation on some enzymes of nucleotide sugar metabolism was studied. While large changes were observed in the activities of the regulatory enzymes, glucosamine-6-phosphate synthase and UDP-GlcNAc 2-epimerase, no substantial change was observed in the activity of the nonregulatory enzyme, CMP-NeuAc synthase. Although it is difficult to interpret information from *in vitro* assays of the enzymes in crude preparations, especially when they are under feedback control, it is of interest to note that both regulatory enzymes showed elevated activities at 8-24 h after inflammation when liver glycoprotein biosynthesis is increasing rapidly (Jamieson *et al*., 1975). In addition, it is clear that the response of the nucleotide sugar pools to inflammation is very rapid with substantial increases in UDP-N-acetylhexosamine and GDP-Man pools being evident as early as 4 h after inflammation. Glycoprotein biosynthesis is just starting to show some stimulation at 4 h after inflammation (Jamieson *et al*., 1975) so changes in hepatic pools of UDP-N-acetylhexosamines and GDP-Man appear to precede elevated protein synthesis. Thus, the results presented in this study, while adding to our knowledge on changes that accompany the acute phase response to inflammation, also emphasize the importance of recognizing early biochemical changes in liver and suggest that short time course studies may be crucial in achieving a full understanding of the acute phase response of glycoproteins to inflammation.

STUDIES ON THE EFFECT OF INFLAMMATION ON THE FORMATION OF SUGAR-LIPID INTERMEDIATES AND THE ACTIVITIES OF OLIGOSACCHARIDE PROCESSING ENZYMES IN RAT LIVER

The results from the present studies indicate that inflammation results in increased glycosylation of lipid intermediates of the dolichol cycle and proteins in liver rough membrane fractions. Previous studies in our laboratory (Jamieson *et al.*, 1983) have determined that there is enhanced liver glycoprotein biosynthesis following inflammation. Thus, it is not surprising that there are changes in oligosaccharide chain synthesis at a time of elevated glycoprotein biosynthesis.

In a study similar to one presented in this thesis, Coolbear and Mookerjea (1981) used an *in vitro* system to study the effect of inflammation on the incorporation of radioactive sugars, from nucleotide sugars, into dolichol phosphate derivatives and proteins. The results from their study indicated that inflammation caused increased glycosylation of sugar-lipid intermediats as was found in the *in vitro* studies in the present work. However, Coolbear and Mookerjea (1981) stated that the incorporation of the labeled sugars into endogenous proteins in their assay system was very low and, these values were not reported. Coolbear and Mookerjea (1981) determined that the increased glycosylation of the sugar-lipid intermediates was due to elevated levels of Dol-P as a result of the increased availability of dolichol and the increased activity of CTP-dependent dolichol phosphokinase following inflammation; as previously mentioned in the Introduction, CTP-dependent dolichol phosphokinase is responsible for catalyzing the phosphorylation of dolichol.

Increased levels of Dol-P have also been linked to increased glycoprotein biosynthesis in studies of other systems, such as after estrogen-induced differentiation of chick oviduct (Lucas and Levin, 1977; Burton *et al*., 1981; Singh and Lucas 1981; DeRosa and Lucas, 1982) and just prior to the gastrulation stage of embryonic development in sea urchins (Carson and Lennarz, 1979,1981; Heifetz and Lennarz, 1979; Rossignol *et al*., 1981). In the latter studies by Lennarz's group, the increase in Dol-P levels was found to be due to elevated CTP-dependent dolichol phosphokinase activity, as was the case in the study by Coolbear and Mookerjea (1981) described above. Thus, increased synthesis of Dol-P is apparently required during times of enhanced N-linked glycoprotein biosynthesis.

Results from *in vivo* incorporation studies in the present work indicated that over the time course examined nearly twice as much [$^{14}$C]Man-label and [$^{3}$H]GlcN-label was incorporated into proteins from hepatic rough membrane fractions prepared from 12 h inflamed rats compared to controls (Fig. 40). Throughout the same time period, little difference was observed between control and 12 h experimental samples in the formation of [$^{14}$C]Man-P-Dol, and [$^{14}$C]Man-labeled and [$^{3}$H]GlcN-labeled oligosaccharide-P-P-Dol (Figs. 38-39); formation of [$^{3}$H]GlcN-labeled $(GlcNAc)_{1-2}$-P-P-Dol in the 12 h experimental samples was twice that of controls (Fig. 38). The increased incorporation of radioactive sugars into proteins from 12 h experimental rough membrane fractions was not surprising since a previous study in our laboratory (Jamieson *et al*., 1975) had shown that the mean rate of synthesis of rat $\alpha_1$-acid glycoprotein, a major acute phase glycoprotein, was twice that of controls at 12 h after inflammation. As

previously mentioned in the Introduction, glucosamine must first enter the hexosamine pathway to form UDP-GlcNAc before eventually being incorporated into glycoproteins. Since there is a substantial increase in the hepatic pool size of UDP-GlcNAc following inflammation (Fig. 26), the increase in incorporation of [$^{3}$H]GlcN-label, and presumably [$^{14}$C]Man-label, into proteins from 12 h experimental rough membrane fractions is most likely due to elevated protein glycosylation and not to an increase in specific radioactivity as a result of altered intracellular sugar pools. Coolbear and Mookerjea (1981) showed that there was no significant elevation in the formation of Dol-P from endogenous dolichol _via_ CTP-dependent dolichol phosphokinase activity until 24 h after onset of inflammation. Thus, there appears to be sufficient Dol-P present at 12 h after inflammation to meet the demands for elevated glycoprotein biosynthesis at that time. Thus, the increased formation of $(GlcNAc)_{1-2}$-P-P-Dol in 12 h experimental livers may have arisen from a salvage pathway involving the recycling of increased amounts of Dol-P-P resulting from an increased rate of transfer of lipid-derived oligosaccharides to proteins. Although this salvage pathway has yet to be demonstrated, evidence in support of its existence comes from the results shown in Figs. 39-40. Figure 39 shows that there was a more rapid decline in the radioactive pulse in oligosaccharide-P-P-Dol at 15-60 min after injection of radioactive sugars in 12 h experimental livers compared to controls. Figure 40 shows that there was increased incorporation of labeled sugars into proteins from 12 h experimental livers. Taken together, these results suggest a more rapid turnover of the lipid-linked oligosaccharides in the 12 h experimental livers. The elevated

amounts of Dol-P-P generated by increased rates of protein glycosylation may then reenter the dolichol cycle, after removal of the terminal phosphate group, to begin the synthesis of more GlcNAc-P-P-Dol and, hence, more oligosaccharide-P-P-Dol. It is interesting to note that while the synthesis of $(GlcNAc)_{1-2}$-P-P-Dol is greater in 12 h experimental livers than in controls, the formation of Man-P-Dol was similar. If the increase in $(GlcNAc)_{1-2}$-P-P-Dol synthesis is a result of the existence of a salvage pathway like the one described above, this would imply that the recycled Dol-P is not used for Man-P-Dol synthesis. This is not surprising since there is some evidence that in rat liver different pools of Dol-P are utilized in the synthesis of Man-P-Dol and GlcNAc-P-P-Dol (Godelaine *et al.*, 1979). Another explanation for the elevated $(GlcNAc)_{1-2}$-P-P-Dol levels is that there may be increased transfer of GlcNAc-P from UDP-GlcNAc to Dol-P (see Introduction) in the 12 h experimental livers. Clearly, more studies would be required to help explain the differences observed in the glycosylation of dolichol phosphate derivatives and proteins following inflammation.

The results from *in vitro* experiments in absence of a cell-free protein synthesizing system in this thesis indicate that the incorporation of [$^{14}$C]Man from GDP-[$^{14}$C]Man into dolichol phosphate derivatives and proteins was greater in 24 h experimental rough membrane fractions than in controls. The increased glycosylation of the lipid intermediates can be explained by the increased level of Dol-P in the 24 h inflamed samples as determined by Coolbear and Mookerjea (1981) in the study outlined above. A low concentration of Triton X-100, a nonionic detergent, was found to stimulate the

mannosylation of lipid-linked intermediates and proteins from both control and 24 h experimental hepatic rough membrane fractions. Similar observations on the stimulation of glycosylation of dolichol phosphate derivatives and proteins in rough endoplasmic reticulum from rat liver in the presence of low levels of Triton X-100 have been made by others (Nilsson *et al*., 1978; Coolbear *et al*., 1979). Previous studies (Czichi and Lennarz, 1977; Chen and Lennarz, 1978; Das and Heath, 1980) have shown that the enzymes involved in the lipid-linked pathway of glycoprotein biosynthesis are associated with the rough endoplasmic reticulum membrane. Also, studies on the topology of sugar-lipid biosynthesis in microsome fraction vesicles indicated a luminal location for the lipid-linked substrate, while GDP-Man and UDP-GlcNAc, located in the cytoplasm (see Introduction), were unable to penetrate the vesicle membranes (Hanover and Lennarz, 1982). The results from these studies further implied that the synthesis of the lipid-linked sugar intermediates occurred *via* a transmembrane enzyme complex (see also Introduction). Thus, the stimulation of glycosylation of the sugar-lipid intermediates and proteins by detergent could be explained by an increase in the accessibility of the enzymes for their substrates once the enzymes are released from the rough membrane fraction membranes.

