EFFECTS OF INFLAMMATION AND DEXAMETHASONE ON VARIOUS ASPECTS OF GLYCOPROTEIN BIOSYNTHESIS

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MOHAN LAL SARKAR

EFFECTS OF INFLAMMATION AND DEXAMETHASONE ON VARIOUS ASPECTS OF GLYCOPROTEIN BIOSYNTHESIS

BY

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in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

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ABSTRACT

The concentration of acute-phase proteins in plasma is significantly increased in response to inflammatory agents. Almost all acute-phase proteins are glycoproteins in which the carbohydrate moiety is attached to the peptide through an asparagine nitrogen and are synthesized *via* dolichol-linked intermediates. Dexamethasone, a potent synthetic glucocorticoid, has been shown to influence the biosynthesis of these acute-phase glycoproteins in experiments with animals and *in vitro*.

This thesis concerns various aspects of the glycosylation of acute-phase proteins during inflammation and dexamethasone treatment in the rat.

Turpentine-induced inflammation caused increased sialyl and galactosyltransferase activities in the liver, while in serum, only the sialyltransferase activity was increased.

The formation of several dolichol-linked intermediates such as dolichol phosphate mannose, dolichol pyrophosphate N-acetyl chitobiose and dolichol pyrophosphate oligosaccharides was increased in cultured hepatocytes, or their homogenates, isolated from inflamed rats. Dexamethasone treatment of hepatocytes from control and inflamed animals also caused an increased formation of these intermediates. The increase in the formation of dolichol-linked intermediates in *inflammation* was attributed to increased endogenous dolichol phosphate levels. In contrast, the increased levels of the intermediates

in dexamethasone treatment were not due to the endogenous dolichol phosphate but were most likely due to the induction of glycosyltransferases involved in glycoprotein biosynthesis. These conclusions were based on the results obtained from the following experiments: 1) estimation of the endogenous dolichol phosphate, 2) formation of dolichol phosphate mannose in presence of increasing amounts of exogenous dolichol phosphate, and 3) formation of dolichol and dolichol phosphate from mevalonate.

Experiments in hepatocytes with actinomycin D and cycloheximide suggested that the increase in dolichol-linked intermediates was dependent on the increased synthesis of glycosylatable polypeptides of the acute-phase proteins.

Nucleotide sugar pyrophosphatase activities were increased only in dexamethasone treated hepatocytes, whereas, the nucleotide sugar levels remained unaltered during both inflammation and dexamethasone treatment. The present study showing increased synthesis of dolichol-linked intermediates during the biosynthesis of acute-phase proteins in response to inflammation and dexamethasone treatment has provided new information on the role of the dolichol pathway in glycoprotein synthesis.

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PUBLICATIONS

Papers published and in preparation.

1. "Key role of dolichol phosphate in glycoprotein biosynthesis:" S. Mookerjea, T. Coolbear & Mohan Lal Sarkar; Can. J. Biochem. & Cell Biology 61, 1032-40 (1983).

2. "Effect of dexamethasone on mannolipid synthesis by hepatocytes prepared from control and inflamed rats:" Mohan Sarkar & S. Mookerjea; Biochem. J. 219, 429-36 (1984).

3. "Increase of sialyltransferase activity in the serum and liver of inflamed rats:" I.H. Fraser, T. Coolbear, Mohan Sarkar & S. Mookerjea; Biochimica et Biophysica Acta, 799, 102-105 (1984).

4. "Synthesis and secretion of serum phosphorylcholine binding protein by rat hepatocytes:" Joan Collins, Uday Saxena, Mohan Sarkar, Arun Nagpurkar and Sailen Mookerjea; Biochimica et Biophysica Acta. 799, 97-101 (1984).

5. Effect of dexamethasone on the synthesis of dolichol-linked saccharide and glycoproteins in hepatocytes prepared from control and inflamed rats: Mohan Sarkar & S. Mookerjea; Biochem. J. 227, 675-82 (1985).

6. "Recovery of dolichol-P-P-oligosaccharide in methanolic aqueous phase prepared from rat liver microsomes;" Mohan Sarkar & S. Mookerjea; Biochem. J. 236, 913-916 (1986).

