University of Manitoba

# The Effect of Inflammation on Rat Liver Glycosidases and Glycosyltransferases.

by

HOWARD ARTHUR KAPLAN

A Thesis Submitted to the Faculty of Graduate Studies in Partial Fulfilment of the Requirements for the Degree of Master of Science.

Department of Chemistry

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a Master's thesis entitled: The Effect of Inflammation on Rat Liver Glycosidases and Glycosyltransferases

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# THE EFFECT OF INFLAMMATION ON RAT LIVER GLYCOSIDASES AND GLYCOSYLTRANFERASES

BY

HOWARD ARTHUR KAPLAN

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

MASTER OF SCIENCE

In loving memory

of

my father

ACKNOWLEDGEMENTS

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Table of Contents

## ABBREVIATIONS

| | |
|---|---|
| GlcNAc | N-Acetylglucosamine |
| Man | Mannose |
| Gal | Galactose |
| NANA | N-Acetylneuraminic Acid |
| SA | Sialic acid |
| Asn | Asparagine |
| Ser | Serine |
| Thr | Threonine |
| CMP | Cytidine 5'-monophosphate |
| CDP | Cytidine 5'-diphosphate |
| CTP | Cytidine 5'-triphosphate |
| UDP | Uridine 5'-diphosphate |
| ATP | Adenosine 5'-triphosphate |
| GTP | Guanosine 5'-triphosphate |
| RNA | Ribonucleic acid |

## ABSTRACT

Reduced activities of β-galactosidase and β-N-Acetylglucosaminidase were found in rat livers following experimentally induced inflammation. The greatest reduction in glycosidase activities was found 24 h after inflammation. The lysosomal fraction accounted for the bulk of both glycosidase activities in experimental and control rats. Kinetic experiments showed that inflammation did not affect $K_m$ values, but did result in a significant reduction in $V_{max}$ values. One suggestion to explain the results is that inflammation causes a reduction in biosynthesis of the two glycosidases in rat liver.

Increased activities of sialyltransferase and galactosyltransferase were found in microsome fractions from rat livers following experimentally induced inflammation. The greatest increase in glycosyltransferase activities was found 24 h after inflammation. It is suggested that the increase in glycosyltransferase activities is characteristic of the response of liver to inflammation and reflects changes in the biosynthetic capacity of liver for $\alpha_1$-acid glycoprotein and other acute phase serum glycoproteins.

Studies on the incorporation of N-acetylglucosamine into nascent polypeptides showed that incorporation occurred into nascent polypeptides from both free and membrane-bound polyribosome preparations, with the membrane-bound preparation showing a significantly greater degree of incorporation. Nascent polypeptides were released from free and membrane-bound polyribosome preparations and used as acceptors for N-acetylglucosamine in an assay system for N-acetylglucosaminyltransferase activity.

Results showed that there was greater incorporation of N-acetylglucosamine into nascent polypeptides from the membrane-bound polyribosome preparation. These results confirmed earlier work that glycoproteins were synthesized mainly on membrane-bound polyribosomes.

An exogenous crude lipid fraction containing dolichol phosphate enhanced N-acetylglucosamine incorporation into nascent polypeptide acceptors, thus suggesting that the transfer of N-acetylglucosamine from UDP-N-acetylglucosamine to nascent polypeptides may involve a lipid-linked intermediate.

# INTRODUCTION

## The acute inflammatory response

Inflammation in mammals caused by chemical inflammatory agents, neoplastic diseases, bacterial infections, rheumatoid arthritis and a variety of other conditions results in characteristic changes in plasma protein concentrations (Table 1) (1, 2). Proteins which increase in concentration during the acute inflammatory process are referred to as acute phase reactants (2). Most of the acute phase reactants are glycoproteins. In spite of the well documented response of serum glycoproteins to the inflammatory stimulus, little is known about the mechanism that leads to the increased content of certain glycoproteins in serum during inflammation. With a view to obtaining some information on the response of serum glycoproteins to inflammation, several groups of workers have studied the events that take place in the body in response to inflammation.

Glenn et al. (3) suggested that the process of inflammation should be separated into two reactions, the local reaction and the systemic reaction (Fig. 1). The local reaction refers to the events occurring in the immediate area of tissue damage, whereas the systemic reaction describes events induced by the local reaction. Glenn et al. (3) suggested that local events were "contributory", or events which initiated the overall process, whereas the systemic responses were "protective" or "inhibitory"; that is, events which protected the body or slowed down the overall inflammatory process. The first event in the inflammatory process is venular dilation (4). This is followed by platelet aggregation, the formation of thrombi and the migration of lymphocyte

TABLE 1

Proteins of Human Plasma Showing Altered Concentration after Trauma

| Concentration | Proteins | Concentrations in Plasma (% of Preoperative Values) |
|---|---|---|
| Increased | Fibrinogen | >200 |
| | Haptoglobin | 206 |
| | Orosomucoid | >200 |
| | C-Reactive Protein | >200 |
| | $\alpha_1$-Antitrypsin | >200 |
| | Slow β-globulin | 173 |
| | Inter α-globulin | 189 |
| | Complement C'3 | 122 |
| | Caeruloplasmin | 124 |
| | Easily precipitable glycoprotein | 140 |
| Unchanged | More than 30 other proteins | |
| Decreased | Thyroxine binding prealbumin | 69 |
| | β-Lipoprotein | 77 |
| | Transferrin | 78 |
| | Albumin | 80 |

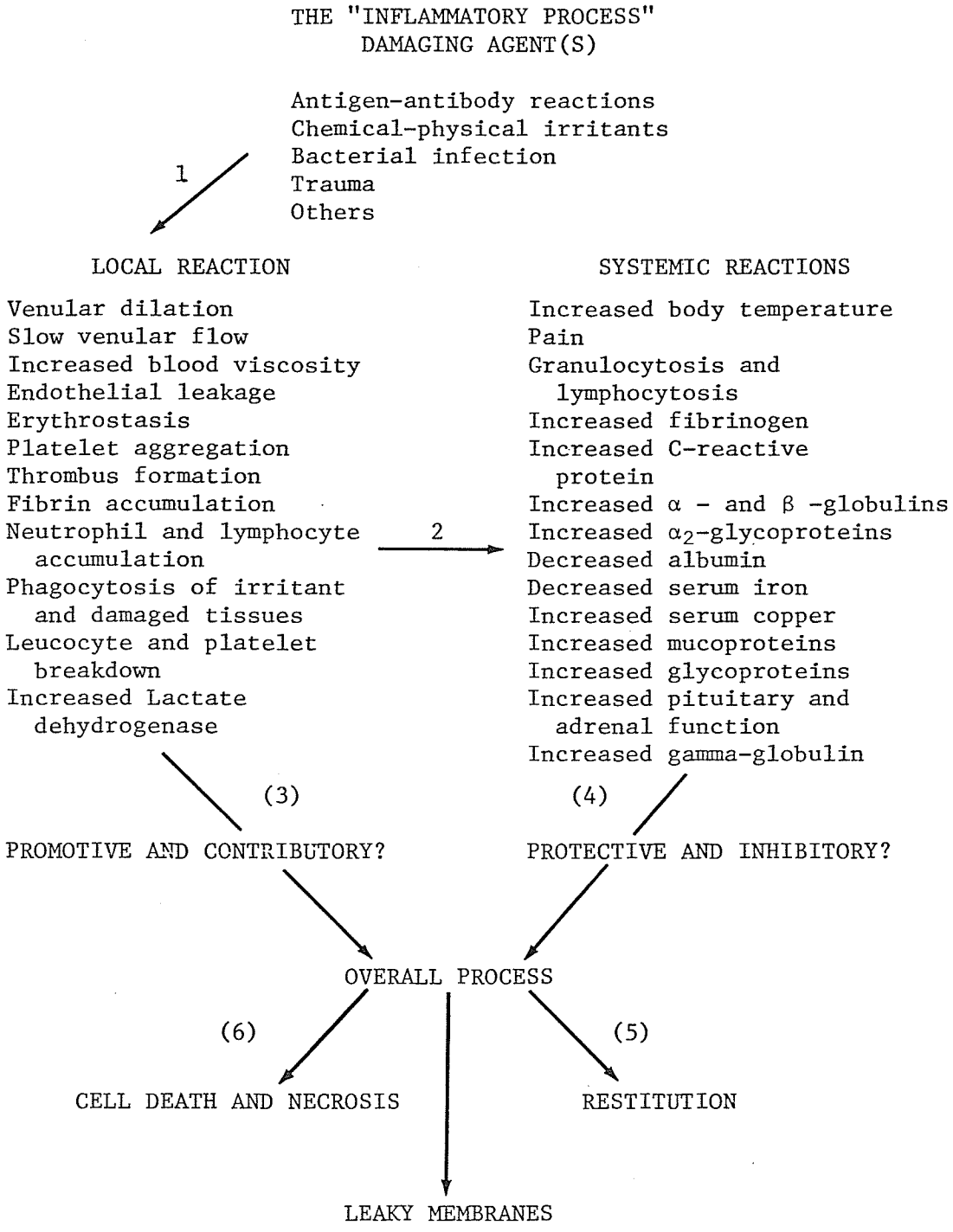


Fig. 1 Schematic representation of the inflammatory process. The numbers in parenthesis describe the order in which the events are believed to occur.

and neutrophil cells into the area of tissue damage (5). This process is thought to be accompanied by the release of cytoplasmic and lysosomal enzymes into the extracellular compartment of the tissue (6). It has been suggested that the lysosomal enzymes released activate cutaneous collagenase which degrades collagen in structural components of the tissue into soluble breakdown products which then drain away from the area of tissue damage (7). The end point of the acute inflammatory response is cell necrosis resulting mainly from the local reaction which may or may not be followed by repair of the damaged tissue (6).

According to the scheme put forward by Glenn et al. (3) (Fig. 1), the local reaction, by some unknown mechanism, induces the systemic reaction which consists primarily of increases in the levels of serum glycoproteins (Fig. 1). The most important of these acute phase reactants in human serum are orosomucoid*, ceruloplasmin, haptoglobin, transferrin, fibrinogen, $\alpha_2$-macroglobulin and some of the $\alpha_1$-globulins (1). In addition to the above acute phase reactants, new serum proteins not normally present in serum make their appearance; these include the C-reactive protein in man (8) and $\alpha_2$-(acute phase) globulin in rats (9).

The response of an $\alpha_1$-acid glycoprotein corresponding to orosomucoid and the $\alpha_2$-(acute phase) globulin to experimentally induced inflammation in rats has been studied by several groups of coworkers (10-14). Darcy

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*Orosomucoid is an $\alpha_1$-acid glycoprotein which is the major component of the seromucoid fraction of serum. The seromucoid fraction consists of those serum proteins that are soluble in 0.6 M perchloric acid, but are precipitated from the perchloric acid soluble fraction by 5% phosphotungstic acid.

(11, 12) found that the content of an $\alpha_1$-acid glycoprotein in rat serum increased 7-fold during turpentine-induced inflammation and 20-fold in Walker tumor-bearing rats. It was originally suggested by Darcy (15) that the increased content of this $\alpha_1$-acid glycoprotein in serum was a response to substances released from damaged necrotic cells. The presence of $\alpha_1$-acid glycoprotein in the area of tissue damage prompted the suggestion that the serum glycoprotein response to inflammation resulted from the release of glycoproteins synthesized locally in the inflammed tissue (16). However, it has since been established that the liver is the site of synthesis of most acute phase reactants including $\alpha_1$-acid glycoprotein (17-21). It is now believed that hormonal factors, that may be released from the site of inflammation, stimulate the liver, either to synthesize new glycoproteins found in serum, or to increase the synthesis rates of glycoproteins that are normally present in serum (9, 15, 22-24). This is presumably accomplished by a "switching on" and "switching off" mechanism affecting the synthesis of acute phase globulins in mammalian liver (25). The exact "switching on" and "switching off" mechanism is not understood at the present time, but is currently being investigated. Moreover, the function that acute phase reactants perform in the body is still not clear. It has been suggested that they act as carrier proteins, either by removing small molecules from the site of tissue damage, or by carrying small molecules to the site of tissue damage for purposes of repair (26). Clearly, an understanding of the function of acute phase reactants may have to await results from studies on the response of well characterized acute phase globulins to the inflammatory stimulus.

Structure of serum glycoproteins

Glycoproteins are normally defined as proteins that contain carbohydrate groups with a relatively low number of sugar residues covalently bound to the polypeptide chain (27). Glycoproteins constitute approximately 43% of plasma proteins by weight (28); serum albumin is the only major serum protein which is free of carbohydrate (29).