The results from *in vitro* studies in the presence of a cell-free protein synthesizing system in this thesis indicated that cell sap preparations from experimental livers have the ability to stimulate glycosylation of oligosaccharide-P-P-Dol and proteins, as well as stimulate protein synthesis, in hepatic rough membrane fractions from control and 12 h experimental rats (Tables 13-14).

It was further determined that a high molecular weight-enriched fraction of 12 h experimental cell sap, prepared by ultrafiltration (see Experimental), still retained the ability to stimulate the glycosylation of oligosaccharide-P-P-Dol and proteins from hepatic rough membrane fractions, but had lost the ability to stimulate protein synthesis (Table 15). The factors in experimental cell sap preparations capable of stimulating the glycosylation of lipid-linked oligosaccharides and proteins from control and 12 h experimental rough membrane fractions are not identified. Dol-P has been found in liver cell sap preparations (Adair and Keller, 1982). However, the high molecular weight-enriched fraction of experimental cell sap should contain little or no Dol-P since this molecule is of a relatively low molecular weight, well below the lower molecular weight retention limit of the ultrafiltration membrane used in the preparation of this fraction of experimental cell sap (see Experimental). It is interesting to note that the high molecular weight fraction of 12 h experimental cell sap stimulated protein glycosylation without stimulating protein synthesis. Carson _et al_. (1981) observed stimulation of glycosylation of N-linked glycoproteins in hen oviduct and bovine pancreas tissue slice cultures supplemented with Dol-P, while observing no stimulation in protein synthesis. These workers suggested that Dol-P availability was a limiting factor in the _in vivo_ glycosylation of proteins in the two systems. Thus, in the present work, if elevated levels of Dol-P in experimental cell sap preparations are responsible for the increase in glycosylation of oligosaccharide-P-P-Dol and proteins in control rough membrane fractions, then the stimulation of glycosylation due to the high

molecular weight fraction of 12 h experimental cell sap could be explained by the retention of Dol-P during the ultrafiltration procedure by specific or nonspecific association of Dol-P with the high molecular weight components in the cell sap. The stimulation of protein synthesis by experimental cell sap preparations plus the lack of stimulation of protein synthesis by the high molecular weight-enriched fraction of 12 h experimental cell sap are also not understood. The inability of the high molecular weight preparation of experimental cell sap to stimulate protein synthesis may possibly be explained by the loss of an essential stimulatory low molecular weight component(s) or the loss of activity of a high molecular weight stimulatory factor(s) during the ultrafiltration procedure. While the identity of the possible high molecular weight stimulatory factor(s) is not known at this time, possible candidates for the low molecular weight stimulatory factor(s) are the polyamines: putrescine, spermidine and spermine. Polyamines have been shown to be able to stimulate overall *in vitro* protein synthesis (Takeda, 1969; Igarashi *et al*., 1971; Lotfield *et al*., 1981). A key step in the pathway of polyamine biosynthesis is the formation of putrescine from ornithine by the enzyme ornithine decarboxylase (Kuehn and Atmar, 1982; Pegg *et al*., 1982; Tabor *et al*., 1982). During the acute phase response, there are elevated levels of ornithine decarboxylase and polyamines in liver (Raina and Janne, 1970). Thus, increased levels of polyamines in experimental cell sap preparations could be responsible for the accelerated *in vitro* protein synthesis observed in the present study. Clearly, more work would be required to elucidate the factors in experimental cell sap preparations

responsible for stimulating glycosylation and protein synthesis in hepatic rough membrane fractions.

Gel filtration elution profiles of lipid-derived oligosaccharides isolated from control and 12 h experimental liver slices incubated with radioactive sugars were similar (Fig. 49). The largest labeled oligosaccharide species that could be identified, based on available information on elution constants of known high-mannose oligosaccharides (see Appendix I), was $Man_4GlcNAc_2$ (Table 16). The reason why larger labeled oligosaccharides were not isolated is unclear. One explanation is that the apparent accumulation of small oligosaccharide-lipid complexes might represent nonuniform labeling.

Inhibition of protein synthesis in canine kidney cells by puromycin and cycloheximide has been shown to result in the inhibition of synthesis of lipid-linked oligosaccharides (Schmitt and Elbein, 1979). The addition of puromycin to slice incubations in the present work appeared to have little effect on the gel filtration elution profiles of lipid-derived oligosaccharides from control and 12 h experimental liver slices (Figs. 51-52). Indeed, the elution profiles were similar to those obtained in absence of puromycin, with the largest labeled oligosaccharide identified being $Man_4GlcNAc_2$ (Tables 17-18). Unfortunately, the slice incubations in absence and presence of puromycin were performed using radioactive sugars with very different initial specific radioactivities (see Experimental). Thus, a quantitative comparison of the amount of the sugars incorporated into oligosaccharide-lipid complexes in the presence and absence of puromycin was not possible. It is clear from the results that the conditions used for labeling of oligosaccharide-lipid complexes

in the slice assays did not permit a good comparison of oligosaccharide-lipid assembly between control and experimental livers, as the larger oligosaccharide-lipid complexes contained very little label. Obviously, more work would be required to optimize the conditions to obtain a more uniform labeling of the oligosaccharide-lipid complexes, thereby providing a better basis for comparison of their assembly between control and experimental livers.

Except for a moderate increase in α-mannosidase activity in rough membrane fractions, the activities of the oligosaccharide processing α-mannosidases and α-glucosidases in rough and smooth membrane fractions and Golgi complex remained fairly constant or decreased following inflammation (Tables 19-20). One explanation for the decreased specific activities of the processing glycosidases in the Golgi complex is that the Golgi membrane is proliferative, as is known to occur in inflammation (Earp, 1975; Turchen et al., 1977; Lombart et al., 1980). It is interesting, if not surprising, that substantial increases in the activities of the processing glycosidases were not observed following inflammation when there is elevated hepatic glycoprotein biosynthesis. The use of artificial substrates, such as p-nitrophenyl glycosides, to measure the processing glycosidase activities, means that the results must be viewed with caution. The different processing α-mannosidases have very different activities towards p-nitrophenyl α-mannoside (Tulsiani et al., 1977, 1981; Opheim and Touster, 1978; Tabas and Kornfeld, 1979; Bischoff and Kornfeld, 1983). As previously mentioned, Friesen and Jamieson (1980) isolated a form of $\alpha_1$-acid glycoprotein from hepatic rough endoplasmic reticulum which contained high mannose oligosaccharide

chains. This protein, as well as other partially processed forms which might be isolated from the smooth endoplasmic reticulum and Golgi complex, should be a more natural substrate and might provide a more valid measurement of the activities of the processing glycosidases during inflammation.

The decrease in the activities of the lysosomal α-glucosidase and α-mannosidase following inflammation was expected in view of the earlier study by Kaplan and Jamieson (1977) in which decreases were observed following inflammation in the activities of the lysosomal glycosidases, β-galactosidase and β-N-acetylhexosaminidase. In that study, Kaplan and Jamieson (1977) suggested that the decreased lysosomal glycosidase activities could be due to a diminished biosynthesis of intracellular glycoproteins, such as the lysosomal glycosidases, at a time when the liver is committed to the biosynthesis of secretable glycoproteins. However, in recent studies in our laboratory on the effect of inflammation on liver and serum glycosidase activities, Jamieson _et al_. (1983) suggest that during inflammation there is a defect in the formation of the mannose-6-phosphate signal, which is required to target the molecule from the Golgi complex to the lysosome (see Introduction). This would result in a loss of the enzymes from liver into serum, an observation that is currently under investigation.

It is evident from the results presented in this thesis that the acute phase response in liver is accompanied by a number of alterations in the biosynthetic pathway of the carbohydrate moieties of N-linked glycoproteins. There were changes in nucleotide sugar

biosynthesis, as well as changes in their pool sizes. These nucleotide sugars serve as precursors for the synthesis of sugar-lipid intermediates. While there were changes in the *in vivo* and *in vitro* glycosylation of oligosaccharide-lipid intermediates, only minor changes were observed in their assembly as determined in preliminary experiments in which gel elution profiles of lipid-derived labeled oligosaccharides from control and experimental rats were compared. In both *in vivo* and *in vitro* sugar incorporation studies, increases were observed in the rate of *en bloc* transfer of the precursor lipid-derived oligosaccharides to acceptor proteins in rough membrane fractions. The processing α-glucosidase and α-mannosidase activities either remained constant or decreased, with the exception of α-mannosidase activity in rough membrane fractions which was found to increase; the corresponding lysosomal enzyme activities decreased. Sialyl and galactosyl transferase activities of liver and serum increased following onset of inflammation. Although the origin of the serum glactosyl transferase activity was not identified, strong evidence has been presented to support the idea that there is a preferential release of Galβ1→4GlcNAcα2→6 sialyl transferase from liver into serum in inflammation.