7. Regulation of glycoprotein synthesis by dexamethasone: Effects on dolichol-linked sugar intermediates and nucleotide sugar pyrophosphatase activity: Mohan Sarkar & S. Mookerjea (submitted).

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List of Abbreviations

ATP	Adenosine 5'-triphosphate
ASN	Asparagine
CMP	Cytidine 5'-monophosphate
CTP	Cytidine 5'-triphosphate
cm	Centimetre
^{14}C	Carbon 14
Ci	Curie
DEAE	Diethylaminoethyl
Dol-P	Dolichol monophosphate
Dol-P-P	Dolichol pyrophosphate
dpm	Disintegration per minute
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetracetate
Fru-6-P	Fructose-6-phosphate
Fuc	Fucose
Gal	Galactose
GERL	Golgi-endoplasmic reticulum lysosome
GlcN	Glucosamine
GlcNAc	N-acetylglucosamine
GalNAc	N-acetylgalactosamine
Gal-1-P	Galactose-1-phosphate
Glc	Glucose
Glc-6-P	Glucose-6-phosphate
Glc-P-Dol	Dolichol monophosphate glucose
GlcNAc-P-P-Dol	Dolichol pyrophosphate N-acetylglucosamine
$(GlcNAc)_2$-P-P-Dol	Dolichol pyrophosphate N-acetylchitobiose
GDP	Guanosine 5'-diphosphate
g	Gravitational force unit
gm	Gram
h	Hour
HSF	Hepatocyte stimulating factor

IL-1	Interleukin-1
Km	Michaelis constant
mRNA	Messenger ribonucleic acidmCimillicurie
MES	2-(N-morpholino) ethanesulphonic acid
ml	Millilitre
mg	Milligram
mM	Millimolar
M	Molar
Man	Mannose
Man-6-P	Mannose-6-Phosphate
mmol	Millimole
min.	Minute
mm	Millimeter
Man-P-Dol	Dolichol monophosphate mannose
NADPH	Reduced nictinamide adenine dinucleotide phosphate
NeuAc	N-acetylneuraminic acid
nmol	Nano mole
pH	- log of hydrogen ion concentration
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
Ser	Serine
SRP	Signal recognition particle
SA	Sialic acid
Thr	Threonine
Tris	Tris (hydroxymethyl) aminomethane
UDP	Uridine 5'-diphosphate
UV	Ultraviolet
v/v	Volume/volume
Vmax	Maximum velocity
w/v	weight/volume
µg	Microgram
µl	Microlitre

PAGE

µM	Micromolar
µCi	Microcurie

CHAPTER I

INTRODUCTION

1.1. INFLAMMATION

Systemic response to inflammation, the acute inflammatory process.

Inflammation in mammals may be caused by various factors, which include: local injection of inflammatory agents (1,2), thermal or mechanical injury (3), major surgery (4), bacterial infection or endotoxin injection (5,6) and neoplastic growth (5,7).

The local reaction of tissue to injury or infection is termed acute inflammation, and the systemic and metabolic changes that occur during inflammation are termed, the acute-phase response (8). The local and systemic reactions that occur during acute and chronic inflammation have been described by Glenn et al., (9) in a scheme shown in fig. 1.

The "Inflammatory Process"