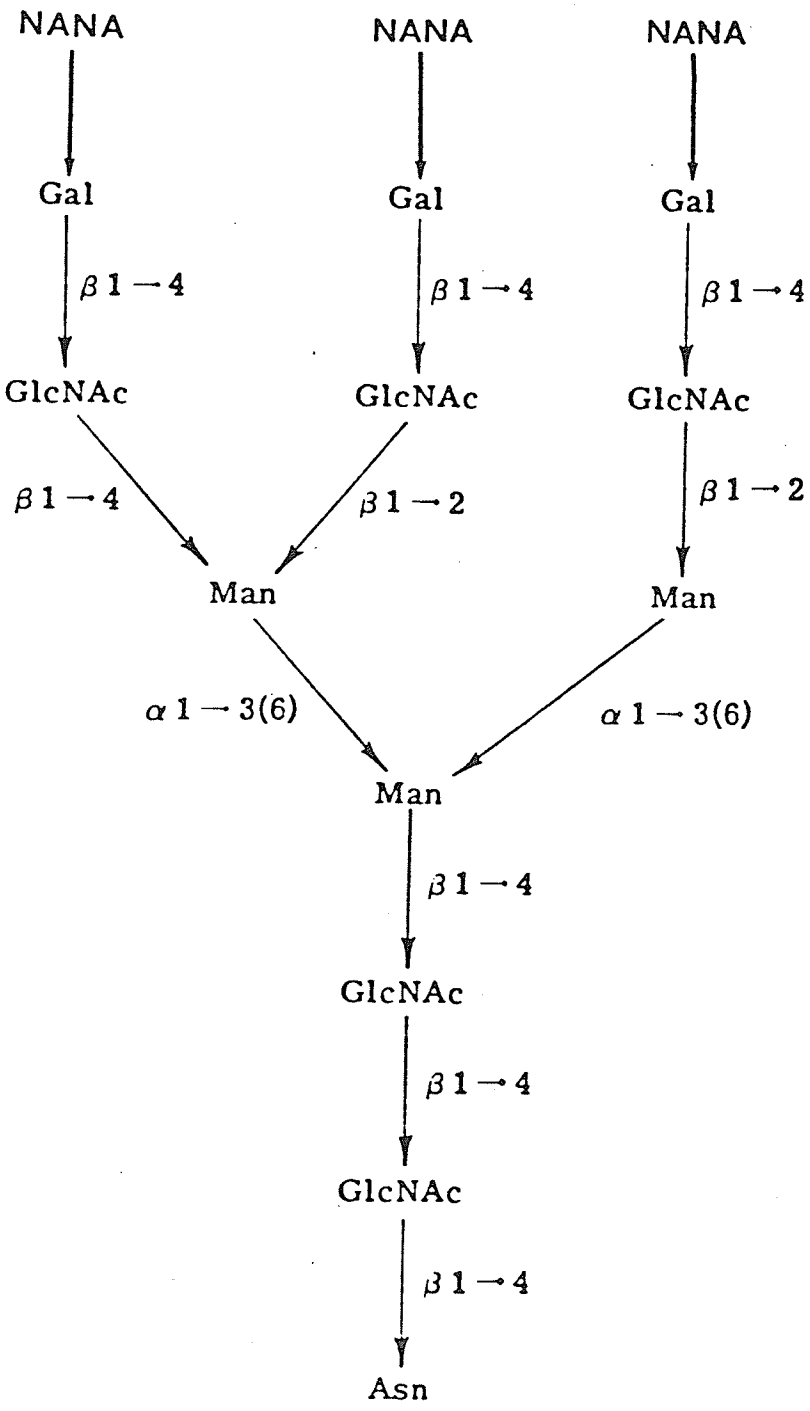
Carbohydrate analysis of serum glycoproteins indicates the presence of characteristic groups of sugars; the neutral sugars are usually D-galactose, D-mannose and the deoxy sugar, L-fucose; the amino sugars are D-glucosamine and D-galactosamine, usually as their N-acetylated derivatives; and the acidic sugars are the sialic acids, a term used to describe the various derivatives of neuraminic acid (N-acetyl, N-glycolyl, N-acetyl-O-diacetyl, and N,O-diacetyl); in a few cases D-glucose, D-xylose and L-arabinose have been found. In the serum type of glycoprotein, carbohydrate chains are attached to the polypeptide in the form of oligosaccharide units linked to polypeptide via a glycosylamine bond involving the reducing group of N-acetylglucosamine and the amide group of asparagine. The number of carbohydrate chains is quite variable and can range from 2 for transferrin (30), 5 for human $\alpha_1$-acid glycoprotein (31), up to as many as 31 for human $\alpha_2$-macroglobulin (32). The sequence of the oligosaccharide units has been determined in some cases by enzymic degradation of a glycopeptide derived from the glycoprotein utilizing purified glycosidases. These studies have indicated that sialic acid or occasionally fucose occupied terminal positions on the carbohydrate chains and there is a characteristic terminal trisaccharide sequence -N-acetylglucosamine-galactose-sialic acid with perhaps 2 or 3 such trisaccharides attached

to an inner core of sugars consisting of mannose and N-acetylglucosamine (33). The precise structure and sequence of sugars in a carbohydrate chain has been elucidated for one serum glycoprotein and this chain is believed to be fairly representative of the carbohydrate chains of the serum type of glycoprotein (34) (see Fig. 2).

Biosynthesis of serum glycoproteins

The site of biosynthesis of serum glycoproteins was shown to be the liver by the classical work of Miller and his associates (35). These workers showed that the isolated perfused liver was capable of incorporating labelled amino acids into all electrophoretically separated fractions of serum except the γ-globulins. Subsequent work (36) has shown that the microsome fraction of liver was the subcellular site of synthesis of the proteins in question. Possible biosynthetic mechanisms include: (A) synthesis of the protein and carbohydrate units separately and then combination at the site of formation of the polypeptide (i.e. the polyribosomes), (B) incorporation of the carbohydrate groups after polypeptide has left the ribosome and is in the lumen of the endoplasmic reticulum, (C) a combination of the two possibilities, with part of the carbohydrate incorporated while the polypeptide is still attached to the ribosome and the remainder incorporated after release of the completed polypeptide from the ribosome. Serum glycoproteins appear to follow the second or third possibility as suggested by the "pipeline" scheme of Peters et al. (37) and Redman and Cherian (38). According to this scheme, sugars are added to the protein part of the molecule beginning at the ribosome and continuing as the protein passes through its intracellular route until

Fig. 2. Proposed structure for a glycopeptide isolated from human $\alpha_1$-acid glycoprotein (34).


NANA
NANA
NANA
Gal
Gal
Gal
β 1 → 4
β 1 → 4
β 1 → 4
GlcNAc
GlcNAc
GlcNAc
β 1 → 4
β 1 → 2
β 1 → 2
Man
Man
α 1 → 3(6)
α 1 → 3(6)
Man
β 1 → 4
GlcNAc
β 1 → 4
GlcNAc
β 1 → 4
Asn

it is secreted from the cell. Although the first sugar may be added as a single transfer, there is evidence that the inner core may be added as a block with the sugars in the terminal triplet added in a stepwise manner (39). The precise mechanism by which the chains are built up is not clear, but it seems that the first sugar, usually N-acetylglucosamine, is added to the incomplete polypeptide during assembly in association with rough endoplasmic reticulum, subsequent sugars are added during secretion via the channels of the endoplasmic reticulum, and terminal N-acetylneuraminic acid is attached mainly in the Golgi complex (Fig. 3). The synthesis of the oligosaccharide units is controlled by the presence of appropriate glycosyltransferases which transfer sugar molecules from nucleotide sugar derivatives to suitable glycoprotein acceptors. The sugar nucleotides can all be derived from glucose; the pathway of their metabolism is shown in Figs. 4 and 5 (40). The attachment of the first N-acetylglucosamine residue to polypeptide is not believed to be coded for by RNA but seems to be fixed by the amino acid sequence of the polypeptide backbone of the glycoprotein. The amino acid sequence which codes for the attachment of carbohydrate to polypeptide has recently been referred to as the sequon (41). The sequon for the serum type of glycoprotein is believed to be Asn-X-Ser(Thr), a sequence which has been found in the linkage region of many serum glycoproteins. The nature of X appears to be unimportant as a variety of amino acids have been shown to occupy the intermediate position between asparagine and serine or threonine in the sequon (41).

Introduction to the present work

The work presented in this thesis forms part of a study on the

Fig. 3

SERUM GLYCOPROTEIN SYNTHESIS AND SECRETION IN LIVER
INVOLVING RER→SER→GOLGI PIPELINE

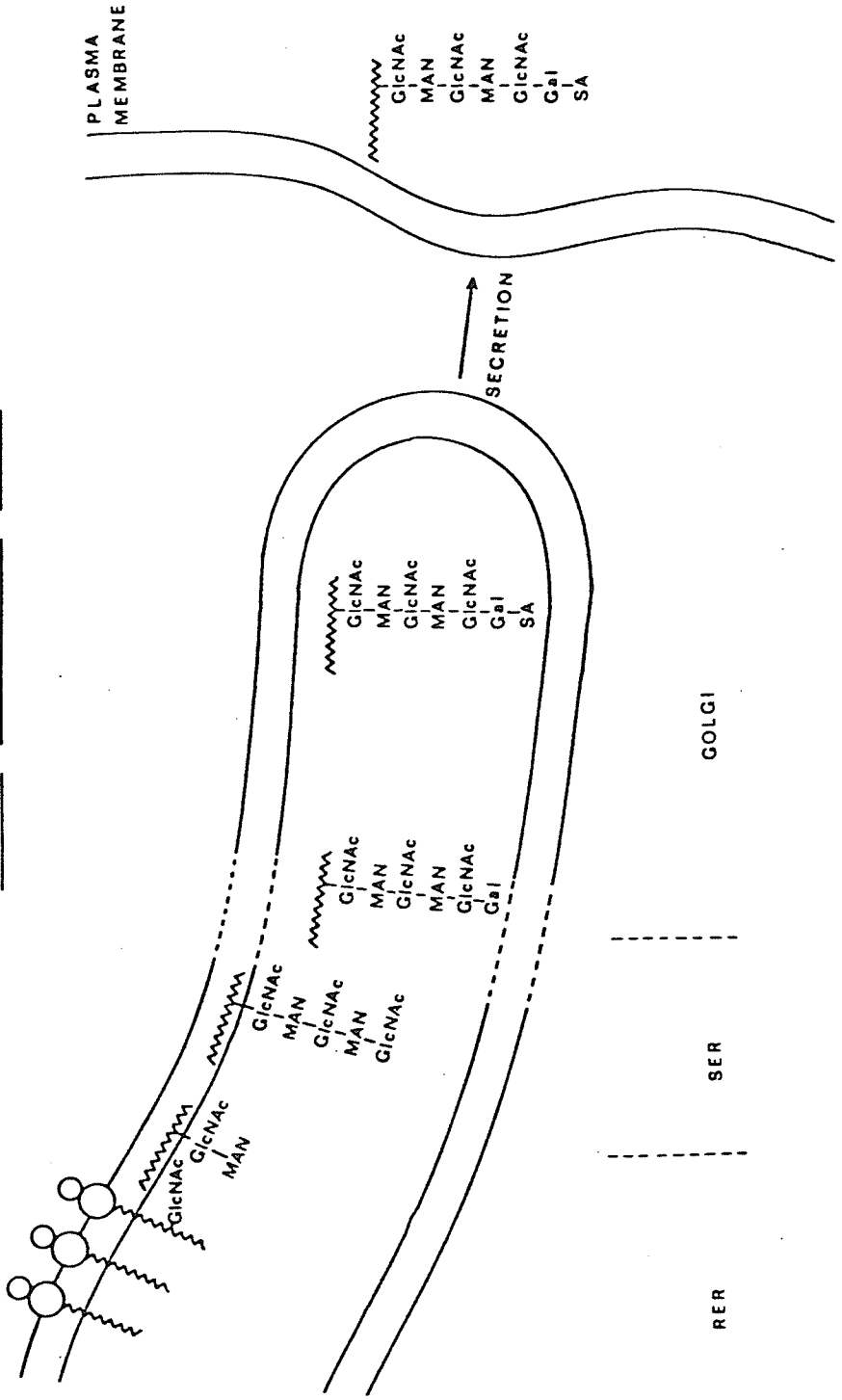
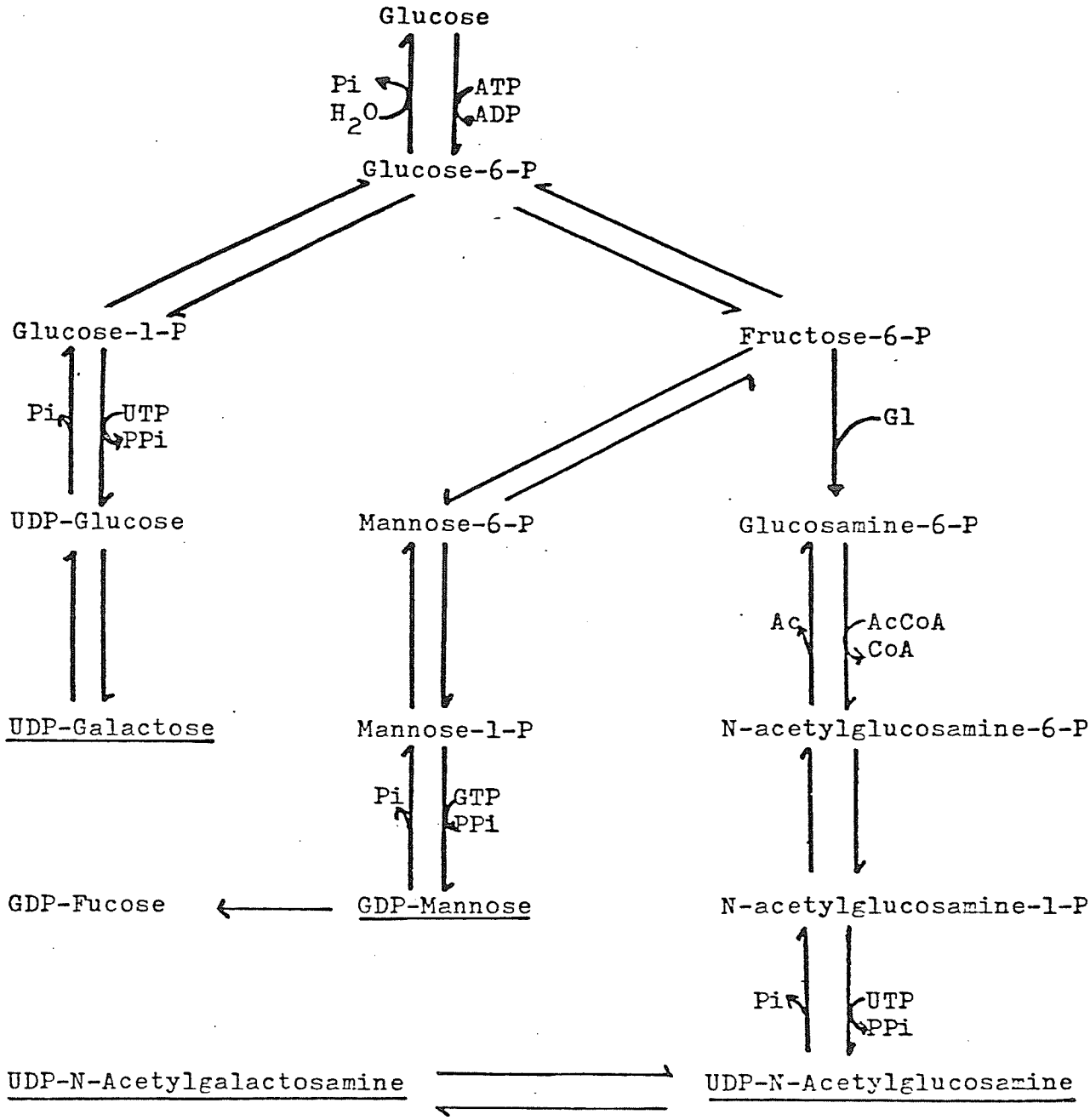
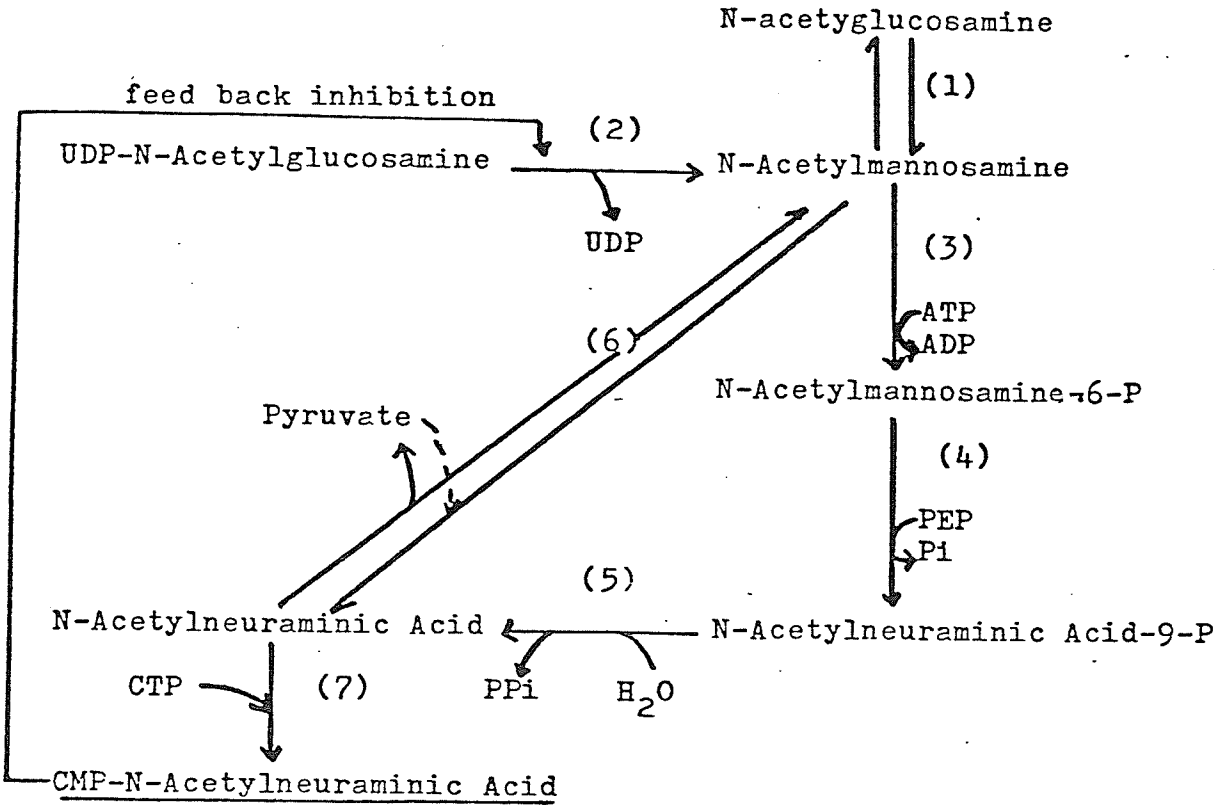