Many different processes are involved in the biosynthesis of the carbohydrate moieties of acute phase glycoproteins. The information obtained from the present studies provides a greater understanding of some of these processes and of the nature of their interactions in glycoprotein biosynthesis during the acute phase response to inflammation.

Appendix I. Reported relative elution constant values of standard lipid-derived oligosaccharides.

| $K_d$ Value | (Oligo)saccharide |
|---|---|
| 0.280 | $Glc_3Man_9GlcNAc_2$ |
| 0.300 | $Glc_2Man_9GlcNAc_2$ |
| 0.332 | $Glc_1Man_9GlcNAc_2$ |
| 0.361 | $Man_9GlcNAc_2$ |
| 0.390 | $Man_8GlcNAc_2$ |
| 0.418 | $Man_7GlcNAc_2$ |
| 0.448 | $Man_6GlcNAc_2$ |
| 0.474 | $Man_5GlcNAc_2$ |
| 0.511 | $Man_4GlcNAc_2$ |
| 0.559 | $Man_3GlcNAc_2$ |
| 0.596 | $Man_2GlcNAc_2$ |
| 0.640 | $Man_1GlcNAc_2$ |
| 0.805 | $Man_1GlcNAc_1$ |
| 0.873 | GlcNAc |

Values shown and assignments are those of Hubbard and Robbins (1980). The relative elution constant ($K_d$) values for the homologous series of high-mannose oligosaccharides, chromatographed on Bio-Gel P4 (minus 400 mesh) filtration columns, were calculated as follows:

$$K_d = \frac{V_x - V_e}{V_i - V_e}$$

where $V_x$ is the elution volume (fraction number) of the oligosaccharide peak, $V_e$ is the exclusion volume, and $V_i$ is the inclusion volume (Etchison *et al.*, 1977).

Abraham, H.D., and Howell, R.R. (1969) J. Biol. Chem. 244, 545-550.

Adair, W.L., and Keller, R.K. (1982) J. Biol. Chem. 257, 8990-8996.

Adrian, G.S., and Keenan, R.W. (1979) Biochim. Biophys. Acta 575, 413-438.

Allen, C.M., Kalin, J.R., Sack, J., and Verizzo, D. (1978) Biochemistry 17, 5020-5026.

Allen, M.B., and Walker, D.G. (1980) Biochem. J. 185, 577-582.

Aronson, N.N. (1972) in Glycoproteins (Gottschalk, A., ed.), vol. 5, pp. 1211-1227, Elsevier Publishing Company, Amsterdam.

Ashton, F.E., Jamieson, J.C., and Friesen, A.D. (1970) Can. J. Biochem. 48, 841-850.

Atkins, E., and Bodel, P. (1979) Fed. Proc. 38, 57-63.

Balegno, H.F., and Neuhaus, O.W. (1970) Life Sci. 9, 1039-1044.

Baltz, M.L., deBeer, F.C., Feinstein, A., Munn, E.A., Milstein, C.P., Fletcher, T.C., March, J.F., Taylor, J., Bruton, C., Clamp, J.R., Davies, A.J.S., and Pepys, M.B. (1982) Ann. N.Y. Acad. Sci. 389, 49-75.

Banerjee, D., Manning, C.P., and Redman, C.M. (1976) J. Biol. Chem. 251, 3887-3892.

Barnabei, O., and Sereni, F. (1964) Biochim. Biophys. Acta 91, 239-247.

Bates, C.J., Adams, W.R., and Handschumacher, R.E. (1966) J. Biol. Chem. 241, 1705-1712.

Bauer, C., Kottgen, E., and Reutter, W. (1977) Biochem. Biophys. Res. Commun. 76, 488-494.

Bauer, C.H., Hassels, B.F., and Reutter, W.G. (1976) Biochem. J. 154, 141-147.

Baumann, H., and Doyle, D. (1982) in The Glygoconjugates (Horowitz, M.I. ed.), vol. 4, pp. 105-153, Academic Press, New York.

Baxter, A., and Durham, J.P. (1979) Anal. Biochem. 48, 95-101.

Behrens, N.H., and Leloir, L.H. (1970) Proc. Natl. Acad. Sci. U.S.A. 66, 153-159.

Beisel, W.R. (1975) Ann. Rev. Med. 26, 9-20.

Beisel, W.R. (1977) Am. J. Clin. Nutr. 30, 1236-1247.

Beisel, W.R. (1980) Fed. Proc. 39, 3105-3108.

Bella, A., and Kim, Y.S. (1971) Biochem. J. 125, 1157-1158.

Bennet, M., and Schmidt, K. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 6109-6113.

Benson, M.D., and Kleiner, E. (1980) J. Immunol. 124, 495-499.

Bergman, L.W., and Kuehl, N.M. (1977) Biochemistry 16, 4490-4497.

Bergman, L.W., and Kuehl, N.M. (1978) Biochemistry 17, 5174-5180.

Bernacki, R.J. (1975) Eur. J. Biochem. 58, 477-481.

Bernacki, R.J., and Kim, U. (1977) Science 195, 577-580.

Bernheim, H.A., Block, L.H., and Atkins, E. (1979) Ann. Int. Med. 91, 261-270.

Beyer, T.A., and Hill, R.L. (1982) in The Glycoconjugates (Horowitz, M.I., ed.), vol. 3, pp. 25-45, Academic Press, New York.

Beyer, T.A., Sadler, J.E., Rearick, J.I., Paulson, J.C., and Hill, R.L. (1981) Adv. Enzymol. 52, 23-175.

Bischoff, J., and Kornfeld, R. (1983) J. Biol. Chem. 258, 7907-7910.

Bley, R., Okubo, H., and Chandler, A.M. (1973) Proc. Soc. Exp. Biol. Med. 144, 134-140.

Blobel, G., and Dobberstein, G. (1975a) J. Cell Biol. 67, 835-851.

Blobel, G., and Dobberstein, G. (1975b) J. Cell Biol. 67, 852-862.

Bornstein, D.L. (1982) Ann. N.Y. Acad. Sci. 389, 323-337.

Brown, D.H., Brown, B.I., and Jeffrey, P.L. (1972) Methods Enzymol. 28, 805-813.

Burns, D.M., and Touster, O. (1982) J. Biol. Chem. 257, 9991-10000.

Butterworth, P.H.W., and Hemming, F.W. (1968) Arch. Biochem. Biophys. 128, 503-508.

Burton, W.A., Lucas, J.J., and Waechter, C.J. (1981) J. Biol. Chem. 256, 632-635.

Burton, W.A., Scher, M.G., and Waechter, C.J. (1979) J. Biol. Chem. 254, 7129-7136.

Cabezas, J.A. (1973) Rev. Exp. Fisiol. 29, 307-322.

Canonico, P.G., Ayala, E., Rill, W.L., Little, J.S. (1977) Am. J. Clin. Nutr. 30, 1359-1363.

Carey, D.J., Sommers, L.W., and Hirschberg, C.B. (1980) Cell 19, 597-605.

Caro, L.G., and Palade, G.E. (1964) J. Cell Biol. 20, 473-495.

Carson, D.D., Earles, B.J., and Lennarz, W.J. (1981) J. Biol. Chem. 256, 11552-11557.

Carson, D.D., and Lennarz, W.J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 5709-5713.

Carson, D.D., and Lennarz, W.J. (1981) J. Biol. Chem. 256, 4679-4686.

Chapman, A., and Kornfeld, R. (1979) J. Biol. Chem. 254, 816-823.

Chen, W.W., and Lennarz, W.J. (1978) J. Biol. Chem. 253, 5774-5779.

Chiu, K.M., Mortensen, R.F., Osmand, A.P., and Gewurz, H. (1977) Immunology 32, 997-1005.

Chojnacki, T., and Dallner, G. (1983) J. Biol. Chem. 257, 916-922.

Claude, A. (1970) J. Cell Biol. 47, 745-766.

Clemens, M.J., and Korner, A. (1970) Biochem. J. 119, 629-634.

Coates, S.W., Gurney, T., Sommers, L.W., Yeh, M., and Hirschberg, C.B. (1980) J. Biol. Chem. 255, 9225-9229.

Cohn, D.V., and MacGregor, R.R. (1981) Endocrine Rev. 2, 1-26.

Coolbear, T., Mookerjea, S., and Hemming, F.W. (1979) 184, 391-397.

Coolbear, T., and Mookerjea, S. (1981) J. Biol. Chem. 256, 4529-4535.

Czichi, U., and Lennarz, W.J. (1977) J. Biol. Chem. 252, 7901-7904.

Dallner, G. (1963) Acta Path. Microbiol. Scand. 166 (Suppl.), 1-94.

Dallner, G. (1974) Methods Enzymol. 31, 191-201.

Damsky, C.H., Levy-Benshimol, A., Buck, C.A. and Warren, L. (1979) Exptl. Cell Res. 119, 1-13.

Darcy, D.A. (1965) Brit. J. Exptl. Path. 46, 155-163.

Das, R.C., and Heath, E.C. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 3811-3815.

Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404-427.

DeRosa, P.A., and Lucas, J.J. (1982) J. Biol. Chem. 257, 1017-1024.