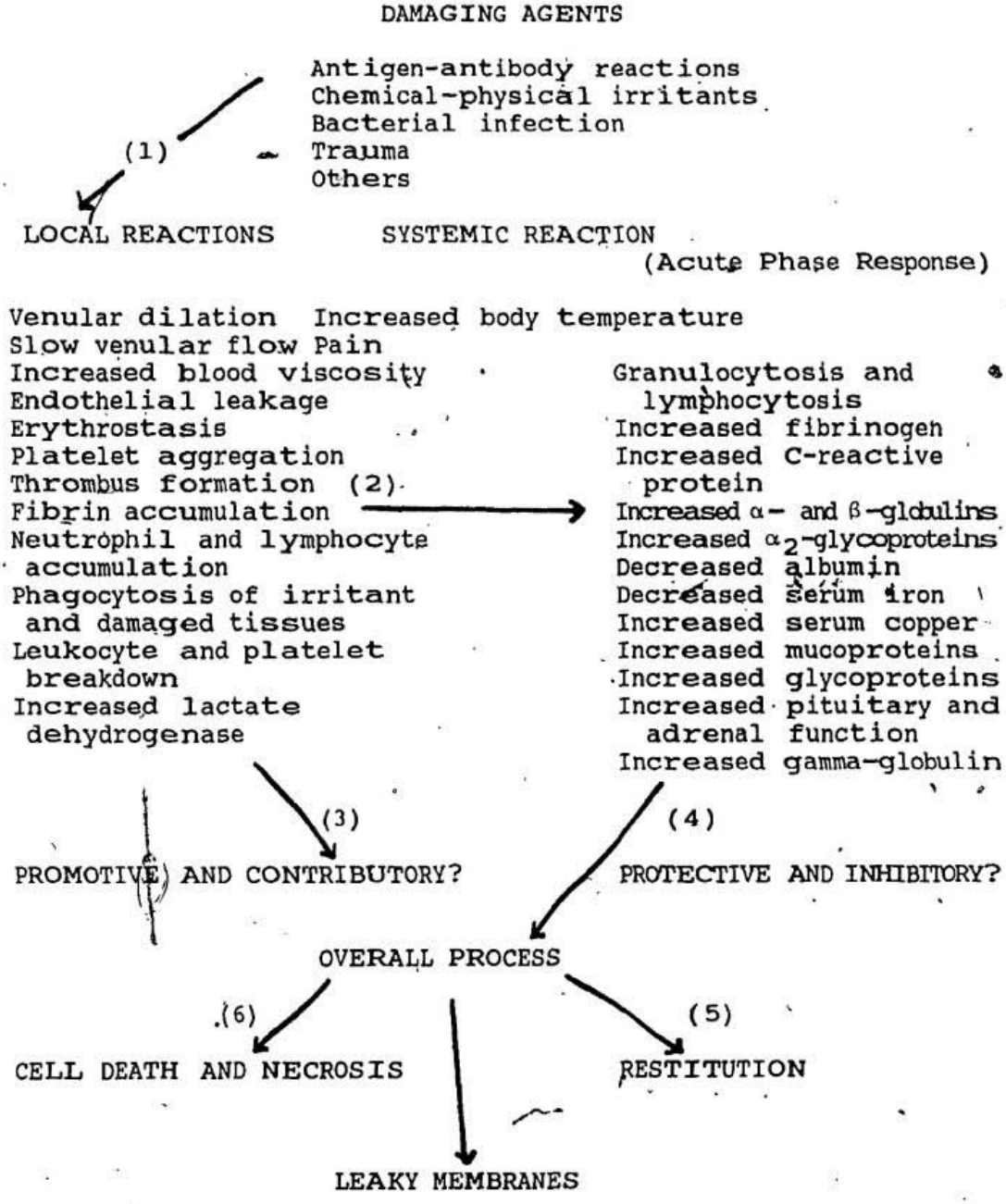

Fig. 1. Schematic diagram of inflammatory process. The numbers in parenthesis indicate the order in which the events are believed to occur. Reproduced from Glenn et al. (9).

Glenn et al (9) have suggested that the systemic reaction is induced by the local reaction, which includes phenomena such as venular dilation, endothelial leakage and oedema, platelet aggregation, fibrin formation, leucocyte accumulation, release of lysosomal enzymes from leucocytes and tissues, formation and release of small molecular weight mediators (histamine, 5-hydroxytryptamine, kinins), mesenchymal cell proliferation and others.

Systemic response includes fever, pain, leucocytosis, increased level of acute-phase proteins, increased function of the pituitary-adrenal system and decreased level of serum iron. Various forms of inflammation are associated with an increased permeability of lysosomal membranes and release of acid hydrolases (10,11). The release of lysosomal enzymes has been referred to by Weissmann (12) as the "final common pathway" in inflammation.