Fig. 4. Pathways for biosynthesis of nucleotide derivatives of sugars found in glycoproteins




| | |
|---|---|
| AcCoA | acetylcoenzyme A |
| CoA | coenzyme A |
| Pi | inorganic phosphate |
| PPi | inorganic pyrophosphate |
| Gl | glutamine |
| PeP | phosphoenol pyruvate |




Enzymes:

(1) N-acetylglucosamine-2-epimerase
(2) UDP-N-acelylglucosamine -2-epimerase
(3) N-acetylmannosamine kinase
(4) N-acetylneuraminate-9-phosphate synthetase
(5) N-acetylneuraminic acid-9-phosphatase
(6) N-acetylneuraminate pyruvate-lyase
(7) CMP-sialic acid synthetase

Fig. 5. Biosynthetic pathways of nucleotide derivatives of sugars found in glycoproteins

control of biosynthesis and catabolism of a specific acute phase reactant isolated from rat serum (14, 42, 43), $\alpha_1$-acid glycoprotein. This protein was found to increase in content in serum by about 6-fold at 48-72 h after induction of experimental inflammation (14). The protein was characterized with respect to its chemical, physical and immunological properties (43) and was found to have similar properties to those of human $\alpha_1$-acid glycoprotein, a major acute phase reactant in human serum for which no known function has yet been established.

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 (21). Subsequent quantitative studies revealed that the content of $\alpha_1$-acid glycoprotein associated with the microsome fraction was approximately 4-fold greater in animals suffering from induced inflammation (21). 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 (21). 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 (44). Thus, this protein appeared to utilize the pipeline scheme described earlier for the secretion of proteins from cells (Fig. 3).

Although the above biosynthetic studies explain the increase in $\alpha_1$-acid glycoprotein in response to inflammation, the increase could also be explained, at least in part, by diminished catabolism of $\alpha_1$-acid glycoprotein.

Therefore, as part of a study on this aspect of the overall work, part of my studies was aimed at determining the effect of inflammation on some enzymes that were likely to be involved in glycoprotein catabolism. Sialic acid-free serum glycoproteins have been shown to bind to liver and subsequently appear associated with liver lysosomes where they are finally degraded by lysosomal glycosidases and proteases (45). It is believed that this mechanism represents the main pathway for catabolism of serum glycoproteins. In order to determine if changes in catabolism of glycoproteins might be occurring during inflammation, my studies were aimed at determining the effect of inflammation on two key liver glycosidases - galactosidase and N-acetylglucosaminidase. These two enzymes were chosen because they have been well studied lysosomal glycosidases about which information on chemical and physical properties was available. Also, in view of the relatively high contents of galactose and N-acetylglucosamine in serum glycoproteins, it was likely that of all the glycosidases present in lysosomes those were likely to be the most important enzymes involved in the catabolism of the carbohydrate chains of glycoproteins taken up from the circulation.

The second part of the work described in this thesis was concerned with enzymes involved in biosynthesis of carbohydrate chains of glycoproteins. The *in vivo* studies described above provided much information on the biosynthesis of $\alpha_1$-acid glycoprotein. However, for the studies undertaken in this thesis, a cell free system was chosen over an *in vivo* system in order to exclude specific and non-specific regulatory effects which could interfere with the biosynthetic and catabolic processes under study.

Part of the aim of the studies presented in this thesis was to determine the effect of induced inflammation on factors involved in the biosynthesis of $\alpha_1$-acid glycoprotein. Therefore, the effect of induced inflammation on sialyltransferase and galactosyltransferase from rat liver microsome fractions was determined. Also, since the capacity for synthesis of $\alpha_1$-acid glycoprotein was found to be greater in animals suffering from induced inflammation (23), it was proposed to isolate nascent polypeptides from polyribosome preparations from experimental animals. These nascent polypeptides were to be used as acceptors for N-acetylglucosamine in order to determine whether two different enzymes were required for the transfer of N-acetylglucosamine to acceptor molecules, one with the specificity for the polypeptide (initial glycosylation) and the other with the specificity for the oligosaccharide (glycosylation in the core region or the terminal trisaccharide).

# EXPERIMENTAL

## Materials

### Radioactive Compounds

Cytidine 5'-monophospho[$^{14}$C]sialic acid (260 mCi/mmole), uridine diphospho-N-acetyl-D-[U-$^{14}$C]glucosamine (269 mCi/mmole), uridine diphospho-D-[U-$^{14}$C]galactose (272 mCi/mmole), N-[1-$^{14}$C]hexadecane, Amersham Searle, Toronto, Ontario.

### Chemicals for liquid scintillation counting

2,5-Diphenyloxazole (PPO) and 1,4-bis-2-(5-phenyloxazolyl) benzene (POPOP), Packard Instrument Co., Inc., Illinois; Bio-Solv Solubilizer (BBS-3), Beckman Instruments Inc., Toronto, Ontario.

### Proteins

Bovine serum albumin (crystalline), pyruvate kinase (Type II: Crystalline), Sigma Chemical Co., St. Louis, Mo. Rat $\alpha_1$-acid glycoprotein was prepared as previously described (43).

### Sugars, nucleotide sugars, and other sugar derivatives

N-Acetylneuraminic acid (Type IV), D(+) galactose, uridine 5'-diphosphogalactose (sodium salt), uridine 5'-diphospho-N-acetylglucosamine (sodium salt), p-nitrophenyl-N-acetyl-β-D-glucosaminide, p-nitrophenyl-β-D-galactoside, Sigma Chemical Co., St. Louis, Mo. CMP-NANA was prepared according to the method described by van den Eijinden and van Dijk (46); CMP-NANA synthetase was prepared from calf brain. This enzyme was incubated with CTP and NANA and the resulting CMP-NANA was isolated by preparative paper chromatography using $^{14}$C-labelled CMP-NANA as a marker.

Nucleotides

Adenosine 5'-triphosphate (crystalline disodium salt), cytidine 5'-triphosphate (type I: Sodium salt), guanosine 5'-triphosphate (type I: sodium salt), Sigma Chemical Co., St. Louis, Mo.

Chromatographic media

Sephadex G-200, DEAE-Sephadex A-50, Pharmacia, Uppsala, Sweden.

Other chemicals were obtained as follows: Trizma Base, glycine (crystalline), phosphoenol pyruvic acid (trisodium salt), dithiothreitol, 2[N-morpholino]ethane sulfonic acid (MES), Triton X-100, deoxycholic acid (sodium salt), CDP-choline, Sigma Chemical Co., St. Louis, Mo.; puromycin dihydrochloride, N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid (HEPES), Nutritional Biochemical Corporation, Cleveland, Ohio; Turpentine oil (double rectified), Fisher Scientific Co., Toronto, Ontario. Other chemicals were of analytical reagent grade obtained from local suppliers.

Physical and chemical methods

Absorbances in the visible region of the spectrum were measured with a Gilford 2400-2 spectrophotometer. Measurements of pH were made with a Radiometer model 28b pH meter. Radioactivity was determined with a Packard model 3003 Tri-carb liquid scintillation spectrometer. For measurement of radioactivity for galactosyltransferase (see below) and N-acetylglucosaminyltransferase assays (see below), aqueous solutions of protein (up to 0.5 ml and 3 mg protein) were added to 10 ml of scintillation cocktail containing 0.7% PPO, 0.036% POPOP, 10 ml BBS-3

and 90 ml toluene (42). For measurement of radioactivity for sialyltransferase assays (see below), 4 x 4 cm strips of Whatman 3 MM paper (containing up to 1 mg protein) were added to 15 ml of scintillation cocktail described above. The green channel was set at a pulse height of 100-1000 divisions (4.5% gain) to count $^{14}C$. The efficiency, as determined with standard $^{14}C$-hexadecane, was 55% for $^{14}C$ in the green channel. Quenching was tested for using the automatic external standard of the machine. With few exceptions the standard deviation of the net count rate was not greater than $\pm$ 5%.

Protein was assayed as described by Lowry et al. (47), but with modified reagents and volumes described by Miller (48). Crystalline bovine serum albumin was used as the standard.