Dinarello, C.A., and Wolff, S.M. (1978) N. Engl. J. Med. 298, 607-612.

Doljanski, F. (1982) in The Glycoconjugates (Horowitz, M.I., ed.), vol. 4, pp. 155-187, Academic Press, New York.

Doolittle, R.F. (1975) in The Plasma Proteins (Putnam, F.W., ed.), vol. 2, pp. 110-162, Academic Press, New York.

Dorland, L., Haverkamp, J., Vliegenthart, J.F.G., Spik, G., Fournet, B., and Montreuil, J. (1979) Eur. J. Biochem. 100, 569-574.

Earp, H.S. (1975) Am. J. Physiol. 228, 1183-1187.

Eaton, J.W., Brandt, P., and Mahoney, J.R. (1982) Science 215, 691-692.

Eddington, C.L., Upchurch, H.F., and Kampschmidt, R.F. (1972) Proc. Soc. Exp. Biol. Med. 139, 565-569.

Edwards, K., Nagashima, M., Dryburgh, H., Wykes, A., and Schreiber, G. (1979) FEBS Lett. 100, 269-272.

Egdahl, R.H., Meguid, M.M., and Aun, F. (1977) Crit. Care Med. 5, 257-263.

Ehrlich, H.P., and Bornstein, P. (1972) Nat. New Biol. 238, 257-260.

Ehrlich, H.P., Ross, R., and Bornstein, P. (1974) J. Cell Biol. 62, 390-405.

Elbein, A.D. (1979) Ann. Rev. Plant Physiol. 30, 239-272.

Eppler, C.M., Morre, D.J., and Keenan, T.W. (1980a) Biochim. Biophys. Acta 619, 318-331.

Eppler, C.M., Morre, D.J., and Keenan, T.W. (1980b) Biochim. Biophys. Acta 619, 332-343.

Etchison, J.R., and Holland, J.J. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4011-4014.

Etchison, J.R., Robertson, J.S., and Summers, D.F. (1977) Virology 78, 375-392.

Etzel, K.R., and Cousins, R.J. (1981) Proc. Soc. Exp. Biol. Med. 167, 223-236.

Evans, W.H., Hood, D.O., and Gurd, J.W. (1973) Biochem. J. 135, 819-826.

Falchuk, K.H. (1977) N. Engl. J. Med. 296, 1129-1134.

Farquhar, M.G. (1978) J. Cell Biol. 77, R35-R42.

Feingold, D.S. (1972) in Biochemistry of the Glycosidic Linkage (Piras, R., and Pontis, H.G., eds.), pp. 79-112, Academic Press, New York.

Feldmann, G. (1982) Ann. N.Y. Acad. Sci. 389, 446-447.

Feldmann, G., and Maurice, M. (1975) Biol. Gastroenterol. 8, 269-274.

Feldmann, G., Maurice, M., Sapin, C., and Benhamou, J.P. (1975) J. Cell Biol. 67, 237-242.

Fiedel, B.A., Simpson, R.M., and Gewurz, H. (1982) Ann. N.Y. Acad. Sci. 389, 263-273.

Fraser, I.H., and Mookerjea, S. (1976) Biochem. J. 156, 347-355.

Friend, D.S. (1965) J. Cell Biol. 25, 563-576.

Friesen, A.D., and Jamieson, J.C. (1979) Abstracts, XI Int. Congr. Biochem., Toronto, p. 170.

Friesen, A.D., and Jamieson, J.C. (1980) Can. J. Biochem. 58, 1101-1111.

Fujita-Yamaguchi, Y., and Yoshida, A. (1981) J. Biol. Chem. 256, 2701-2706.

Fuller, G.M., and Ritchie, D.S. (1982) Ann. N.Y. Acad. Sci. 389, 308-322.

Ghosh, S., Blumenthal, H.J., Davidson, E., and Roseman, S. (1960) J. Biol. Chem. 235, 1265-1273.

Gibson, R., Schlesinger, S., and Kornfeld, S. (1979) J. Biol. Chem. 254, 3600-3607.

Ginsburg, V. (1964) Adv. Enzymol. 26, 35-88.

Glabe, C.G., Hanover, J.A., and Lennarz, W.J. (1980) J. Biol. Chem. 255, 9236-9242.

Glaumann, H. (1970) Biochim. Biophys. Acta 224, 206-218.

Gleeson, P.A., and Schachter, H. (1983) J. Biol. Chem. 258, 6162-6173.

Gleeson, P., Vella, G., Narasimhan, S., and Schachter, H. (1982) Fed. Proc. 41, 1147.

Glenn, E.M., Bowman, B.J., and Koslowski, T.C. (1968) Biochem. Pharmac., Supplement. pp. 27-38, Permagon Press, Great Britain.

Godelaine, D., Beaufay, H., and Wibo, M. (1979) Eur. J. Biochem. 96, 27-34.

Goldstein, I.M., Kaplan, H.B., Edelson, H.S., and Weissman, G. (1982) Ann. N.Y. Acad. Sci. 389, 368-379.

Gonzalez-Noriega, A., Grubb, J.H., Talkad, V., and Sly, W.S. (1980) J. Cell Biol. 85, 839-852.

Gordon, A.H. (1970) in Plasma Protein Metabolism (Rothschild, M.A., and Waldman, T., eds.), pp. 351-368, Academic Press, New York.

Gordon, H.T., Thornkurg, W., and Werum, L.N. (1956) Anal. Chem. 28, 849-855.

Gorog, P., and Kovacs, I.B. (1969) in Inflammation Biochemistry and Drug Interaction (Bertelli, A., and Houck, J.C., eds.), pp. 197-203, Excerpta Medica Foundation, Amsterdam.

Gorski, J., and Mueller, G.C. (1963) Arch. Biochem. Biophys. 102, 21-25.

Gottschalk, A. (1972) in Glycoproteins (Gottschalk, A., ed.), Pt. A, pp. 141-157, Elsevier Publishing Co., New York.

Greenberg, G.R. (1975) J. Cell Biol. 64, 269-288.

Gregoriadis, G. (1975) in Lysosomes in Biology and Pathology (Dingle, J.T., and Dean, R.T., eds.), vol. 4, pp. 265-294, North-Holland Publishing Company, Amsterdam.

Grinna, L.S., and Robbins, P.W. (1979) J. Biol. Chem. 254, 8814-8818.

Hagopian, A., and Eylar, E.H. (1968) Arch. Biochem. Biophys. 128, 422-433.

Hand, A.R., and Oliver, C. (1981) Meth. Cell Biol. 23, 137-153.

Hanover, J.A., and Lennarz, W.J. (1979) J. Biol. Chem. 254, 9237-9246.

Hanover, J.A., and Lennarz, W.J. (1980) J. Biol. Chem. 255, 3600-3604.

Hanover, J.A., and Lennarz, W.J. (1982) J. Biol. Chem. 257, 2787-2794.

Hanover, J.A., Lennarz, W.J., and Young, J.D. (1980) J. Biol. Chem. 255, 6713-6716.

Hardingham, T.E., and Phelps, C.F. (1968) Biochem. J. 108, 9-16.

Harms, E., Kreisel, W., Morris, H.P., and Reutter, W. (1973) Eur. J. Biochem. 32, 254-262.

Harpaz, N., and Schachter, H. (1980a) J. Biol. Chem. 255, 4885-4893.

Harpaz, N., and Schachter, H. (1980b) J. Biol. Chem. 255, 4894-4902.

Haugen, T.H., Hanley, T.M., and Heath, E.C. (1981) J. Biol. Chem. 256, 1055-1057.

Heifetz, A., and Lennarz, W.J. (1979) J. Biol. Chem. 254, 6119-6127.

Hemming, F.W. (1974) MTP Int. Rev. Sci. Biochem. Ser. 4, 39-98.

Hemming, F.W. (1977) Biochem. Soc. Trans. 5, 1223-1231.

Henderson, M., and Kessel, D. (1977) Cancer 39, 1129-1134.

Hers, H.G. (1976) Ann. Rev. Biochem. 45, 176-187.

Hickman, S., and Kornfeld, S. (1978) J. Immunol. 121, 990-996.

Hickman, S., Kulczycki, A., Lynch, R.G., and Kornfeld, S. (1977) J. Biol. Chem. 252, 4402-4408.

Houck, J.C., Johnston, J., and Jacob, R.A. (1968) Biochem. Pharmac., Supplement, pp. 19-26, Permagon Press, Great Britain.

Hubbard, S.C., and Ivatt, R.J. (1981) Ann. Rev. Biochem. 50, 555-583.

Hubbard, S.C., and Robbins, P.W. (1980) J. Biol. Chem. 255, 11782-11793.

Hudgin, R.L., Murray, R.K., Pinteric, L., Morris, H.P., and Schachter (1971) Can. J. Biochem. 49, 61-70.

Hudgin, R.L., and Schachter, H. (1971) Can. J. Biochem. 49, 829-837.

Hudgin, R.L., and Schachter, H. (1972) Can. J. Biochem. 50, 1024-1028.

Hudig, D., and Sell, S. (1979) Mol. Immunol. 16, 547-554.