Fig. 1 shows that the systemic response is accompanied by various metabolic, humoral and physiologic alterations. Fever is considered a part of the systemic response to different types of stimuli, which provides pertinent growth conditions for invasive microorganisms (8,13). Fever causes increased utilization of nutrients to meet the elevated energy requirements of body cells. Increased gluconeogenesis and glycogenolysis, elevation of amino acid degradation with accelerated ureogenesis and ammoniogenesis, increased catabolism of somatic proteins, reduced ketogenesis and

hyperglyceridemia (8,13,14,15) are observed due to the high energy requirement during elevated body temperature.

The systemic response is characterized by elevation of a number of hormones, e.g. insulin, glucagon, adrenocorticotropic hormone (ACTH), cortisol, catecholamines, growth hormone, thyroxine, thyroid stimulating hormone, vasopressin and aldosterone (15,16,17). The precise role of these hormones in initiating the acute-phase response has yet to be determined.

One of the important changes occurring during the acute-phase response is in the levels of some plasma proteins. Table 1 lists the changes in the levels of some important plasma proteins that occur in response to experimental inflammation. Plasma proteins which increase following inflammation are referred to as "acute-phase proteins" (18), whereas, proteins such as albumin which decrease in concentration, have been described as "negative acute-phase proteins" (8). At least two common features have been ascribed to the acute-phase proteins, i) almost all are glycoproteins and ii) they are synthesized by the liver (18,19,20,21,22).

Table 1. Effect of injection of croton oil into rats*.

Components analyzed	Response
Total Protein	No change
Albumin	Decrease
Fibrinogen	Increase
Glycoprotein	Increase
Mucoprotein	Increase
Alpha-globulins	Increase
Beta-globulins	Increase
Alpha-2-glycoprotein	Increase

*The information in this table is taken from Glenn et al (23).

When the principal events between the occurrence of inflammation and the appearance of newly synthesized acute-phase proteins in the blood are considered, they may be presented in the form of the following chain of events as shown in Fig. 2.

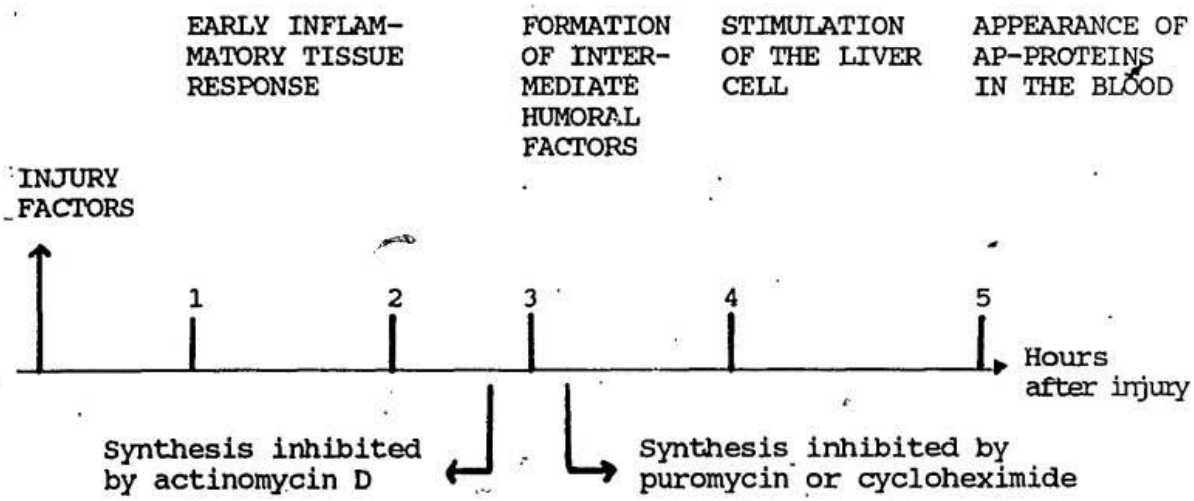

Fig. 2. Sequence of events in the acute-phase protein response.