Isolation of β-galactosidase from jack bean meal

The isolation of β-galactosidase from jack bean meal was based on a method described by Li and Li (49). A suspension of jack bean meal in 3 litres distilled water was stirred at room temperature for 1 h. The suspension was strained through cheesecloth and the turbid filtrate adjusted to pH 5.5 at room temperature with 1.5 M sodium citrate buffer, pH 2.7. The solution was then centrifuged at 5000 r.p.m. (4080 $g_{av}$) for 30 min in the Sorvall RC-2B fitted with the GSA rotor. The volume of the supernatant was measured and solid ammonium sulfate was added to 30% saturation. This solution was allowed to stand for 2 h and the precipitate formed was removed by centrifugation as above. The volume of the supernatant was measured and solid ammonium sulfate was added to 60% saturation final concentration. The solution was allowed to stand

overnight at 4°, then centrifuged as aboye. The supernatant was aspirated and discarded; the precipitate was dissolved in a total volume of 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° and centrifuged at -10° as above to remove the precipitate. The supernatant was warmed to room temperature and adjusted to pH 4.9 by addition of 1.5 M sodium citrate buffer, pH 2.7. The mixture was cooled to -10° and kept at this temperature overnight. The mixture was then centrifuged at -10° as aboye to collect the precipitate. The precipitate was dissolved in 20 ml 0.1 M sodium phosphate buffer, pH 7.0. This was the β-N-acetylglucosaminidase-rich fraction containing some β-galactosidase. The β-N-acetylglucosaminidase-rich fraction was applied to a Sephadex G-200 column (30 x 5 cm) which had been 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° overnight. The precipitate, obtained by centrifugation as previously described, was dissolved in 10 ml 0.05 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 DEAE-Sephadex A-50 column (30 x 5 cm) 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, concentrated and stored at -20° until required. This fraction contained 138

units of enzyme activity per ml (see below). It did not contain any α-mannosidase or β-N-acetylglucosaminidase activity as determined using p-nitrophenyl glycosides as substrates (see below).

Preparation of sialic acid-free and sialic acid-galactose-free $\alpha_1$-acid glycoprotein

For the preparation of sialic acid-free $\alpha_1$-acid glycoprotein, 35 mg of rat $\alpha_1$-acid glycoprotein in 6.0 ml 0.05 N $H_2SO_4$ was heated in a glass-stoppered tube at 90° for 60 min. The hydrolysate was dialyzed overnight against 2 x 500 volumes of distilled water, and then freeze-dried.

For the preparation of sialic acid-galactose-free $\alpha_1$-acid glycoprotein, 20 mg of sialic acid-free $\alpha_1$-acid glycoprotein, prepared as above, was dissolved in 6.0 ml 0.05 M sodium citrate buffer, pH 3.5, containing 800 units of β-galactosidase isolated from jack bean meal (see above). The solution was incubated at 37° for 48 h. The release of galactose was determined quantitatively by the method of Gordon et al. (50). 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 18 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 4.5 h. The chromatogram was dried and sprayed with 0.1 M 2-aminobiphenyl hydrogen oxalate to detect the galactose, which appeared as yellow-brown spots. These spots were cut out, eluted with 5 ml methanol, and the absorbances read at 400 nm.

Subcellular fractionation

Male hooded rats of 200-250 g body weight were obtained locally and

were maintained on a diet of Purina Laboratory Chow and tap water and were starved for 16 h prior to sacrifice. Inflammation was induced by subcutaneous injection of 0.5 ml oil of turpentine per 100 g body weight as previously described (44). Rats used as controls received injections of sterile 0.15 M NaCl. At appropriate times rats were killed by severing the jugular veins and livers were rapidly transferred to ice-cold 0.25 M sucrose. Unless otherwise stated, a Beckman L5-50 ultracentrifuge equipped with Ti-60, Ti-50 and SW27.1 rotors was used for all subcellular fractionation procedures. All procedures were performed at 0-4°.

For experiments in which distribution of glycosidase activities in subcellular fractions was determined, total liver homogenates were prepared by homogenizing 10 g liver with 30 ml 0.25 M sucrose using five strokes of a Potter-Elvehjem homogenizer as described by Jamieson and Ashton (21). Subcellular fractions were prepared by a method based on that described by Touster et al. (51). Nuclear fractions were prepared by centrifuging at 2000 r.p.m. (500 $g_{av}$) for 10 min in the Sorvall RC-2B fitted with the SS-34 rotor. A maximum amount of supernatant was removed and a lysosomal-enriched fraction was prepared by centrifuging at 22000 r.p.m. (33000 $g_{av}$) for 7.5 min in the Ti-60 rotor. Two-thirds of the supernatant were removed and microsome and supernatant fractions were prepared by centrifuging at 40000 r.p.m. (112000 $g_{av}$) for 90 min in the Ti-60 rotor. Nuclear and lysosomal-enriched fractions were washed by resuspension in 20 ml and 10 ml 0.25 M sucrose, respectively, and centrifuging as above. Nuclear, lysosomal-enriched and microsome fractions were resuspended in 20 ml, 10 ml and 5 ml 0.25 M sucrose, respectively,

and stored at -20° until required.

For the preparation of a microsome fraction for assay of sialyl-, galactosyl- and N-acetylglucosaminyl-transferase activities, 5 g liver were homogenized with 25 ml 0.25 M sucrose using eight strokes of a Potter-Elvehjem homogenizer as before (21). The homogenate was centrifuged at 13500 r.p.m. (10000 $g_{av}$) for 20 min in the Ti-60 rotor. The supernatant was aspirated and centrifuged at 40000 r.p.m. (112000 $g_{av}$) for 90 min in the Ti-60 rotor. The soluble cell sap fraction was decanted and the pellet of microsome material was resuspended in 1.5 ml 0.25 M sucrose.

The preparation of a rough endoplasmic reticulum fraction for N-acetylglucosaminyltransferase assay was performed by a method based on that described by Palamarczyk and Hemming (52). Total liver homogenate was prepared by homogenizing 5 g of rat liver from a 24 h experimental animal in 25 ml 0.25 M sucrose in medium A (50 mM Tris-maleate buffer, pH 7.1, containing 5 mM $MgCl_2$) and centrifuged at 13500 r.p.m. (10000 $g_{av}$) for 20 min in the Ti-60 rotor. The supernatant was aspirated and 15 ml volumes were layered over 15 ml 1.3 M sucrose in medium A and centrifuged at 30000 r.p.m. (63500 $g_{av}$) for 3.5 h in the SW 27.1 rotor. The pellet was resuspended in 0.4 M NaCl in medium B (50 mM Tris-maleate, pH 7.4, containing 5 mM $MgCl_2$) and recovered by centrifuging at 40000 r.p.m. (112000 $g_{av}$) for 1.5 h in the Ti-60 rotor. The pellet was then suspended in a buffer containing 50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$ and 0.1 mM dithiothreitol (TKMD buffer) to a final protein concentration of 50 mg/ml.

The preparation of free and membrane-bound polyribosomes was per-

formed by a method based on that described by Ikehara and Pitot (53). Total liver homogenate was prepared by homogenizing 40 g of liver in two volumes 0.44 M sucrose in medium C (50 mM Tris-HCl buffer, pH 7.4, containing 25 mM KCl and 10 mM $MgCl_2$) using six strokes of a Potter-Elvehjem homogenizer at 2000 r.p.m. The homogenate was centrifuged at 13500 r.p.m. (10000 $g_{av}$) for 10 min in the Ti-60 rotor. The upper two-thirds of the supernatant were removed, mixed with 1.47 volumes 2.0 M sucrose in medium C, and 20 ml volumes were layered on 10 ml 2.0 M sucrose and centrifuged at 42000 r.p.m. (120000 $g_{av}$) for 6 h in the Ti-60 rotor. The pellet consisted mainly of free polyribosomes. The rough endoplasmic reticulum fraction at the interface was aspirated and Triton X-100 was added to a final concentration of 0.5% to solubilize the membranes. The resulting solution was layered in 20 ml volumes on 10 ml 2.0 M sucrose in medium C and centrifuged at 42000 r.p.m. (120000 $g_{av}$) for 6 h in the Ti-60 rotor. The pellet consisted of polyribosomes which were released from membranes by detergent treatment; these will subsequently be referred to as bound polyribosomes. Each polyribosome preparation was washed with high salt solution consisting of 50 mM Tris-HCl, pH 7.4, containing 10 mM $MgCl_2$, 500 mM $NH_4Cl$ and 0.44 M sucrose, as described by Ikehara and Pitot (52) to remove contamination with non-specific protein. Each polyribosomal preparation was then suspended in TKMD buffer to a final protein concentration of 7.5 mg/ml.

Preparation of a dolichol phosphate-containing lipid fraction

A crude lipid fraction containing dolichol phosphate was prepared by a method based on that of Behrens and Leloir (54) and Folch et al. (55).

Porcine liver, 1.1 kg, was ground with 200 ml of acetone in a Waring blender, and filtered. The dry residue was extracted with 3 volumes of chloroform-methanol (2:1, v/v), and filtered. The extract was made 0.1 N with respect to NaOH, incubated at 37° for 15 min, and then neutralized by addition of HCl. The extract was then gently refluxed for 15 min, allowed to cool, and 0.2 volumes of distilled water were added. The mixture was mixed thoroughly and then allowed to separate into two phases. The upper phase was aspirated and the lower phase was collected. The solvent in the lower phase was removed by vacuum distillation at 37°. The crude lipid extract was stored at 4°.

Enzyme Assays

(1) Glycosidases

(a) β-galactosidase and β-N-acetylglucosaminidase

The assay for galactosidase contained: 52 mM citric acid-54 mM sodium dihydrogen phosphate buffer, pH 3.7; 2 mM p-nitrophenyl galactoside; 0.2 M KCl; 0.1% Triton X-100 (final concentration); 50 µℓ enzyme preparation (containing up to 1 mg protein depending on the liver fraction) in a final volume of 500 µℓ. The assay for N-acetylglucosaminidase was similar except that the buffer was 58 mM citric acid-41 mM sodium dihydrogen phosphate, pH 4.2, 2 mM p-nitrophenyl-N-acetylglucosaminide was the substrate and KCl was omitted. Incubations were normally carried out at 30° for 30 min for assay of galactosidase and 10 min for assay of N-acetylglucosaminidase. Both assays were stopped with 1.5 ml 0.2 M glycine adjusted to pH 9.5 with NaOH. Samples were centrifuged at 25000

r.p.m. (42000 $g_{av}$) for 30 min using the Ti-50 rotor in the Beckman L5-50 ultracentrifuge and optical densities of the supernatants determined at 400 nm. Any deviation from the above conditions is described in the Results section. In some control experiments up to 5% turpentine (v/v) was added to samples of total homogenates and lysosomal fractions prior to determination of enzyme activities. Mixing experiments were also performed in which volumes of total homogenates from control and 24 h experimental animals containing the same amount of protein were assayed for enzyme activities. Mixed lysosomal fractions from these homogenates were also assayed.

One unit of enzyme activity is defined as the amount of enzyme which hydrolyzes 1 μmole p-nitrophenyl glycoside per minute at 30°; the molar extinction coefficient of p-nitrophenyl under the assay conditions employed is $1.77 \times 10^4$ $M^{-1}$ $cm^{-1}$.

(2) Glycosyltransferases

All glycosyltransferase assays were carried out on the same day that the microsome fractions were prepared. Each assay was conducted under optimum conditions such that there was a linear relationship between product formation with respect to time and quantity of enzyme protein in the incubation media. All assays were done in duplicate to verify the results obtained. Control incubations were conducted on all samples in the absence of glycoprotein acceptor. Endogeneous radioactivity was subtracted in the calculation of enzyme activities.

(a) Sialyltransferase

The assay system for sialyltransferase was based on that described

by Schachter et al. (56). The incubation mixture contained: 250 µg sialic acid-free rat $\alpha_1$-acid glycoprotein; 5 nanomoles $^{14}$C-labelled CMP-sialic acid (50 nCi); 2.5 µmoles N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid buffer, pH 6.7; 1% Triton X-100 (final concentration); 1 mg of enzyme (microsome fraction) in a final volume of 100 µl. Incubations were carried out at 37° for 20 min. The reaction was stopped by adding 50 µl of 2% sodium tetraborate, and 75 µl aliquots were subjected to high voltage paper electrophoresis in 1% sodium tetraborate on Whatman No. 3 MM paper at 2000 volts for 90 min. The equipment used was a Savant model FP22A flat plate high voltage electrophoresis apparatus utilizing a Savant model HV3000A power supply. The protein-bound radioactive sialic acid remained at the origin and this segment of the paper was dried and counted.

(b) galactosyltransferase

The assay mixture was based on that described by Mookerjea et al. (57). The incubation mixture contained: 100 µg sialic acid-galactose-free rat $\alpha_1$-acid glycoprotein; 5 nanomoles UDP-$^{14}$C-galactose (50 nCi); 1.25 µmoles $MnCl_2$; 0.1 µmole dithiothreitol; 12.5 µmoles 2-(N-morpholino) ethane sulfonate buffer, pH 6.8; 0.4 µmoles CDP-choline; 1% Triton X-100 (final concentration); 1 mg of enzyme (microsome fraction) in a final volume of 200 µl. Incubations were carried out at 37° for 20 min. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid-5% phosphotungstic acid, and the protein was washed as described by Simkin and Jamieson (58). Suitable volumes were removed for the determination of protein and radioactivity.