Hultin, J., Arrhenius, E., Low, H., and Magee, P.N. (1960) Biochem. J. 76, 109-116.

Igarashi, K., Matsuzaki, K., and Takeda, Y. (1971) Biochim. Biophys. Acta 254, 91-103.

Ikehara, Y., Oda, K., and Kato, K. (1976) Biochem. Biophys. Res. Comm. 72, 319-326.

Ip, C., and Dao, T. (1978) Cancer Res. 38, 723-728.

Jabbal, I., and Schachter, H. (1971) J. Biol. Chem. 246, 5154-5161.

Jamieson, J.C. (1977) Can. J. Biochem. 55, 408-414.

Jamieson, J.C. (1983) in Plasma Protein Secretion by the Liver (Glaumann, H., ed.), pp. 257-284, Academic Press, New York.

Jamieson, J.C., and Ashton, F.E. (1973a) Can. J. Biochem. 51, 1034-1045.

Jamieson, J.C., and Ashton, F.E. (1973b) Can. J. Biochem. 51, 1281-1291.

Jamieson, J.C., Ashton, F.E., Friesen, A.D., and Chou, B. (1972b) Can. J. Biochem. 50, 871-880.

Jamieson, J.C., Friesen, A.D., Ashton, F.E., and Chou, B. (1972a) Can. J. Biochem. 50, 856-870.

Jamieson, J.C., Kaplan, H.A., Woloski, B.M.R.N.J., Hellman, M.A., and Ham, K. (1983) Can. J. Biochem. Cell Biol. 61, 1041-1048.

Jamieson, J.C., Morrison, K.E., Molasky, D., and Turchen, B. (1975) Can. J. Biochem. 53, 401-414.

Jamieson, J.D., and Palade, G.E. (1967) J. Cell Biol. 34, 577-596.

John, D.W., and Miller, L.L. (1969) J. Biol. Chem. 244, 6134-6142.

Judah, J.D., and Quinn, P.S. (1976) Trends Biochem. Sci., 1, 107-109.

Kampschmidt, R.F., Pulliam, L.A., and Upchurch, H.F. (1973) Proc. Soc. Exp. Biol. Med. 144, 882-886.

Kampschmidt, R.F., Upchurch, H.F., and Pulliam, L.A. (1982) Ann. N.Y. Acad. Sci. 389, 338-353.

Kaplan, H.A., and Jamieson, J.C. (1977) Life Sci. 21, 1311-1316.

Kaplan, M.H., and Volanakis, J.E. (1974) J. Immunol. 112, 2135-2147.

Kassell, B., and Kay, J. (1973) Science 180, 1022-1027.

Kean, E.L. (1970) J. Biol. Chem. 245, 2301-2308.

Kean, E.L., and Roseman, S. (1966) J. Biol. Chem. 241, 5643-5650.

Keegstra, K., Sefton, B., and Burke, D. (1975) J. Virol. 23, 613-620.

Keenan, R.W., and Kruczek, M.E. (1976) Biochemistry 15, 1586-1591.

Keenan, T.W., Morre, D.J., and Basa, S. (1974) J. Biol. Chem. 249, 310-315.

Keller, R.K., Jehle, E., and Adair, W.L. (1982) J. Biol. Chem. 257, 8985-8989.

Kessel, D., and Allen, J. (1975) Cancer Res. 35, 670-672.

Kessel, D., Chou, T., and Allen, J. (1978) Eur. J. Biochem. 82, 535-541.

Kessel, D., Samson, M.K., Shah, P., Allen, J., and Baker, L.H. (1976) Cancer 38, 2132-2134.

Kiely, M.L., McKnight, G.S., and Schimke, R.T. (1976) J. Biol. Chem. 251, 5490-5495.

Kikuchi, K., Kikuchi, H., and Tsuiki, S. (1971) Biochim. Biophys. Acta 252, 357-368.

Kim, Y.S., Perdomo, J., Whitehead, J.S., and Curtis, K.J. (1972) J. Clin. Invest. 51, 2033-2039.

Kobata, A., Ginsburg, V., and Tsuda, M. (1969) Arch. Biochem. Biophys. 130, 509-513.

Koj, A. (1974) in Structure and Function of Plasma Proteins (Allison, A.C., ed.), pp. 73-125, Plenum Press, New York.

Koj, A., Dubin, A., Kasperczyk, K., Bereta, J., and Gordon, A.H. (1982) Biochem. J. 206, 545-533.

Kornfeld, R. (1967) J. Biol. Chem. 242, 3135-3141.

Kornfeld, R., and Ginsburg, V. (1966) Biochim. Biophys. Acta 117, 79-87.

Kornfeld, R., and Kornfeld, S. (1976) Ann. Rev. Biochem. 45, 217-237.

Kornfeld, R., and Kornfeld, S. (1980) in The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W.J., ed.), pp. 1-34, Plenum Press, New York.

Kornfeld, S., Kornfeld, R., Neufeld, E.F., and O'Brien, P.J. (1964) Proc. Natl. Acad. Sci. U.S.A. 53, 371-379.

Kornfeld, S. (1982) in The Glycoconjugates (Horowitz, M.I., ed.), vol. 3, pp. 3-23, Academic Press, New York.

Kornfeld, S., Li, E., and Tabas, I. (1978) J. Biol. Chem. 253, 7771-7778.

Kreil, G. (1981) Ann. Rev. Biochem. 50, 317-348.

Kuehn, G.D., and Atmur, V.J. (1982) Fed. Proc. 41, 3078-3083.

Kuhlenschmidt, M.K., Peters, S.P., Pinkard, O.D., Glew, R.H., and Sharp, H. (1976) Biochim. Biophys. Acta 429, 359-373.

Kulka, J.P. (1964) Ann. N.Y. Acad. Sci. 116, 1018-1044.

Kuo, S.C., and Lampen, J.O. (1976) Biochem. Biophys. Res. Commun. 58, 287-295.

Kushner, I. (1982) Ann. N.Y. Acad. Sci. 389, 39-48.

Kushner, I., and Feldmann, G. (1978) J. Exp. Med. 148, 466-477.

Labrie, F., Gauthier, M., Pelletier, G., Borgeat, P., Lemay, A., and Gouge, J.J. (1973) Endocrinology 93, 903-914.

Lacy, P.E., Howell, D.A., Young, C., and Fink, J. (1968) Nature 219, 1177-1179.

Lacy, P.E., Walker, M.M., and Fink, C.J. (1972) Diabetes 21, 987-998.

Langstaff, J.M., Burton, D.N., and Jamieson, J.C. (1980) Arch. Biochem. Biophys. 204, 294-301.

Laurell, C.B., and Jeppsson, J.O. (1975) in The Plasma Proteins (Putnam, F.W., ed.), vol. 1, pp. 229-264, Academic Press, New York.

Leavitt, R., Schlesinger, S., and Kornfeld, S. (1977a) J. Virol. 21, 375-385.

Leavitt, R., Schlesinger, S., and Kornfeld, S. (1977b) J. Biol. Chem. 252, 9018-9023.

Ledford, B.E., and Davis, D.F. (1983) J. Biol. Chem. 258, 3304-3308.

Lejeune, N., Thines-Sempoux, D., and Hers, H.G. (1963) Biochem. J. 86, 16-21.

Le Marchand, Y., Singh, A., Assimacopoulos-Jeannet, F., Orci, L., Rouiller, C., and Jeanrenaud, B. (1973) J. Biol. Chem. 248, 6862-6870.

Le Marchand, Y., Patzelt, C., Assimacopoulos-Jeannet, F., Loten, E.G., and Jeanrenaud, B. (1974) J. Clin. Invest. 53, 1512-1517.

Li, E., and Kornfeld, S. (1978) J. Biol. Chem. 253, 6426-6431.

Li, Y., and Li, S. (1972) Methods Enzymol. 28, 702-713.

Liang, C.J., Yamashita, K., Mullenberg, C.G., Schichi, H., and Kobata, A. (1979) J. Biol. Chem. 254, 6414-6418.

Lingappa, V.R., Devillers-Thiery, A., and Blobel, G. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 2432-2436.

Lingappa, V.R., Lingappa, J.R., Prasad, R., Ebner, K.E., and Blobel, G. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 2338-2342.

Little, J.S. (1981) Proc. Soc. Exp. Biol. Med. 167, 284-289.

Lombart, C., Sturgess, J., and Schachter, H. (1980) Biochim. Biophys. Acta 629, 1-12.

Lotfield, R.B., Eigner, E.A., and Pastuszyn, A. (1981) J. Biol. Chem. 256, 6729-6735.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-276.

Lucas, J.J., and Levin, E. (1977) J. Biol. Chem. 252, 4330-4336.

Macbeth, R.A.L., Bekesi, J.G., Sugden, E., and Bice, S. (1965) J. Biol. Chem. 240, 3707-3713.

Magee, S.C., Geren, C.R., and Ebner, K.E. (1976) Biochim. Biophys. Acta 420, 187-194.

Magee, S.C., Mawal, R., and Ebner, K.E. (1973) J. Biol. Chem. 248, 7565-7569.