Hormone-like factors, originating at the site of tissue injury, are believed to be transported by blood to the liver where they stimulate increased synthesis of the acute-phase proteins (14,18). Work by Woloski et al (24) and others (14,25,26,27,28) have suggested that leucocytes may exert an indirect effect on the liver to stimulate acute-phase protein synthesis by formation of such chemical mediators, as kinins, pyrogen, histamine and cytokines. Woloski (29) has shown that monocyte derived factor(s) are able to stimulate the process characteristic of the acute-phase response in liver, including elevated glycoprotein synthesis. The mechanism by which these chemical mediators stimulate hepatic synthesis of the acute-phase proteins is unknown. Although cytokines are able to stimulate the synthesis of the acute-phase protein, the response appears to be lower than that found following trauma (24,29).

Trauma stimulates the pituitary-adrenal system, as well as other endocrine glands and it has been suggested that hormones, such as corticosteroids transported by blood, stimulate the synthesis of acute-phase proteins in the liver. Corticosteroids have been reported to be involved in stimulating the acute-phase response resulting in enhanced synthesis of glycoproteins in inflammation (30,31). John and Miller (32) using a liver perfusion system, have shown that cortisol elevated synthesis of haptoglobin, fibrinogen and an α_1-globulin. Adrenalectomy caused a pronounced decrease

in the synthesis of some of the acute-phase proteins in response to turpentine-induced inflammation. These were restored to normal by replacement therapy with cortisol (33,34). A small increase of acute-phase proteins in adrenalectomized rats after turpentine induced inflammation, suggests that a second effector system other than corticosteroid can mediate an enhanced synthesis of these proteins.

Thus, although hormones may be involved in the stimulation of elevated, hepatic acute-phase protein synthesis, current ideas suggest that cytokines and other mediators are also required for full expression of elevated synthesis of these proteins. Clearly, the acute inflammatory process represents a coordinated system to limit, modulate or otherwise direct host response during periods of intense inflammation and tissue destruction.

1.2. Acute-phase proteins

This category includes several proteins with diverse physicochemical properties (Table 2). Almost all acute-phase proteins contain significant amounts of carboydrate and all are synthesized in liver parenchymal cells. When the physicochemical properties of proteins listed in Table 2 are compared, it appears that their carbohydrate content decreases with increasing isoelectric point. The oligosaccharide chains of the acute-phase proteins are mainly of the asparagine linked (N-linked) complex type (35) and are synthesized via

Table 2. Physical and chemical properties of typical acute-phase proteins.*

The data refer to human plasma except α_1 and α_2 acute-phase globulins from the rat and C_x-reactive protein from rabbit.

Protein	Amount in normal plasma mg/100 ml	Molecular weight	Isoelectric point pI	Carbohydrate content %
α_1-acid glycoprotein	75-100	44 000	2.7	41.4
Haptoglobin (Hp 1-1)	30-190	85 000	4.1	19.3
α_1-AT globulin (rat)	120	45 000	4.5	16
α_1-antitrypsin	210-287	45 000	4,0	12.4
α_2-AP globulin (rat)	0	950 000 approx.		
Ceruloplasmin	27-63	160 000	4.4	8.0
C-reactive protein	0	138 000	?	?
C_x-reactive protein (rabbit)	0	120 000	?	?
Fibrinogen	200-600	341 000	5.8	2.5
Seromucoid	61	Heterogenous fraction		25 approx.

*The information in this table was taken from Koj. A (18).

dolichol linked intermediates (36,37,38). Alteration in the carbohydrate moieties of some acute-phase proteins following an inflammatory challenge has been observed (39).

Acute-phase proteins are of considerable interest in practical and experimental medicine. Changes in their plasma concentration are regarded as a sensitive (although rather non-specific) test for diagnostic and prognostic assessments. The biological activities of some of the acute-phase proteins are well defined. Haptoglobin (HP), combines with haemoglobin (Hb) to give a complex (HP.Hb) thus removing hemoglobin from circulation (40). The involvement of the adrenal gland in regulating the serum haptoglobin level in response to inflammation has been established. The response of serum haptoglobin to inflammation is impaired in the absence of the adrenals (41). Ceruloplasmin is believed to protect cells from damage by generating superoxide anion radicals at the site of tissue damage (42). Inflammation, pregnancy, rheumatoid arthritis (43) and injections of estradiol to chickens (44) produce a significant increase in serum ceruloplasmin.