(c) N-acetylglucosaminyltransferase

(i) Use of polyribosome preparations as acceptors for N-acetylglucosamine

Free and bound polyribosomes were prepared as previously described (see Experimental). The incubation mixture was based on that of Letts and Schachter (59). The incubation mixture contained: 0.5-1.5 mg of ribosomal protein preparation (consisting of polyribosomes with attached nascent chains); 0.2 nanomoles $^{14}$C-labelled UDP-N-acetylglucosamine (50 nCi); 1.0 mg enzyme protein (microsome fraction-prepared as previously described from a 24 h experimental rat, see Experimental); 0.4 µmoles CDP-choline; 1% Triton X-100 (final concentration); TKMD buffer (50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$ and 0.1 mM dithiothreitol) in a final volume of 380 µl. Incubations were carried out at 37° for 20 min. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid 5% phosphotungstic acid, and the protein was washed as described by Simkin and Jamieson (58). Suitable volumes were removed for the determination of protein and radioactivity.

(ii) Use of polyribosome preparations in presence of puromycin as acceptors for N-acetylglucosamine

Free and bound polyribosomes were prepared as previously described (see Experimental). The incubation mixture contained: 5.0 mg of ribosomal protein in TKMD buffer, pH 7.4 (see above) (0.5 ml); 1.0 nanomole UDP-N-acetyl-$^{14}$C-glucosamine (250 nCi); 9.5 mg of enzyme protein (microsome fraction-prepared from a 24 h experimental rat, see Experimental); 1% Triton X-100, (final concentration); 10 mM phosphoenol pyruvate; 10 mM pyruvate kinase; 1 mM ATP; 0.025 mM GTP and 0.4 µmoles

CDP-choline in a final volume of 2.0 ml. Incubations were carried out at 37° for 60 min. Aliquots of 0.3 ml were removed at 5, 10, 15, 30 and 60 min and the reaction stopped in these samples by the addition of 0.3 ml 10% trichloroacetic acid-5% phosphotungstic acid. After the 15 min sample was removed, the incubation mixture was divided in two and puromycin was added to one of these tubes to a final concentration of 1 mM, and incubation was continued. Aliquots of 0.3 ml were then taken after a further 15 and 30 min after addition of puromycin. The protein was washed as described by Simkin and Jamieson (58). Suitable volumes were removed for the determination of protein and radioactivity.

(iii) Use of a rough endoplasmic reticulum preparation in presence of exogenous lipid and detergent as an acceptor for N-acetylglucosamine

A rough endoplasmic reticulum fraction, free of β-N-acetylglucosaminidase activity, and a crude lipid fraction, containing dolichol phosphate, were prepared as previously described (see Experimental). The incubation medium contained: 1.2 mg rough endoplasmic reticulum protein; 0.2 nanomoles UDP-N-acetyl-$^{14}$C-glucosamine (50 nCi); 0.4 μmoles CDP-choline; 10 mM pyruvate kinase; 10 mM phosphoenol pyruvate; 1 mM ATP; 0.025 mM GTP; 1 mM puromycin; 0.2% deoxycholate (sodium salt), (final concentration); 25 μl of crude lipid in a final volume of 300 μl. Incubations were performed at 37° for 30 min. The reaction was stopped by the addition of 0.3 ml 10% trichloroacetic acid-5% phosphotungstic acid, and the protein was washed according to the method described by Simkin and Jamieson (58). Appropriate volumes were removed for protein and radioactivity determinations.

## RESULTS

### The effect of inflammation on rat liver β-galactosidase and β-N-acetylglucosaminidase

The pH optima for β-galactosidase and β-N-acetylglucosaminidase in control liver homogenates were found to be 3.7 and 4.2, respectively, as shown in Fig. 6. Assays were performed in the presence of Triton X-100 to disrupt lysosomal material (60). β-Galactosidase was assayed in the presence of potassium chloride which has a stabilizing effect on the enzyme (61). β-N-Acetylglucosaminidase did not require potassium chloride for stability under the assay conditions used. Linear relationships were established between enzyme activities and amount of enzyme protein (Fig. 7); reactions were also linear up to 60 min incubation (Fig. 8).

The effect of experimental inflammation on total activities of β-galactosidase and β-N-acetylglucosaminidase in liver homogenates is shown in Fig. 9. In order to construct Fig. 9 units of enzyme activity were determined per gram wet weight liver under optimum conditions of pH and temperature and all values were then expressed in terms of control livers which were assigned 100% enzyme activity. There was little or no change in liver weight during experimental inflammation. In order to determine whether the decrease in enzyme activities found after induced inflammation (Fig. 9) might be due to a shift in the pH optima of the enzymes, the pH optima of β-galactosidase and β-N-acetylglucosaminidase in 24 h experimental liver homogenates was determined. As shown in Fig. 10,

Fig. 6. pH activity curves for (a) β-N-acetylglucosaminidase and (b) β-galactosidase from control rat liver homogenates. Typical specific activities corresponding to 100% maximal activities were 8.2 units/mg for β-N-acetylglucosaminidase and 1.4 units/mg for β-galactosidase. Results represent mean values from 4-6 experiments. Reproducibility was within $\pm$ 10%.

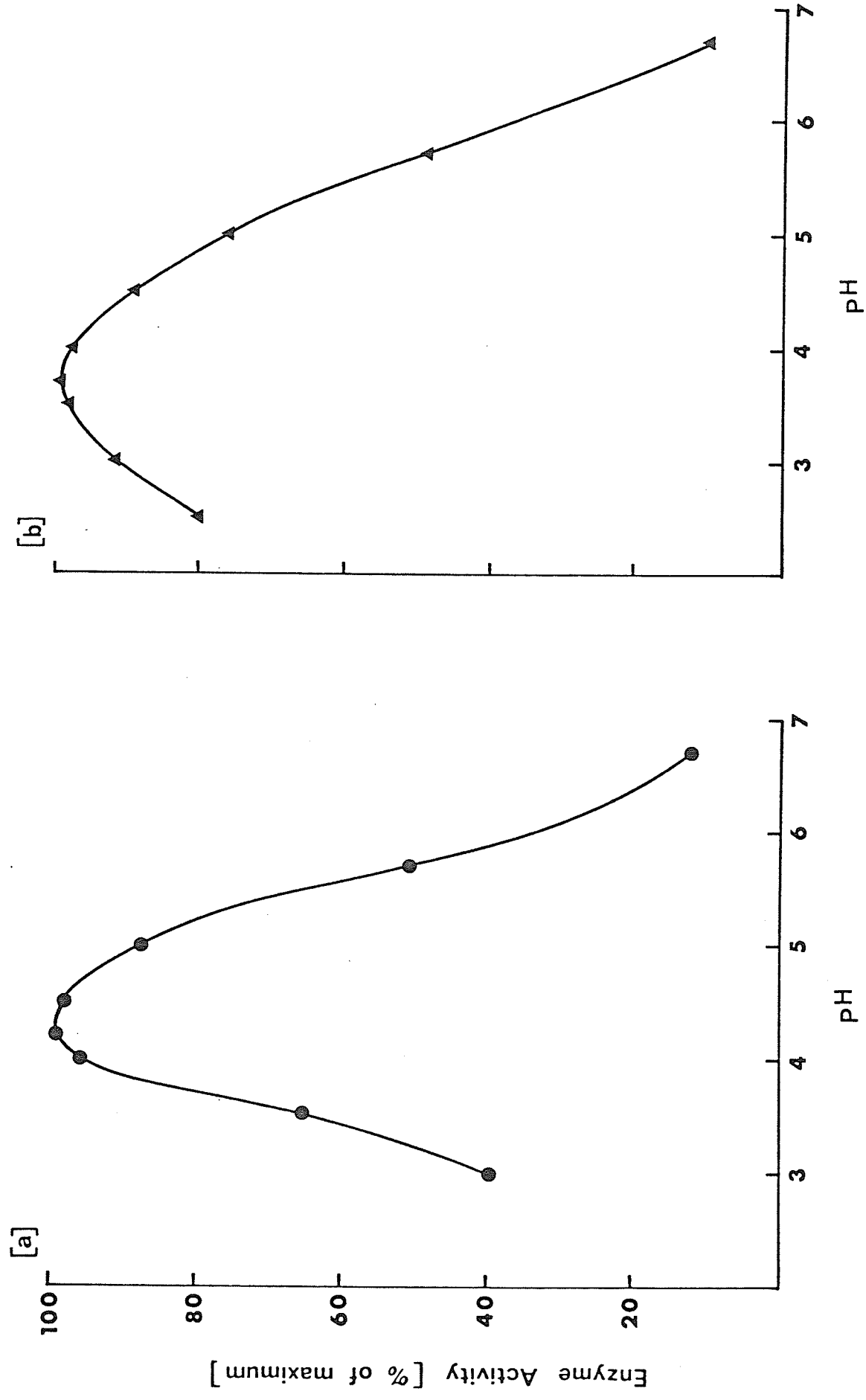

Enzyme Activity [% of maximum]
100
80
60
40
20
[a]
3
4
5
6
7
pH
[b]
3
4
5
6
7
pH

Fig. 7. Effect of amount of protein on rat liver homogenate β-N-acetylglucosaminidase (-●-) and β-galactosidase (-▲-) activities. Results represent mean values from 4-6 experiments. Reproducibility was within ± 10%.

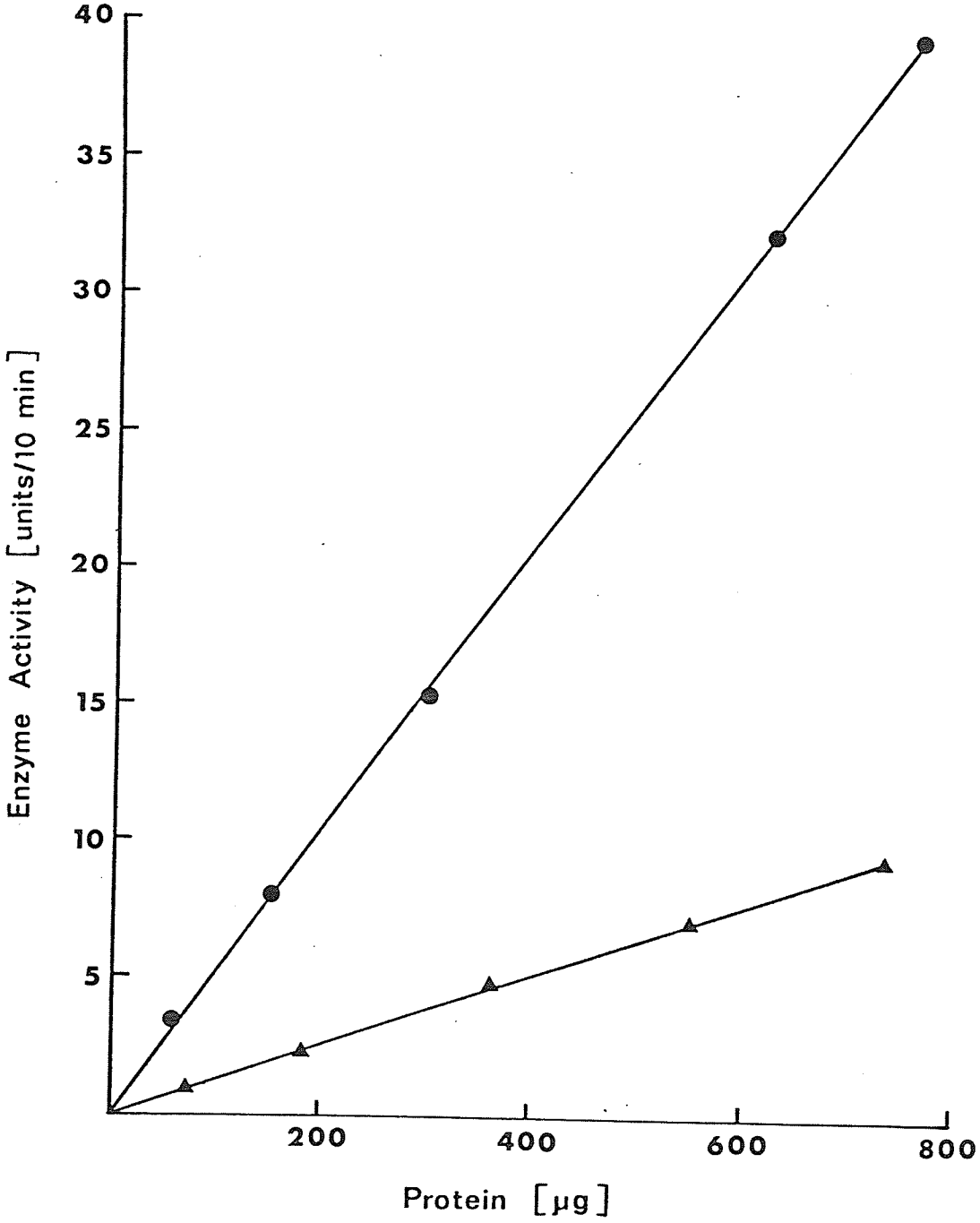

40
35
30
25
20
15
10
5
Enzyme Activity [units/10 min]
200
400
600
800
Protein [µg]

Fig. 8. Demonstration of linearity of rat liver homogenate β-N-acetyl-glucosaminidase (-●-) and β-galactosidase (-▲-) reactions. Results represent mean values from 4-6 experiments. Reproducibility was within ± 10%.