Maley, F., and Maley, G.F. (1959) Biochim. Biophys. Acta 31, 577-578.

Marsh, J.B., and Drabkin, D.L. (1958) J. Biol. Chem. 230, 1073-1081.

Marshall, R.D. (1974) Biochem. Soc. Symp. 40, 17-26.

Marshall, R.D., and Neuberger, A. (1964) Biochemistry 3, 1596-1600.

Maurice, M., Feldmann, G., Bellon, B., and Druet, P. (1980) Biochem. Biophys. Res. Comm. 97, 355-363.

McAdam, K,P.W.J., Li, J., Knowles, J., Foss, N.J., Dinarello, C.A., Rossenwasser, L.J., Selinger, M.J., Kaplan, M.M., Goodman, R., Herbert, P.N., Bausserman, L.L., and Nadler, L.M. (1982) Ann. N.Y. Acad. Sci. 389, 126-136.

McCloskey, M.A., and Troy, F.A. (1980) Biochemistry 19, 2061-2066.

Mendicino, J., and Rao, A.K. (1972) Eur. J. Biochem. 51, 547-556.

Miller, G.L. (1959) Anal. Chem. 31, 964.

Miyagi, T., and Tsuiki, S. (1971) Biochim. Biophys. Acta 250, 51-62.

Mizouchi, T., Yamashita, K., Fujikawa, K., Titani, K., and Kobata, A. (1980) J. Biol. Chem. 255, 3526-3531.

Molnar, J., Robinson, G.B., and Winzler, R.J. (1964) J. Biol. Chem. 239, 3157-3162.

Montreuil, J. (1980) Adv. Carb. Chem. Biochem. 37, 157-223.

Mookerjea, S., and Yung, J.W.M. (1975) Arch. Biochem. Biophys. 166, 223-236.

Morre, D.J., Kartenbeck, J., and Franke, W.W. (1979) Biochim. Biophys. Acta 559, 71-152.

Morrison, T.G., and Lodish, H.G. (1975) J. Biol. Chem. 250, 6955-6962.

Morrow, J.F., Stearman, R.S., Peltzman, C.G., and Potter, D.A. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 4718-4722.

Mortensen, R.F., and Gewurz, H. (1976) J. Immunol. 116, 1244-1250.

Mortensen, R.F., Osmand, A.P., and Gewurz, H. (1975) J. Exp. Med. 141, 821-839.

Munro, J.R., Narasimhan, S., Wetmore, S., Riordan, J.R., and Schachter, H. (1975) Arch. Biochem. Biophys. 169, 269-277.

Murgita, A., and Tomasi, T., (1975) J. Exp. Med. 141, 276-286.

Narasimhan, S. (1982) J. Biol. Chem. 257, 10235-10242.

Natowicz, M., Hallet, D.W., Fier, C., Chi, M., Schesinger, P.H., and Baenzinger, J.U. (1983) J. Cell Biol. 96, 915-919.

Nelson, J.D., Jato-Rodriguez, J.T., and Mookerjea, S. (1973) Biochem. Biophys. Res. Commun. 55, 530-535.

Nickerson, J.M., and Fuller, G.M. (1981a) Biochemistry 20, 2818-2821.

Nilsson, B., Norden, N.E., and Svensson, S. (1979) J. Biol. Chem. 254, 4545-4553.

Nilsson, O.S., DeThomas, M.E., Peterson, E., Bergman, A., Dallner, G., and Hemming, F.W. (1978) Eur. J. Biochem. 89, 619-628.

Novikoff, A.B. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2781-2787.

Okubo, H., and Chandler, A.M. (1976) Exptl. Molec. Path. 24, 91-104.

Olden, K., Pratt, R.M., and Yamada, K.M. (1978) Cell 13, 461-473.

Opheim, D.J., and Touster, O. (1978) J. Biol. Chem. 253, 1017-1023.

Oppenheimer, C.L. ,and Hill, R.L. (1981) J. Biol. Chem. 256, 799-804.

Oppenheim, J.J, Stadler, B.M., Siraganian, R.P., Mage, M., and Mathieson, B. (1982) Fed. Proc. 41, 257-262.

Ovtracht, L., Morre, D.J., Cheetham, R.D., and Mollenhauer, H.H. (1973) J. Microsc. (Paris) 18, 87-102.

Palade, G. (1975) Science 189, 347-358.

Palmiter, R.D., Gagnon, J., and Walsh, K.A. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 94-98.

Parodi, A.J., Behrens, N.H., Leloir, L.F., Carminatti, H. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 3268-3272.

Parodi, A.J., and Leloir, L.F. (1979) Biochim. Biophys. Acta 559, 1-37.

Patzelt, C., Brown, D., and Jeanrenaud, B. (1977) J. Cell Biol. 73, 578-593.

Paulson, J.C., Beranek, W.E., and Hill, R.L. (1977a) J. Biol. Chem. 252, 2356-2362.

Paulson, J.C., Rearick, J.I., and Hill, R.L. (1977b) J. Biol. Chem. 252, 2363-2371.

Pegg, A.E., Seely, J.E., Poso, H., Ragione, F.D., and Zagon, I.S. (1982) Fed. Proc. 41, 3065-3072.

Pekarek, R.S., Wannemacher, R.W., and Beisel, W.R. (1972) Proc. Soc. Exp. Biol. Med. 140, 685-688.

Pestka, S. (1977) in Molecular Mechanisms of Protein Biosynthesis (Weissbach, H., and Pestka, S., eds.), pp. 467-553, Academic Press, New York.

Peters, T. (1977) Clin. Chem. 23, 5-12.

Peters, T., Fleischer, B., and Fleischer, S. (1971) J. Biol. Chem. 246, 240-244.

Phelps, C.F., Hardingham, T.E., and Winterburn, P.J. (1970) Exposes a. Biochim. med. 30, 79-95.

Pierece, M., Turley, E.A., and Roth, S. (1980) Int. Rev. Cytol. 65, 1-47.

Pless, D.D., and Lennarz, W.J. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 134-138.

Podolsky, D.K., and Weiser, M.M. (1975) Biochem. Biophys. Res. Commun. 65, 545-551.

Poncz, L., and Kean, E.L. (1980) Arch. Biochem. Biophys. 201, 527-532.

Poulik, M.D., and Weiss, M.L. (1975) in The Plasma Proteins (Putnam, F.W., ed.), vol. 2, pp. 51-108, Academic Press, New York.

Powell, J.T., and Brew, K. (1974) Eur. J. Biochem. 48, 217-228.

Pricer, W.E., and Ashwell, G. (1971) J. Biol. Chem. 246, 4825-4833.

Pricer, W.E., and Ashwell, G. (1976) J. Biol. Chem. 251, 7539-7544.

Princen, J.M.G., Nieuwenhuizen, W., Mol-Backx, G.P.B.M., and Yap, S.H. (1981) Biochem. Biophys. Res. Commun. 102, 717-723.

Quill, H., and Wolf, G. (1981) Ann. N.Y. Acad. Sci. 359, 331-344.

Quinn, P.S., Gamble, M., and Judah, J.D. (1975) Biochem. J. 146, 389-393.

Raina, A., and Janne, J. (1970) in Biogenic Amines as Physiological Regulators (Blum, J.J., ed.), pp. 275-300, Prentice-Hall, Englewood Cliffs, N.J.

Ravoet, A.M., Amar-Costesec, A., Godelaine, D., and Beaufay, H. (1981) J. Cell Biol. 91, 679-688.

Reaven, E.P., and Reaven, G.M. (1975) J. Clin. Invest. 56, 49-55.

Reaven, E.P., and Reaven, G.M. (1980) J. Cell Biol. 84, 28-39.

Redman, C.M., Banerjee, D., Howell, K., and Palade, G.E. (1975) J. Cell Biol. 66, 42-59.

Redman, C.M., Banerjee, D., Manning, C., Huang, C.Y., and Green, K. (1978) J. Cell Biol. 77, 400-416.

Redman, C.M., Banerjee, D., and Yu, S. (1981) Meth. Cell Biol. 23, 231-245.

Redman, C.M., and Cherian, M.G. (1972) J. Cell Biol. 52, 231-245.

Reid, K.B.M., and Porter, R.R. (1981) Ann. Rev. Biochem. 50, 433-464.

Reitman, M.L., and Kornfeld, S. (1981) J. Biol. Chem. 256, 4275-4281.

Revel, M. (1977) in Molecular Mechanisms of Protein Biosynthesis (Weissbach, H., and Pestka, S., eds.), pp. 246-321, Academic Press, New York.

Revel, M., and Groner, Y. (1978) Ann. Rev. Biochem. 47, 1079-1126.

Ricca, G.A., McLean, J.W., and Taylor, J.M. (1982) Ann. N.Y. Acad. Sci. 389, 88-103.

Richards, J.B., and Hemming, F.W. (1972) Biochem. J. 130, 77-93.

Rip, J.W., and Carroll, K.K. (1980) Can. J. Biochem. 58, 1051-1056.

Rip, J.W., Rupar, C.A., Chaudhary, N., and Carroll, K.K. (1981) J. Biol. Chem. 256, 1929-1934.