Fibrinogen is involved in localizing infections through clot formation (13). α_1-antichymotrypsin, α_1-antitrypsin and α_2-macroglobulin are known as protease inhibitors (45). Some acute-phase proteins are known to inhibit certain lymphocyte responses <u>in vitro</u>; these include C-reacive proteins (46), α_1-acid glycoprotein (47) and α-fetoprotein

(48). C-reactive protein (CRP), a member of acute-phase proteins in humans is normally undetectable in the plasma but appears to increase by as much as 1000 fold in response to inflammation and injury. It is interesting to note that CRP in humans and rabbits is a nonglycosylated acute-phase protein whose physiological function may be related to host defense and repair (49,50).

The liver is the site of synthesis of acute-phase proteins, and the increased concentrations of these plasma proteins have been shown to be accompanied by their increased hepatic synthesis (8,18,35,51). During the acute-phase response, increased synthesis of acute-phase proteins is accompanied by alterations in ultrastructural elements and chemical constituents of the liver. The most important alterations observed are: proliferation of the Golgi complex (52), dilation of the rough endoplasmic reticulum, increased amounts of smooth endoplasmic reticulum and increased synthesis of plasma membranes (53,54). There is also increased synthesis of microtubules (55,56), cytoplasmic actin (57), RNA, particularly ribosomal RNA (58), cholesterol and other lipids by the endoplasmic reticulum (59). These alterations are consistent with increased synthesis, transport and secretion of acute-phase proteins.

There is no doubt that liver is the major organ involved in acute-phase response. The liver is known to respond in several ways during the acute-phase response including

activation of the glycoprotein biosynthetic machinery. This thesis is mainly concerned with the involvement of liver in the regulation of the synthesis of the acute-phase proteins.

1.3. Glycoprotein Biosynthesis

The presence of oligosaccharide chains covalently attached to the peptide backbone is the feature that distinguishes glycoproteins from non-glycosylated proteins and accounts for some of their physical and chemical properties. Glycoproteins are broadly classified into two types: O-glycosidically and N-glycosidically-linked glycoproteins. Virtually, all plasma glycoproteins are N-linked glycoproteins and are synthesized by liver. Therefore, only the synthesis of N-linked glycoprotein will be outlined at length in this thesis. The synthetic pathway can be divided into two parts; (A) the synthesis of polypeptides and (B) the synthesis of carbohydrate moieties.

A. Polypeptide Synthesis.

The rough endoplasmic reticulum (RER) has been postulated to possess a single translation-coupled translocation system (in multiple copies) that effects signal sequence-mediated translocation of all secretory proteins (such as acute-phase proteins), lysosomal proteins and all integral membrane proteins whose port of entry is the rough endoplasmic reticulum

(60,61,62,63). The formulation of an hypothesis for the transfer of proteins across the membranes, is referred to as the signal hypothesis.

The essential feature of the signal hypothesis (Fig. 3) is the occurrence of a unique sequence of codons, located immediately to the right of the initiation codon which is present only in those mRNA's whose translation products are to be transferred across a membrane. No other mRNA's contain this unique sequence. Translation of the signal codon results in a unique sequence of amino acid residues on the amino terminal of the nascent chain. Emergence of this signal sequence of the nascent chain from within a space in the large ribosomal subunit triggers attachment of the ribosome to the membrane, thus providing the topological conditions for the transfer of the nascent chain across the membrane. If the nascent chain lacks the signal sequence, attachment of the ribosome to the membrane will not occur.