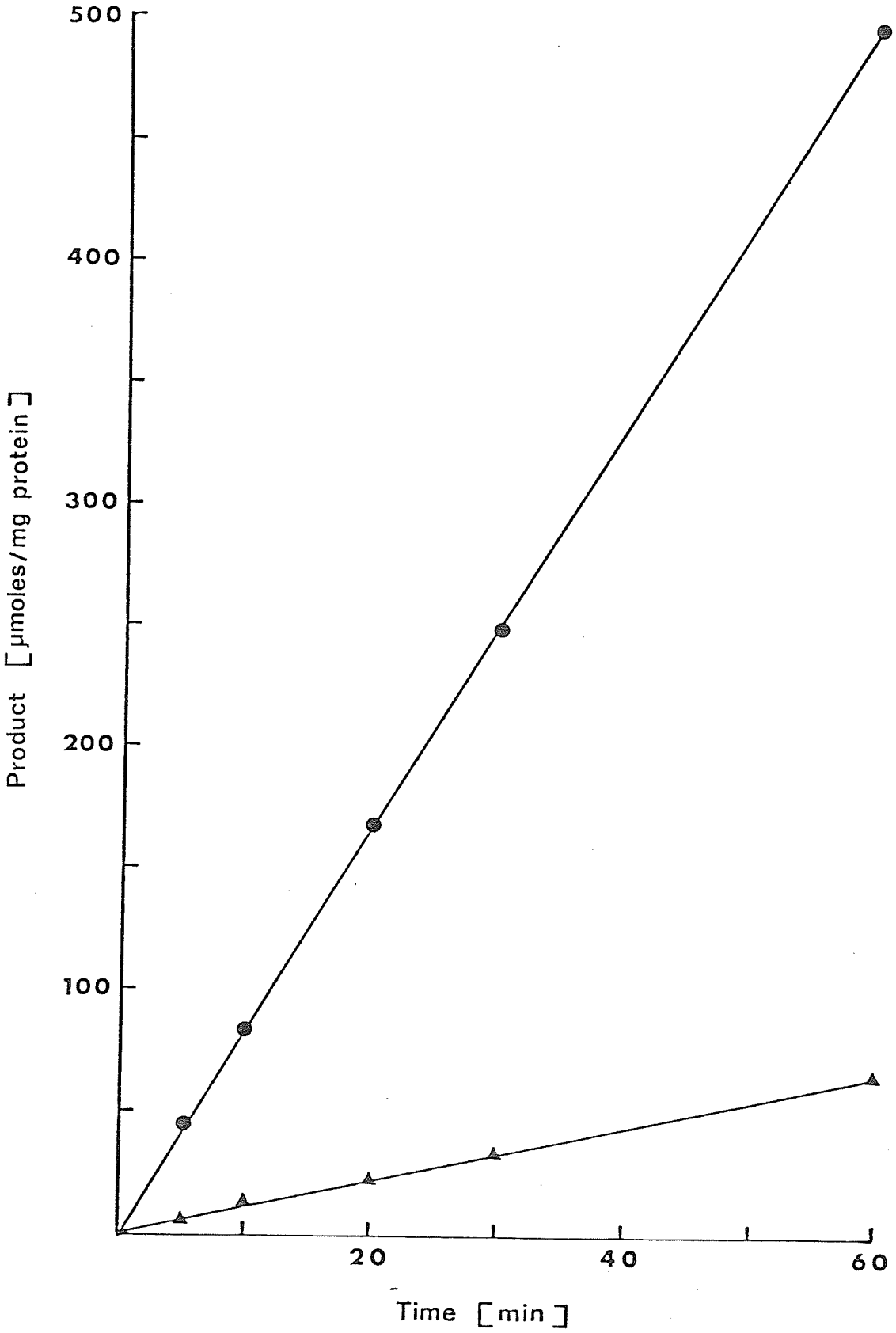

500
400
300
200
100
Product [µmoles/mg protein]
20
40
60
Time [min]

Fig. 9. Effect of inflammation on the activities of β-N-acetylglucosaminidase (-●-) and β-galactosidase (-▲-) in rat liver homogenates. Controls were assigned 100% activities (see Results for details). Typical specific activities for control homogenates were 8.2 units/mg protein for β-N-acetylglucosaminidase and 1.4 units/mg protein for β-galactosidase. Results represent mean values from 4-6 experiments. Reproducibility was within ± 10%.

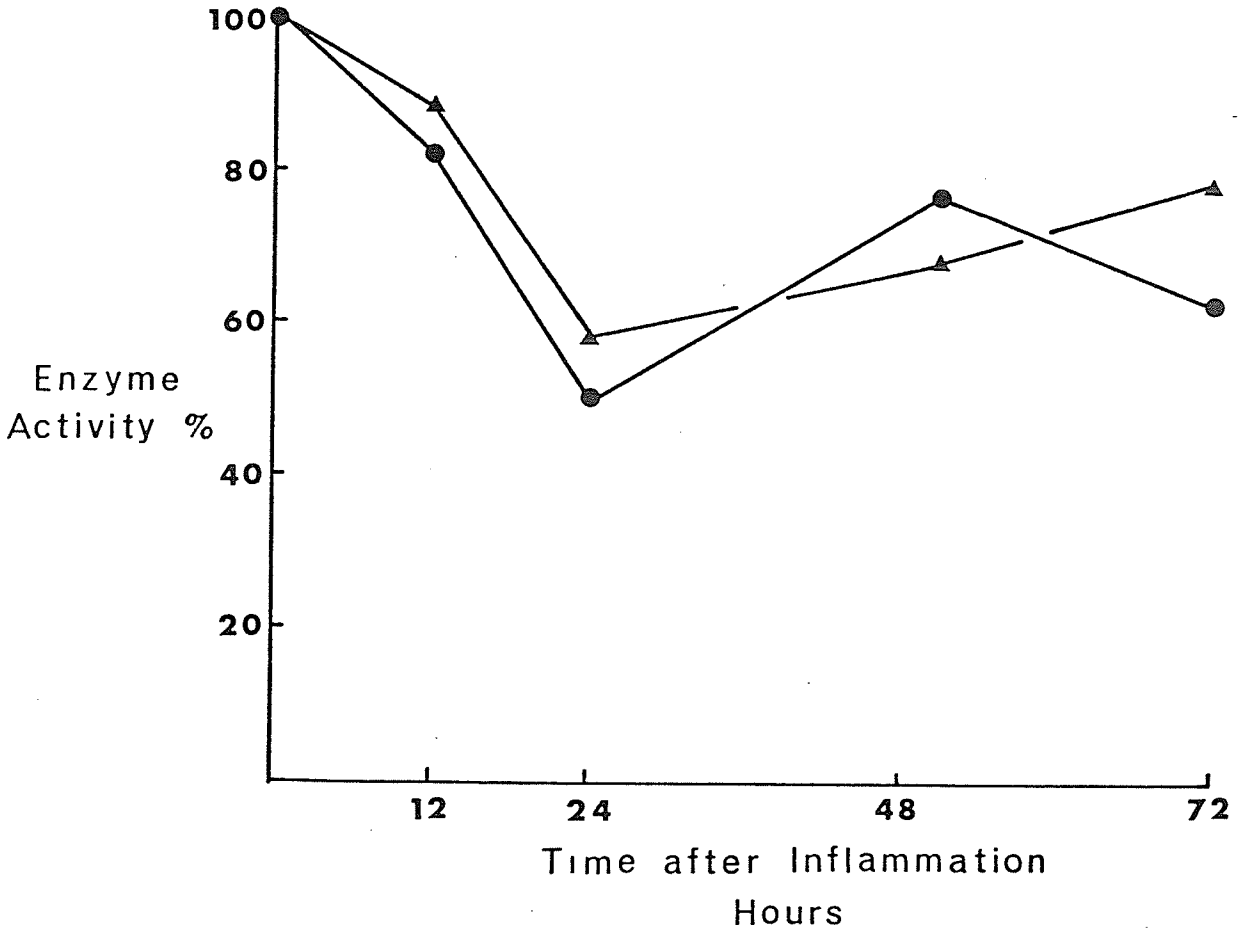

100
80
60
40
20
Enzyme
Activity %
12
24
48
72
Time after Inflammation
Hours

Fig. 10. pH activity curves for (a) β-N-acetylglucosaminidase and (b) β-galactosidase in 24 h experimental rat liver homogenates. Typical specific activities corresponding to 100% maximal activities were 4.1 units/mg protein for β-N-acetylglucosaminidase and 0.85 units/mg protein for β-galactosidase. Results represent mean values from 4-6 experiments. Reproducibility was within $\pm$ 10%.

Enzyme Activity [% of maximum]
100
80
60
40
20
[a]
3
4
5
6
7
pH
[b]
3
4
5
6
7
pH

no apparent change in the pH optima of the enzymes occurred as a result of inflammation. There was also no change in the linear relationship earlier established with respect to time and amount of enzyme protein.

Table 2 shows the distribution of β-galactosidase and β-N-acetylglucosaminidase in liver subcellular fractions from control and 24 h experimental animals. As shown in Table 2, the lysosomal-enriched fraction accounted for the bulk of both glycosidase activities in control and 24 h experimental livers. Table 3 shows the specific activities of both glycosidases in liver subcellular fractions from control and 24 h experimental animals. All subcellular fractions from control livers showed greater specific activities than their counterparts from 24 h experimental livers.

Double reciprocal plots for β-galactosidase and β-N-acetylglucosaminidase are shown in Figs. 11 and 12, respectively, for control and 24 h experimental liver homogenates. It is clear that inflammation resulted in a significant reduction in the magnitude of $V_{max}$ for both enzyme activities, but did not markedly affect values for $K_m$ (Figs. 11-12; Table 4). As shown in Table 4, values for $K_m$ for both enzymes in subcellular fractions were similar to values obtained for the corresponding liver homogenates. However, in fractions from experimental livers $V_{max}$ values were always significantly lower (Table 4).

The specific activities of both enzymes in freshly prepared subcellular fractions from control and 24 h experimental livers were compared with the same subcellular fractions that had been frozen and thawed several times over several weeks. Little or no change in the specific activities and $K_m$ and $V_{max}$ values occurred.

TABLE 2

Distribution of Glycosidases in Liver Subcellular Fractions*

| | β-N-Acetylglucosaminidase | | β-Galactosidase | |
|---|---|---|---|---|
| | Control | Experimental | Control | Experimental |
| Total homogenate | 100 | 100 | 100 | 100 |
| Nuclear | 17 | 16 | 16 | 13 |
| Nuclear wash[/] | 8 | 8 | 9 | 11 |
| Lysosomal | 57 | 52 | 55 | 58 |
| Lysosomal wash | 1 | 1 | 2 | 2 |
| Microsome[≠] | 3 | 2 | - | - |
| Cell Sap | 2 | 1 | 4 | 3 |
| Recovery in fractions | 88 | 80 | 86 | 87 |

*Results are recoveries expressed as a percentage of the total homogenate activities; values are given to the nearest whole number and represent results from a typical experiment.

[/]Nuclear wash fractions contain mainly lysosomal material; total lysosomal activities will be the sum of nuclear wash and lysosomal activities.

[≠]β-Galactosidase activities were negligible.

TABLE 3

Specific Activities of Glycosidases in Liver Subcellular Fractions*

| | β-N-Acetylglucosaminidase | | β-Galactosidase | |
|---|---|---|---|---|
| | Control | Experimental | Control | Experimental |
| Total homogenate | 8.2 | 4.1 | 1.4 | 0.85 |
| Nuclear | 2.9 | 1.5 | 0.46 | 0.28 |
| Lysosomal | 28.1 | 16.2 | 3.7 | 3.0 |
| Microsomal† | 2.1 | 1.1 | - | - |
| Cell Sap | 0.43 | 0.27 | 0.23 | 0.14 |

*Specific activity is expressed as units/mg protein/min; results represent mean values from 4-6 experiments reproducibility was within ± 10%.

†β-Galactosidase activities were negligible.

Fig. 11. Double reciprocal plot for reaction rate versus substrate concentrate for β-galactosidase in control (-●-) and 24 h experimental (-▲-) rat liver homogenates. Results shown are from a typical experiment. Values for v are in arbitrary units/mg protein.