Robinson, G.B., Molnar, J., and Winzler, R.J. (1964) J. Biol. Chem. 239, 1134-1141.

Rodriguez-Boulan, E., Kreibich, G., and Sabatine, D.D. (1978) J. Cell Biol. 78, 874-893.

Rome, L., Garvin, A.J., Allieha, M.M., and Neufeld, E.F. (1979) Cell 17, 143-153.

Ronin, C., and Bouchilloux, S. (1978) Biochim. Biophys. Acta 539, 470-480.

Ronin, C., Bouchilloux, S., Granier, C., and van Rietschoten, J. (1979) FEBS Letters 96, 179-182.

Roseman, S. (1962) Fed. Proc. 21, 1075.

Rosner, M.R., Grinna, L.S., and Robbins, P.W. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 67-71.

Ross, R. (1972) in Inflammation Mechanisms and Control (Lepow, I.H., and Ward, P.A., eds.), pp. 29-43, Academic Press, New York.

Rossignol, D.P., Lennarz, W.J., and Waechter, C.J. (1981) J. Biol. Chem. 256, 10538-10542.

Rothman, J.E., Bursztyn-Pettegrew, H., and Fine, R.E. (1980) J. Cell Biol. 86, 162-171.

Rothman, J.E., and Lenard, J. (1977) Science 195, 743-753.

Rothman, J.E., and Lodish, H.F. (1975) Nature 269, 775-780.

Rupar, C.A., and Carroll, K.K. (1978) Lipids 13, 291-293.

Rupar, C.A., Rip, J.W., Chaudhary, N., and Carroll, K.K. (1982) J. Biol. Chem. 257, 3090-3094.

Sabatini, D.D., Kreibich, G., Morimoto, T., and Adesnik, M. (1982) J. Cell Biol. 92, 1-22.

Sadler, J.E., Rearick, J.I., Paulson, J.C., and Hill, R.L. (1979a) J. Biol. Chem. 254, 4434-4443.

Sadler, J.E., Rearick, J.I., and Hill, R.L. (1979b) J. Biol. Chem. 254, 5934-5941.

Schachter, H. (1974a) Adv. Cytopharmacol. 2, 207-218.

Schachter, H. (1974b) Biochem. Soc. Symp. 40, 57-71.

Schachter, H. (1978) in The Glycoconjugates (Horowitz, M.I., and Pigman, W., eds.), vol. 2, pp. 87-181, Academic Press, New York.

Schachter, H. (1981) in Lysosomes and Lysosomal Storage Diseases (Callahan, J.W., and Lowden, J.A., eds.), pp. 73-93, Raven Press, New York.

Schachter, H., Harpaz, N., Narasimhan, S., Wilson, D., and Longmore, G. (1979) in Proceedings of the Fifth International Symposium on Glycoconjugates (Schauer, R., Boer, P., Buddecke, E., Kramer, M.F., Vliengenthart, J.F.G., and Wiegandt, H., eds.), pp. 305-306, Georg Thieme Verlag, Stuttgart.

Schachter, H., Jabbal, I., Hudgin, R.L., Pinteric, L., McGuire, E.J., and Roseman, S. (1970) J. Biol. Chem. 245, 1090-1100.

Schachter, H., Narasimhan, S., and Wilson, J.R. (1978) in Glycoproteins and Glycolipids in Disease Processes (Walborg, E.F., ed.), pp. 21-46, ACS Symposium Series 80.

Schachter, H., and Roden, L. (1973) in Metabolic Conjugation and Metabolic Hydrolysis (Fishman, W.H., ed.), pp. 1-149, Academic Press, New York.

Schauer, R., Buscher, H.P., and Casals-Stenzel, J. (1974) Biochem. Soc. Symp. 40, 87-116.

Schauer, R., and Wember, M. (1973) Hoppe-Seyler's Z. Physiol. Chem. 354, 1405-1414.

Scher, M.G., and Waechter, C.J. (1981) Biochem. Biophys. Res. Commun. 99, 675-681.

Schmitt, J.W., and Elbein, A.D. (1979) J. Biol. Chem. 254, 12291-12294.

Schreiber, G., Dryburgh, H., Millership, A., Matsuda, Y., Inglis, A., Phillips, J., Edwards, K., and Maggs, J. (1979) J. Biol. Chem. 254, 12013-12019.

Schreiber, G., and Urban, J. (1978) Rev. Physiol. Biochem. Pharmacol. 82, 28-90.

Schreiber, G., Urban, J., and Edwards, J. (1976) J. Theor. Biol. 60, 241-245.

Schwartz, R.T., and Datema, R. (1982) in The Glycoconjugates (Horowitz, M.I., ed.), pp. 47-79, Academic Press, New York.

Schwartz, R.T., Schmidt, M.F.G., Anwer, U., and Klenk, H.D. (1977) J. Virol. 23, 217-226.

Sefton, B.M. (1976) J. Virol. 17, 85-93.

Sefton, B.M. (1977) Cell 10, 659-668.

Sela, B.A., Lis, H., Sharon, N., and Sachs, L. (1970) J. Membrane Biol. 3, 267-279.

Selinger, M.J., McAdam, K.P.W.J., Kaplan, M.M., Sipe, J.D., Vogel, S.N., and Rosenstreich, D.L. (1980) Nature 285, 498-500.

Shafritz, D.A. (1977) in Molecular Mechanisms of Protein Biosynthesis (Weissbach, H., and Pestka, S., eds.), pp. 556-601, Academic Press, New York.

Shah, S.N., and Raghupathy, E. (1977) Proc. Soc. Exp. Biol. Med. 155, 516-518.

Sharon, N., and Lis, H. (1981) Special Report, Chem. Eng. News, March 30, pp. 21-44.

Shetlar, M.R., Capps, J.C., and Hern, D.L. (1964) Biochim. Biophys. Acta 83, 93-101.

Shore, G.C., and Tata, J.R. (1977) Biochim. Biophys. Acta 472, 197-236.

Shoyab, M., and Bachhawat, B.K. (1967) Biochem. J. 102, 13c-14c.

Shur, B.D., and Roth, S. (1975) Biochim. Biophys. Acta 415, 473-512.

Shutler, G.G., Langstaff, J.M., Jamieson, J.C., and Burton, D.N. (1977) Arch. Biochem. Biophys. 183, 710-717.

Silver, H.K.B., Karim, K.A., Archibald, E.L., and Salinas, F.A. (1979) Cancer Res. 39, 5036-5042.

Simkin, J.L., and Jamieson, J.C. (1967) Biochem. J. 103, 153-163.

Simkin, J.L., Seshadri, H.S., and Skinner, E.R. (1964b) Nature 202, 702.

Simkin, J.L., Skinner, E.R., and Seshadri, H.S. (1962a) Biochem. J. 90, 136-321.

Simon, P.L., and Willoughby, W.F. (1982) in Lymphokines (Mizel, S.B., ed.), vol. 6, pp. 47-63, Academic Press, New York.

Singh, B.N., and Lucas, J.J. (1981) J. Biol. Chem. 256, 12018-12022.

Sly, W.S. (1979) Adv. Exp. Biol. Med. 125, 433-451.

Sly, W.S., and Fischer, H.D. (1982) J. Cell. Biochem. 18, 67-85.

Sly, W.S., Fischer, H.D., Gonzalez-Noriega, A., Grubb, J.H., and Natowicz, M. (1981) Meth. Cell Biol. 23, 199-214.

Sly, W.S., Gonzalez-Noriega, A., Natowicz, M., Fischer, H.D., and Chambers, J.P. (1979) Fed. Proc. 38, 467.

Sly, W.S., and Stahl, P. (1978) in Transport of Macromolecules in Cellular Systems (Silverstein, S.C., ed.), pp. 229-244, Dahlem Konferenzen, Berlin.

Snider, M.D., Sultzman, L.A., and Robbins, P.W. (1980) Cell 21, 385-392.

Sobocinski, P.Z., and Canterbury, W.J. (1982) Ann. N.Y. Acad. Sci. 389, 354-367.

Sommar, K.M., and Ellis, D.B. (1972) Biochim. Biophys. Acta 268, 581-589.

Spiro, R.G. (1965) in The Amino Sugars (Balazs, E.A., and Jeanloz, R.W., eds.), vol. 2A, pp. 47-78, Academic Press, New York.

Spiro, R.G. (1973) Adv. Prot. Chem. 27, 349-367.

Spiro, R.G., and Bhoyroo, V.D. (1974) J. Biol. Chem. 249, 5704-5714.

Spiro, M.J., Spiro, R.G., and Bhoyroo, V.C. (1979) J. Biol. Chem. 254, 7668-7674.

Spivak, C.T., and Roseman, S. (1966) Methods Enzymol. 9, 612-613.

Stackhouse, H.L., Chetsanga, C.J., and Tan, C.H. (1968) Biochim. Biophys. Acta 155, 159-168.

Stein, D., and Stein, Y. (1973) Biochim. Biophys. Acta 306, 142-147.

Stein, W.H., and Moore, S. (1954) J. Biol. Chem. 211, 915-926.