The attachment of the ribosome to the endoplasmic reticulum membrane is mediated through an 11s ribonucleoprotein, the so-called signal recognition particle (SRP), which has a receptor on the endoplasmic reticulum membrane, termed SRP receptor. SRP functions in decoding the information contained in the signal peptide of nascent secretory proteins (62,64) for the specific attachment of the translating ribosome to the microsomal membrane (62). In the absence of endoplasmic reticulum membranes, SRP specifically arrests the elongation

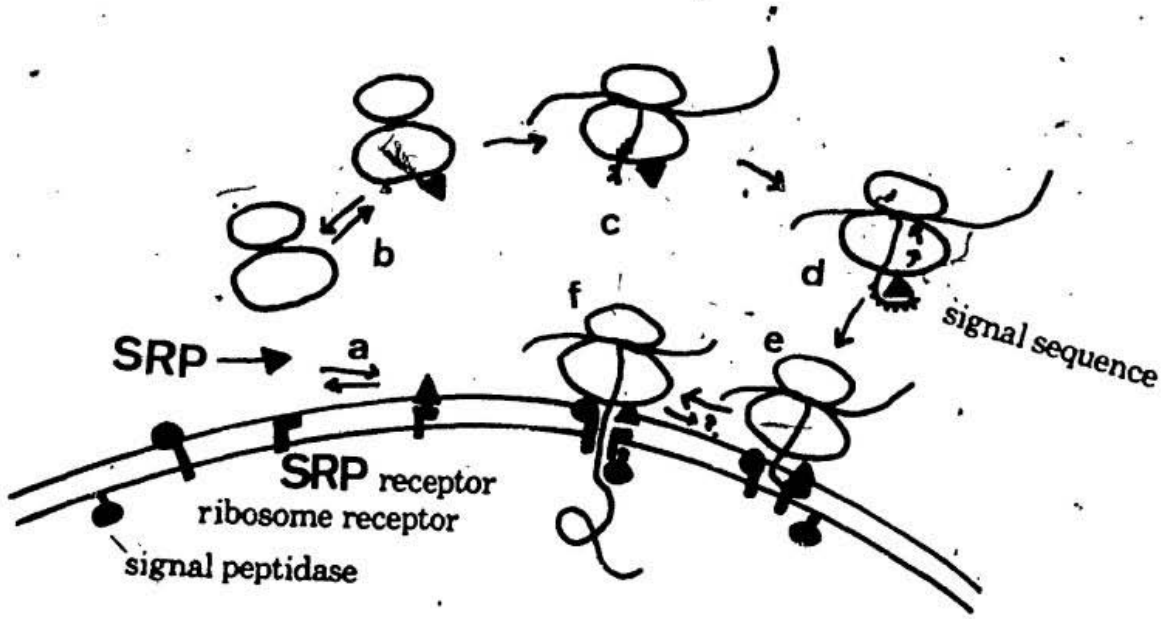

Fig. 3. Schematic diagram for co-translational protein translocation across the rough endoplasmic reticulum membrane. Reproduced from Walter and Blobel (62).

of secretory protein synthesis *in vitro* just after the signal peptide has emerged from the ribosome, thus preventing the completion of pre-secretory protein (many of which may be potentially harmful to the cell) in the cytoplasmic compartment. However, interaction of the arrested ribosomes with the SRP receptor on the microsomal membrane results in the elongation of the nascent chain which is then translocated across the membrane.

The signal peptide is removed by signal peptidase after the protein is transferred to the endoplasmic reticulum membrane.

B. Structure and synthesis of N-linked oligosaccharides in glycoproteins.

Glycoproteins are broadly classified into two types; N-linked and O-linked. Most acute-phase proteins are N-linked glycoproteins in which the carbohydrate chain is linked to the polypeptide chain through an N-glycosidic bond. This N-glycosidic bond is between the C-1 hydroxyl of an N-acetylglucosamine residue in the carbohydrate chain and the amido nitrogen of an asparagine residue in the polypeptide chain. The other type of linkage, which is less common in serum glycoproteins, is the O-glycosidic linkage normally found between N-acetylgalactosamine and either serine or threonine residues on mucin-type glycoproteins (65). The O-glycosidic linkage also occurs in collagen in which galactose is linked

to hydroxylysine residues (66). The oligosaccharide of O-glycosidic linked glycoproteins is formed by direct transfer of individual sugars from the respective nucleotide sugar.

The asparagine linked oligosaccharides have heterogeneous structures but fall into two general classes, i) high mannose type and ii) complex type (35,67,68). Both classes have a common inner core structure at the reducing terminus as shown in Fig. 4.