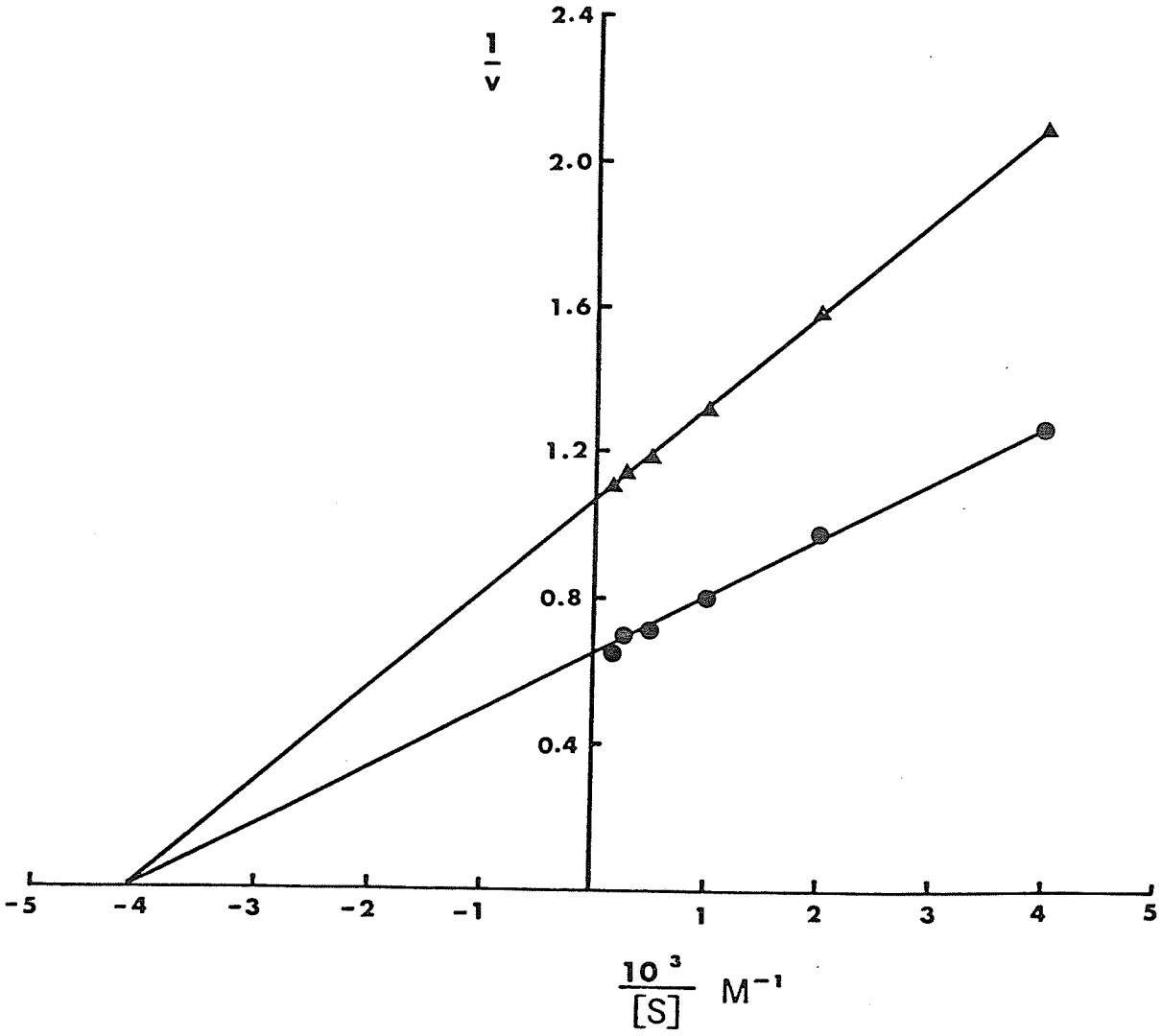

2.4
2.0
1.6
1.2
0.8
0.4
1/v
-5
-4
-3
-2
-1
1
2
3
4
5
10 ³/[S] M⁻¹

Fig. 12. Double reciprocal plot for reaction rate versus substrate concentration for β-N-acetylglucosaminidase in control (-●-) and 24 h experimental (-▲-) rat liver homogenates. Results shown are from a typical experiment. Values for v are in arbitrary units/mg protein.

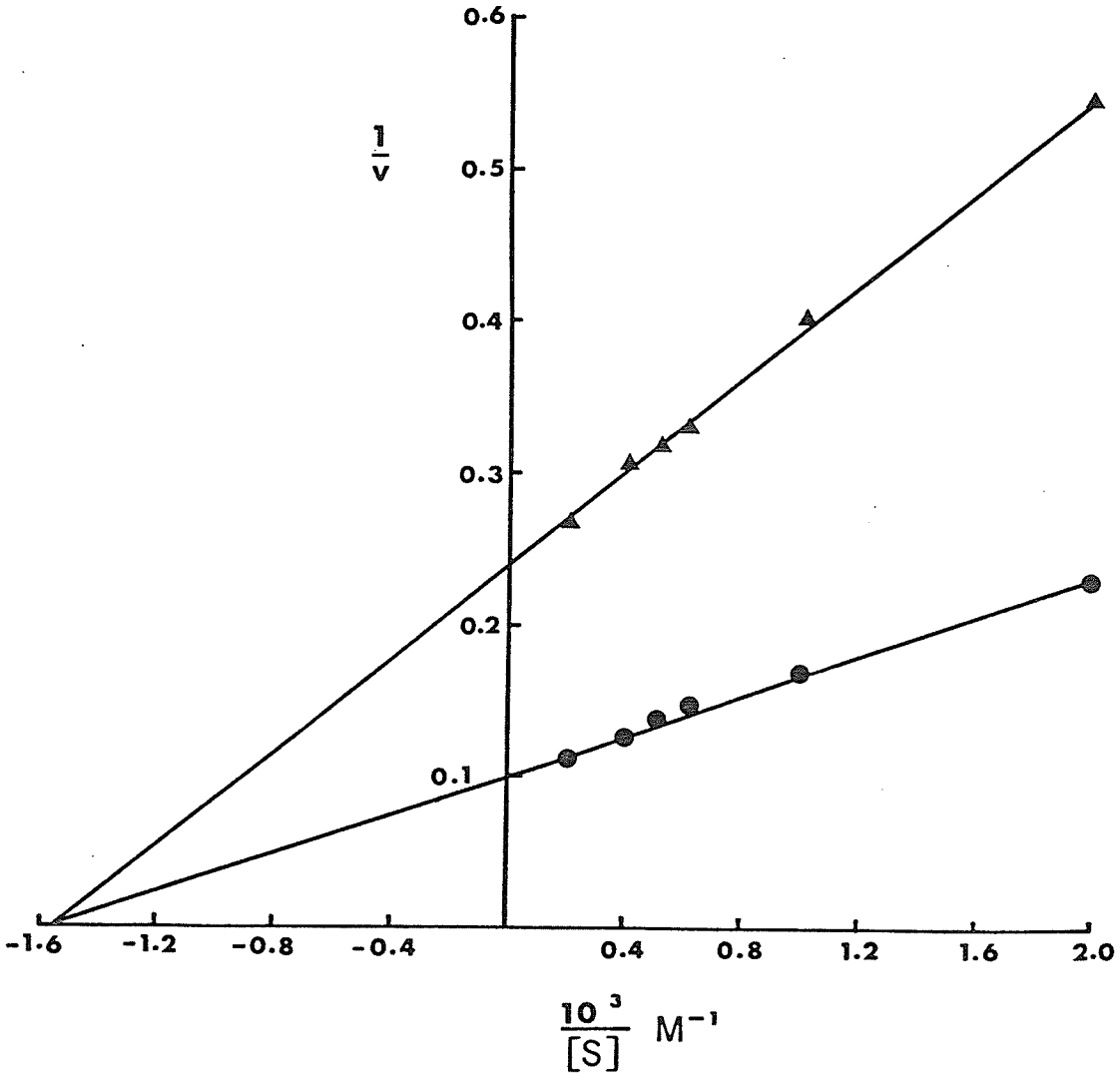

0.6
0.5
0.4
0.3
0.2
0.1
1/v
-1.6
-1.2
-0.8
-0.4
0.4
0.8
1.2
1.6
2.0
10 ³/[S] M⁻¹

TABLE 4

Values of $K_m$ and $V_{max}$ for Glycosidase Activities in Total Homogenate, Lysosomal-Enriched and Microsome Fractions from Control and 24 h Experimental Rat Livers*

| | Control | | Experimental | |
|---|---|---|---|---|
| | $K_m$ (mM) | $V_{max}$ | $K_m$ (mM) | $V_{max}$ |
| β-N-Acetylglucosaminidase | | | | |
| Total homogenate | 0.69 | 10.1 | 0.67 | 4.7 |
| Lysosomal | 0.62 | 32.8 | 0.66 | 22.8 |
| Microsome | 0.66 | 2.9 | 0.68 | 1.5 |
| β-Galactosidase† | | | | |
| Total homogenate | 0.22 | 1.4 | 0.23 | 1.0 |
| Lysosomal | 0.23 | 3.9 | 0.20 | 3.1 |

*Results are from a typical experiment; $V_{max}$ values are given in arbitrary units/mg protein.

†β-Galactosidase activity was negligible in microsome fraction.

Two types of control experiments were performed. In the first type turpentine was added to total homogenates and lysosomal fractions at concentrations up to 5%, homogenized, and enzyme activities determined. However, turpentine had little or no effect on $K_m$ and $V_{max}$ values in these experiments. In the second type of control experiment homogenates from control and experimental livers were mixed and assays performed on the mixtures and lysosomal fractions derived from them. There was no change in $K_m$ values between the mixtures and the corresponding unmixed samples. However, values for $V_{max}$ for both enzyme activities in the mixtures were the averages of the $V_{max}$ values found for the corresponding control and experimental samples.

Effect of inflammation on sialyltransferase and galactosyltransferase from rat liver microsome fractions

Previous work (44) has suggested that the oligosaccharide of rat $\alpha_1$-acid glycoprotein is synthesized during passage of the protein from the rough endoplasmic reticulum to the Golgi complex. Since the amount of $\alpha_1$-acid glycoprotein, and presumably other acute phase proteins, passing out of the liver is elevated in inflammation (14, 44), it was thought that there might be accompanying increases in sialyltransferase and galactosyltransferase activities following inflammation. The effect of experimentally induced inflammation on sialytransferase and galactosyltransferase activities in rat liver microsome fractions is shown in Table 5. The assays for galactosyltransferase were performed in the presence of CDP-choline as it inhibits non-specific pyrophosphatase activity which was found (62) to affect UDP-galactose concentrations in this assay system.

TABLE 5

Effect of Time After Inflammation on Rat Liver Microsome Fraction Sialyltransferase and Galactosyltransferase activities.

| Time After Inflammation (HOURS) | Glycosyltransferase Activities* Sialyltransferase† | Galactosyltransferase≠ |
|---|---|---|
| 0 | 682 | 205 |
| 12 | 1035 | 316 |
| 24 | 1080 | 431 |
| 48 | 976 | 249 |
| 72 | 852 | 225 |

*Results represent mean values from 3-5 experiments. Reproducibility was within ± 10%.

†Sialyltransferase activity is expressed as: pmoles $^{14}$C-labelled sialic acid incroporated into 250 µg acceptor protein/mg microsome fraction protein/min.

≠Galactosyltransferase activity is expressed as: pmoles $^{14}$C-labelled galactose incorporated into 100 µg acceptor protein/mg microsome fraction protein/min.

Both glycosyltransferase assays were performed in the presence of Triton X-100 to solubilize the membranes, thus releasing the enzymes and allowing them easier access to their respective acceptor glycoproteins (63).

N-Acetylglucosaminyltransferase

Assays for sialyltransferase and galactosyltransferase are easy to understand from a mechanistic point of view since sialic acids and galactose always occupy terminal and penultimate positions, respectively, on carbohydrate chains of serum type glycoproteins. The sugars transferred require only one type of acceptor molecule i.e. sialic acid-free $\alpha_1$-acid glycoprotein for sialyltransferase or sialic acid-galactose-free $\alpha_1$-acid glycoprotein for galactosyltransferase. The transfer reaction thus occurs directly from the appropriate nucleoside monophosphate or diphosphate derivative. However, when assays for N-acetylglucosaminyltransferase activities are performed there is the possibility that different activities and different enzymes are being examined and assayed. This occurs because N-acetylglucosamine occupies three distinct positions on the oligosaccharide chain of a typical serum glycoprotein. It is present in the terminal triplet and the inner core and is also involved in linkage with polypeptide (63, 64). Thus, there is the potential for the existence of three distinct activities each with a different acceptor molecule. In addition, lipid intermediates have been implicated in the transfer reactions so that the mechanism is clearly more complex than that described for the transfer of sialic acid and galactose. This part of the work was therefore aimed at determining if there were different types of N-acetylglucosaminyltransferases and, if present, were they increased as a result of experimental inflammation.

Previous studies (14, 21) have indicated an increase rate of synthesis of rat $\alpha_1$-acid glycoprotein as a result of induced inflammation. Thus, it would be expected that ribosomes from the livers from these animals would be synthesizing a proportionately greater number of polypeptides for glycoproteins. Thus, nascent polypeptides, particularly from bound polyribosomes, should be good acceptors for the enzyme responsible for addition of the first N-acetylglucosamine residue to polypeptide. Therefore, free and bound polyribosomes were isolated from 24 h experimental rat livers and the incorporation of $^{14}$C-labelled N-acetylglucosamine into nascent polypeptides attached to these polyribosomes was studied. All N-acetylglucosaminyltransferase assays were performed in the presence of CDP-choline (see above).

The incorporation of $^{14}$C-labelled N-acetylglucosamine was significantly higher into nascent polypeptides attached to bound polyribosomes (Table 6). Puromycin causes premature termination of chain elongation during protein synthesis which results in the release of the nascent polypeptide from the ribosome. The effect of puromycin on the incorporation of $^{14}$C-labelled N-acetylglucosamine into nascent polypeptides from free and bound polyribosomes was also determined. A significant increase in the incorporation of $^{14}$C-labelled N-acetylglucosamine was noted upon addition of puromycin, with nascent polypeptides from bound polyribosomes having the greatest amount of $^{14}$C-labelled N-acetylglucosamine incorporated (Fig. 13).