Steiner, D.F., Kemmler, W., Tager, H.S., and Peterson, J.D. (1974) Fed. Proc. 33, 2105-2115.

Strauss, A.W., Bennet, C.D., Donohue, A.M., Rodkey, J.A., and Alberts, A.W. (1977) J. Biol. Chem. 252, 6846-6855.

Strominger, J.L. (1962) J. Biol. Chem. 237, 1388-1392.

Stroud, R.M., Volankis, J.E., Nagasawa, S., and Lint, T.F. (1979) in Immunochemistry of Proteins (Atassi, M.Z., ed), vol. 3, pp. 167-227, Plenum Publishing Corporation, New York.

Struck, D.K., and Lennarz, W.J. (1980) in The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W.J., ed.), pp. 35-73, Plenum Press, New York.

Struck, D.K., Siuta, P.B., Lane, M.D., and Lennarz, W.J. (1978) J. Biol. Chem. 253, 5332-5337.

Tabas, I., and Kornfeld, R. (1979) J. Biol. Chem. 254, 11655-11663.

Tabas, I., and Kornfeld, S. (1980) J. Biol. Chem. 255, 6633-6639.

Tabor, C.W., Tabor, H., Tyagi, A.K., and Cohn, M.S. (1982) Fed. Proc. 41, 3084-3088.

Takeda, Y. (1969) Biochim. Biophys. Acta 179, 232-234.

Tepperman, H.M., Silver, R., Dewitt, J., and Tepperman, J. (1981) J. Nutr. 111, 1734-1741.

Thompson, W.L., and Wannemacher, R.W. (1973) Biochem. J. 134, 79-87.

Torello, L.A., Yates, A.J., and Thompson, D.K. (1980) J. Chromat. 202, 195-209.

Tulsiani, D.R., Hubbard, S.C., Robbins, P.W., and Touster, O. (1982) J. Biol. Chem. 257, 3660-3668.

Tulsiani, D.R., Opheim, D.J., and Touster, O. (1978) J. Biol. Chem. 253, 1017-1023.

Tulsiani, D.R.P., Hubbard, S.C., Robbins, P.W., and Touster, O. (1981) Fed. Proc. 40, 1883.

Tulsiani, D.R.P., Opheim, D.J., and Touster, O. (1977) J. Biol. Chem. 252, 3227-3233.

Turchen, B., Jamieson, J.C., Huebner, E., and Van Caeseele, L. (1977) Can. J. Zool. 55, 1567-1570.

Turco, S.J., and Robbins, P.W. (1979) J. Biol. Chem. 254, 4560-4567.

Turco, S.J., Stetson, B., and Robbins, P.W. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 4411-4414.

Ugalde, R.A., Staneloni, R.J., and Leloir, L.F. (1979) Biochem. Biophys. Res. Comm. 91, 1174-1181.

Urban, J., Chan, D., and Schrieber, G. (1979) J. Biol. Chem. 254, 10565-10568.

van den Eijnden, H., and van Dijk, W. (1972) Hoppe-Seyler, Z. Physiol. Chem. 353, 1817-1820.

van Obberghen, E.G., Somers, G., Devis, M., Ravazzola, I., Malaise-Lagae, L., Orci, L., and Malaisse, W.J. (1974) Endocrinology 95, 1518-1528.

Varki, A., and Kornfeld, S. (1980a) J. Biol. Chem. 255, 8398-8401.

Varki, A., and Kornfeld, S. (1980b) J. Biol. Chem. 255, 10847-10858.

Varki, A., and Kornfeld, S. (1981) J. Biol. Chem. 256, 9937-9943.

Voisey, J.R., and Winterburn, P.J. (1982) Biochem. Soc. Trans. 10, 275-276.

Volanakis, J.E. (1982) Ann. N.Y. Acad. Sci. 389, 235-250.

Waechter, C.J., and Lennarz, W.J. (1976) Ann. Rev. Biochem. 45, 95-112.

Wagner, R.R., and Cynkin, M.A. (1971) J. Biol. Chem. 246, 143-151.

Wagner, R.R., and Cynkin, M.A. (1971) Biochem. Biophys. Res. Commun. 45, 57-62.

Waheed, A., Pohlmann, R., Hasilik, A., and von Figura, K. (1981a) J. Biol. Chem. 256, 4150-4152.

Waheed, A., Hasilik, A., and von Figura, K. (1981b) J. Biol. Chem. 256 5717-5721.

Walter, P., and Blobel, G. (1981a) J. Cell Biol. 91, 551-556.

Walter, P., and Blobel, G. (1981b) J. Cell Biol. 91, 557-561.

Walter, P., and Blobel, G. (1982) Nature 299, 691-698.

Walter, P., Ibrahimi, I., and Blobel, G. (1981) J. Cell Biol. 91, 545-550.

Warren, L. (1959) J. Biol. Chem. 242, 1971-1975.

Warren, L. (1960) Nature 186, 237.

Warren, L. (1963) Comp. Biochem. Physiol. 10, 153-171.

Warren, L. (1972) in Glycoproteins: Their Composition, Structure and Function (Gottschalk, A., ed.), pt. B, 1097-1126, Elsevier, New York.

Wedgewood, J.F., and Strominger, J.L. (1980) J. Biol. Chem. 255, 1120-1123.

Weinberg, E.D. (1978) Microbiol. Rev. 42, 45-66.

Weinstein, J., de Souza-e-Silva, U., and Paulson, J.C. (1982a) J. Biol. Chem. 257, 13835-13844.

Weinstein, J., de Souza-e-Silva, U., and Paulson, J.C. (1982b) J. Biol. Chem. 257, 13845-13853.

Weiser, M.M., Klohs, W.D., Podolsky, D.K., and Wilson, J.R. (1982) in The Glycoconjugates (Horowitz, M.I., ed.), vol. 4, pp. 301-333, Academic Press, New York.

Weiser, M.M., Podolsky, D.K., and Isselbacher, K. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1319-1322.

Weissbach, H., and Ochoa, S. (1976) Ann. Rev. Biochem. 45, 191-216.

Weissman, G., and Uhr, J.W. (1968) Biochem. Pharmac. Supplement, pp. 5-18, Permagon Press, Great Britain.

Weitzman, S., Grennon, M., and Keegstra, K. (1979) J. Biol. Chem. 254, 5377-5382.

Westcott, K.R., and Hill, R.L. (1982) Fed. Proc. 41, 662.

Whaley, W.G., and Dauwalder, M. (1979) Int. Rev. Cytol. 58, 199-245.

White, B.N., Shetlar, M.R., Shurley, H.M., and Schilling, J.A. (1965) Biochim. Biophys. Acta 101, 259-266.

Wilkinson, G.N. (1961) Biochem. J. 80, 324-332.

Williams, J.A. and Wolff, J. (1972) J. Cell Biol. 54, 157-165.

Wilson, J.R., Williams, D., and Schachter, H. (1976) Biochem. Biophys. Res. Commun. 72, 909-916.

Wilson, L., Bamburg, J.R., Mizel, S.B., Brisham, L.M., and Creswell, K.M. (1974) Fed. Proc. 33, 158-166.

Wilson, M.J. (1981) in Advanced Cell Biology (Schwartz, L.M., and Azar, M.M., eds.), pp. 482-489, Van Nostrand Reinhold Co., New York.

Winterburn, P.J., and Phelps, C.F. (1971) Biochem. J. 121, 701-710.

Winzler, R.J. (1965) in The Amino Sugars (Balazas, E.A., and Jeanloz, W., eds.), pp. 337-352, Academic Press, New York.

Woessner, J.F. (1979) in Handbook of Inflammation I. Chemical Messengers of the Inflammatory Process (Houck, J.C., ed.), pp. 261-284, Elsevier, New York.

Wolf, G., Kiorpes, T.C., Masushige, S., Schreeber, J.B., Smith, M.J., and Anderson, R.S. (1979) Fed. Proc. 38, 2540-2543.

Woloski, B.M.R.N.J. (1983) Ph.D. Thesis, University of Manitoba.

Woloski, B.M.R.N.J., and Jamieson, J.C. (1980) Proc. Can. Fed. Biol. Soc. 23, 99.

Woloski, B.M.R.N.J., Kaplan, H.A., Gospodarek, E., and Jamieson, J.C. (1983a) Biochem. Biophys. Res. Comm. 112, 14-19.

Woloski, B.M.R.N.J., Kaplan, H.A., and Jamieson, J.C. (1983b) Comp Biochem. Physiol. 74A, 813-816.

Yachnin, S. (1975) J. Exp. Med. 141, 242-256.

Yamashita, K., Liang, C-J., Funakoshi, S., and Kobata, A. (1981) J. Biol. Chem. 256, 1283-1289.

Yoshima, H., Matsumoto, A., Mizuochi, T., Kawasaki, T., and Kobata, A. (1981) J. Biol. Chem. 256, 8476-8484.

Zinn, A.B., Plantner, J.J., and Carlson, D.M. (1977) in The Glycoconjugates (Horowitz, M.I., and Pigman, W., eds.), vol. 1, pp. 69-85, Academic Press, New York.