The effect of addition of the dolichol phosphate - containing lipid fraction and detergent on the incorporation of $^{14}$C-labelled N-acetylglucosamine into nascent polypeptides attached to membrane-bound poly-

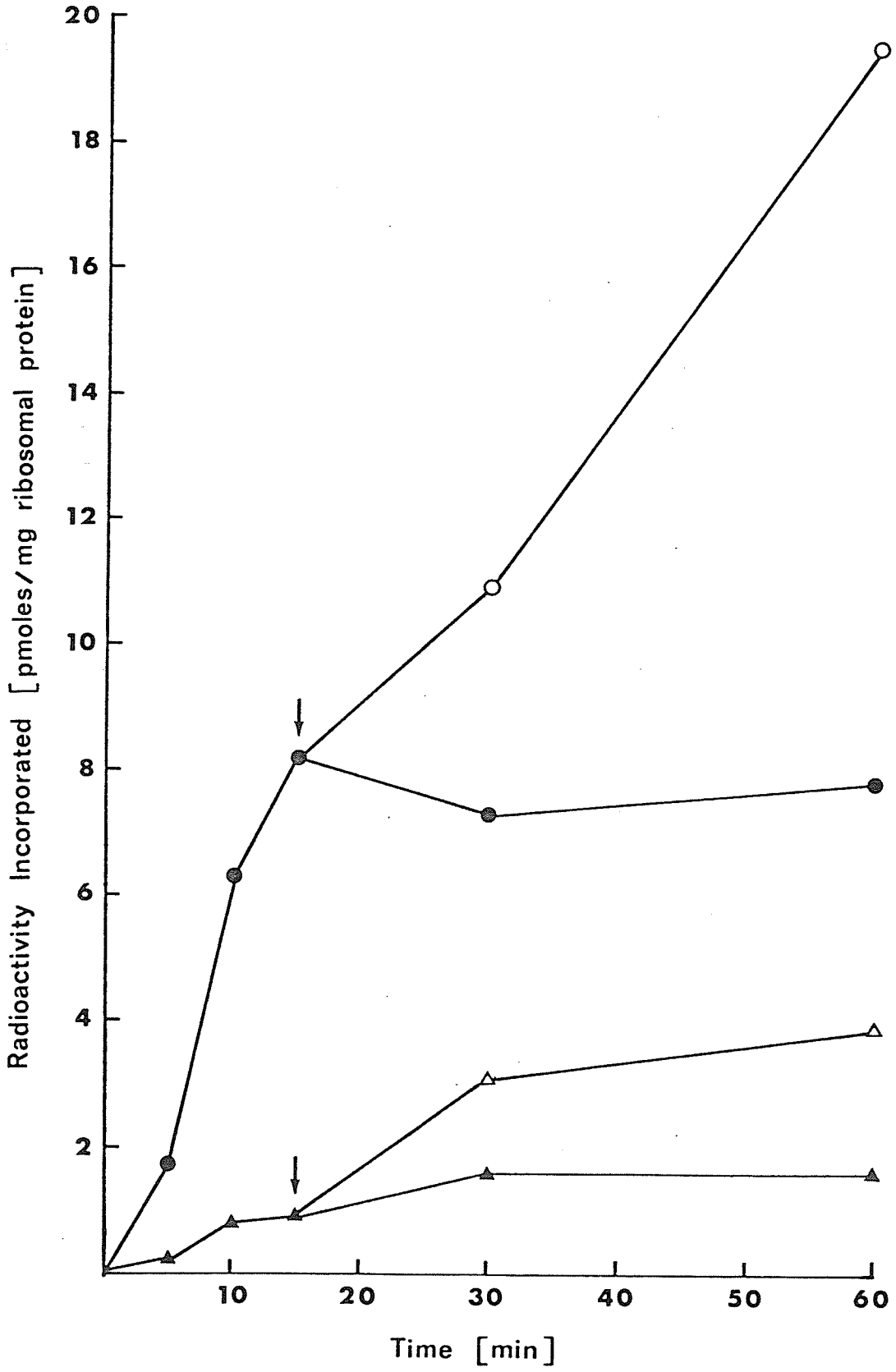
TABLE 6

Incorporation of $^{14}$C-Labelled N-Acetylglucosamine into Nascent Polypeptides Attached to Free and Bound Polyribosomes.

| Total Polyribosome Preparation protein (mg) | $^{14}$C-N-Acetylglucosamine Incorporated* | |
|---|---|---|
| | Free | Bound |
| 0.5 | 17.5 | 68.0 |
| 1.0 | 33.0 | 138.5 |
| 1.5 | 52.5 | 203.0 |

*Results are in pmoles/mg 24 h experimental microsome fraction protein/min. Results represent mean values from 4-6 experiments. Reproducibility was within ± 10%.

Fig. 13. Effect of time of incubation and addition of puromycin (arrows) on the incorporation of $^{14}C$-labelled N-acetyl-glucosamine into nascent chains from bound polyribosomes (without puromycin (-●-); with puromycin (-O-)) and free polyribosomes (without puromycin (-▲-); with puromycin (-△-)). Results represent mean values from 4-6 experiments. Reproducibility was within ± 10%.


Radioactivity Incorporated [pmoles/mg ribosomal protein]
20
18
16
14
12
10
8
6
4
2
10
20
30
40
50
60
Time [min]

ribosomes (prepared as a rough endoplasmic reticulum fraction, see Experimental) was determined. The incorporation of $^{14}$C-labelled N-acetylglucosamine was enhanced in the presence of exogenous lipid and greatly enhanced in the presence of both exogenous lipid and deoxycholate; however, the presence of deoxycholate alone inhibited the incorporation of $^{14}$C-labelled N-acetylglucosamine (Table 7).

Several attempts to use nascent polypeptides, isolated from puromycin-treated free and bound polyribosomes as acceptors, were unsuccessful. The nascent polypeptides were to be used to determine the specificity of N-acetylglucosaminyltransferase in the rough endoplasmic reticulum (responsible for initial glycosylation) and in the Golgi complex (responsible for glycosylation in the terminal trisaccharide) and also to determine changes in enzyme activities during inflammation. However, in all attempts to isolate nascent polypeptides, the polypeptides aggregated and precipitated from solution. Several buffers were employed in the concentration-dialysis procedure, however, the nascent polypeptides precipitated each time. Due to the inability to resolve this problem, this series of experiments was terminated at this stage and will form the starting point for future work.

TABLE 7

Effect of Exogenous Lipid and Deoxycholate (DOC) on the Incorporation of $^{14}$C-Labelled N-Acetylglucosamine into Nascent Polypeptides Contained in a Rough Endoplasmic Reticulum (RER) Fraction from a 24 h Experimental Rat Liver

| Fraction | Radioactivity Incorporated* |
|---|---|
| RER | 89.7 |
| RER + Lipid | 147.5 |
| RER + Lipid + DOC | 224.5 |
| RER + DOC | 37.6 |

*Results are expressed as pmoles/mg rough endoplasmic reticulum protein/min. Results represent mean values from 4-6 experiments. Reproducibility was within $\pm$ 10%.

## DISCUSSION

Livers from both rats and rabbits have the ability to remove serum glycoproteins, such as fetuin, haptoglobin and $\alpha_1$-acid glycoprotein, from the circulation when the penultimate galactose units of their carbohydrate moieties have been exposed by prior removal of the terminal sialic acid residues (65). In vivo experiments (66-68) have shown that soon after their removal and uptake by the liver, the sialic acid-free glycoproteins enter hepatic lysosomes where they are degraded to sugars and amino acids by lysosomal glycosidases and cathepsins, respectively. Digestion of proteins in vitro by lysosomal extracts has also been demonstrated by a number of workers (69, 70).

Other work in our laboratory has indicated that sialic acid-free $\alpha_1$-acid glycoprotein is rapidly removed from the circulation of control rats and localizes in liver lysosomes, presumably for catabolism. In the case of experimental rats, however, the survival times of sialic acid-free $\alpha_1$-acid glycoproteins in the circulation are significantly increased (M. Wong and J. C. Jamieson, unpublished work). It is believed that this is due to elevated levels of circulating sialic acid-free glycoproteins which saturate the binding proteins on the hepatic cell surface. Since these studies suggest changes in catabolism during inflammation, it was of interest to study liver glycosidases, which are probably intimately involved in the catabolism of serum glycoproteins, to determine whether there were any changes during inflammation which could be related to some alteration in catabolism of serum glycoproteins.

Inflammation had no effect on the subcellular distribution and pH optima of the two glycosidases chosen for study (Table 2; Figs. 6, 10).

Inflammation, however, resulted in decreased glycosidase activities with 24 h experimental liver homogenates exhibiting as much as 40% and 50% decreases in β-galactosidase and β-N-acetylglucosaminidase activities, respectively, when compared to activities in control liver homogenates (Fig. 9). Kinetic studies showed that the decrease in the activities of both enzymes following inflammation was due to a change in $V_{max}$ values, since the $K_m$ values for each enzyme remained relatively constant (Figs. 11-12; Table 4).

There are many possible explanations for the decrease in glycosidase activities in liver found following inflammation. For example, it is possible that small amounts of turpentine could be present in the liver and inhibit glycosidase activities although it is believed that little turpentine reaches the liver under conditions where it is administered subcutaneously. However, this explanation seems unlikely in view of the results from control experiments which showed that turpentine did not inhibit glycosidase activities even at concentrations as high as 5%. Another possibility is that experimental livers contain a natural inhibitor of glycosidase activities which is not present in control livers. This also seems unlikely in view of the results of the mixing experiments where one would have expected enzyme activities to be inhibited and give $V_{max}$ values resembling those of the experimental system. The most likely explanation for the decrease in glycosidase activities in liver following inflammation is that there is a decrease in the amount of each enzyme present as a result of inflammation. This could arise, for example, from diminished biosynthesis, reduced conversion of the enzymes to their active

forms, or loss due to secretion into blood. The latter would be difficult to assess because blood contains glycosidases which are not of liver origin and these might be expected to increase under conditions associated with tissue injury. Results from other experiments have shown that the activity of the intracellular enzyme, fatty acid synthetase, is significantly reduced following inflammation (71). The effect on this liver enzyme is accompanied by an increase in the amount of membrane-bound relative to free polyribosomes. Intracellular proteins, such as fatty acid synthetase, appear to be synthesized mainly on free polyribosomes (72), whereas secretable glycoproteins appear to be synthesized mainly on membrane-bound polyribosomes (73). It is possible that the reduced liver glycosidase activities found in these studies have arisen because liver is committed to the biosynthesis of secretable glycoproteins at the expense of intracellular proteins such as fatty acid synthetase and the glycosidases. In any event the results imply that the increased synthesis of glycoproteins such as $\alpha_1$-acid glycoprotein found following inflammation may be accompanied by diminished catabolism of these glycoproteins at least at the lysosomal site. Thus, increased synthesis coupled with some diminished catabolism might be contributing to the elevated levels of circulating glycoproteins found in blood in inflammatory conditions.

The second study undertaken in this work was on the effect of inflammation on glycosyltransferases associated with rat liver microsome fractions. As mentioned previously, sialyltransferase and galactosyltransferase are responsible for the incorporation of the terminal and penultimate sugars, respectively, into the oligosaccharide moiety of $\alpha_1$-acid glycoprotein and other serum glycoproteins. The activities of both

enzymes were significantly increased following inflammation (Table 5). This was not surprising in view of the increased capacity for synthesis of glycoproteins found in liver following induced inflammation (14, 74). In addition, a recent ultrastructural study (75) has revealed that there is a proliferation of smooth endoplasmic reticulum and Golgi complex membranes in liver during inflammation. Time course studies of the incorporation of labelled monosaccharide precursors into the glycoproteins of subcellular organelles of rat liver (76) have revealed that sialyltransferase and galactosyltransferase activities are associated with the membranes of the smooth endoplasmic reticulum and Golgi complex. Thus, it must be assumed that the proliferation of these organelles is accompanied by the presence of increased amounts of the transferase enzymes which probably explains the enhanced activities found in liver in the present work following experimental inflammation.

A third study related to that discussed above was to determine if there was more than one N-acetylglucosaminyltransferase activity in liver microsome material and whether there were changes in activities during inflammation. The first study was to detect a specific enzyme for addition of the first N-acetylglucosamine using nascent chains as acceptor molecules. However, although the isolation of nascent polypeptides for use as acceptors was unsuccessful, some information was obtained using polyribosomes in which the nascent polypeptides were released <u>in situ</u> by incubation with puromycin. It was found that the presence of puromycin stimulated the transfer of N-acetylglucosamine from its nucleotide precursor to nascent polypeptides. The transfer also seemed to be stimulated in the presence of a lipid preparation containing dolichol phosphate suggesting that a lipid

intermediate may be involved in this transfer reaction. However, the detailed mechanism of this transfer and whether it is altered during inflammation was inconclusive due to the lack of availability of good acceptors.

However, work reported while this thesis was in preparation suggests that generation of the acute phase response to injury may well be associated with enhanced asparagine-sequon-N-acetylglucosaminyltransferase activities. In the work in question, Marshall's group (77) used ribonuclease A as acceptor (ribonuclease A has an asparagine sequon with no carbohydrate attached) and showed that regenerating liver had high activities of the enzyme. In his controls, however, which consisted of animals suffering from sham operations, there was an 8-10-fold increase in activities in rough microsome subfractions of liver when compared to normal rat livers. Since these control animals were undoubtedly suffering from acute inflammation, it is possible that similar results would be obtained following turpentine induced inflammation. Thus, experiments similar to those described in this thesis, but using ribonuclease A in place of nascent polypeptides as acceptors, will form the basis of future experimental work.

It is hoped that information gained from these studies will help to elucidate the complex processes that may be functioning within the liver cell to control the synthesis and catabolism of a specific acute phase glycoprotein and which may eventually explain the nature of the acute phase response of serum glycoproteins to inflammation in humans.

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