High mannose type structures contain additional α-linked mannose residues, while complex oligosaccharides have sugars such as N-acetylglucosamine, galactose, fucose and N-acetylneuraminic acid or sialic acid (Fig. 5).

The existence of a common core structure in many of the N-linked glycoproteins suggests a common mechanism of synthesis for at least the internal region of the saccharide chain. Parodi et al (69) reported in 1972 that a glucose-containing lipid-linked oligosaccharide comprising approximately 20 monosaccharide units could be synthesized and transferred to protein in cell-free preparations from rat liver. Synthesis of the oligosaccharide portion, which is formed via dolichol-linked intermediates, is now referred to as the 'dolichol cycle'. After extensive investigation in different laboratories, it has been found that the oligosaccharide contains three glucose, nine mannose and two N-acetylglucosamine residues. The oligosaccharide moiety of this complex is transferred to an acceptor protein which undergoes subsequent

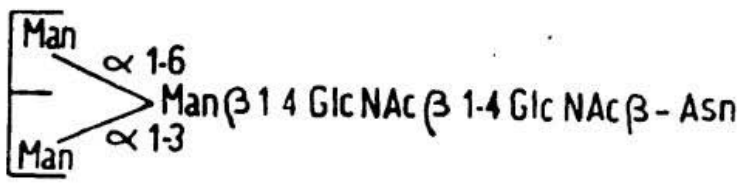

Fig. 4. Core structure of asparagine-linked oligosaccharide chains.

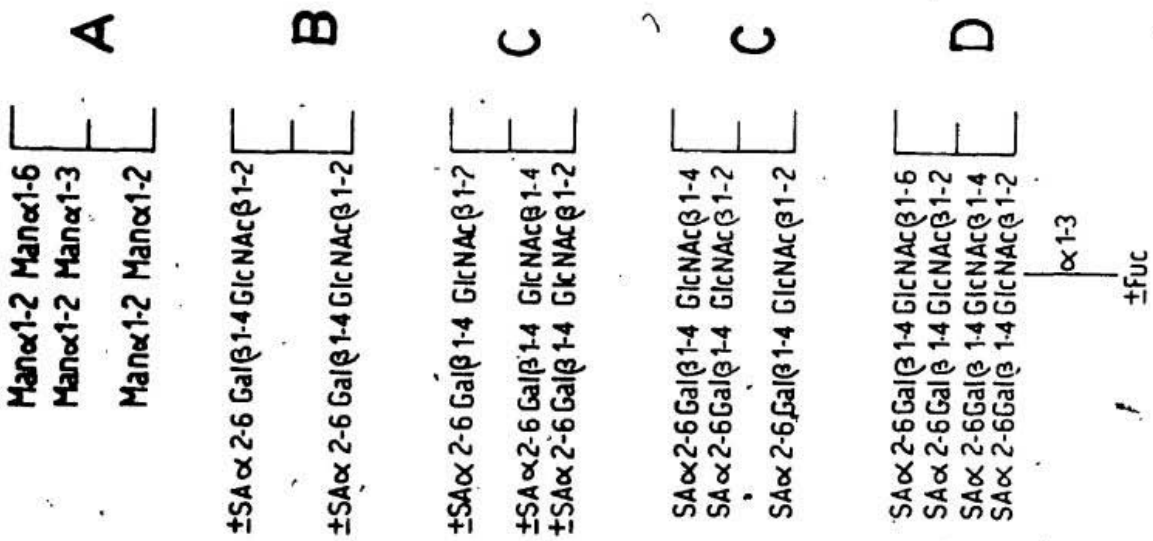

Fig. 5. Structure of N-glycosidically linked oligosaccharide. The high mannose structure (A) shown, is found in bovine thyroglobulin. Complex type of oligosaccharide chains with bi- (B), tri- (C) and tetra (D) antennary structures have also been reported. Reproduced from Staneloni and Leloir (73). The large E represents the core, as illustrated in Fig. 4.

modifications by removal and addition of sugars to produce either high mannose or complex type oligosaccharides. This process collectively is referred to as oligosaccharide processing.

The involvement of lipid in the synthesis of complex glycan in bacteria had been known for some time (70). Behrens and Leloir (71) were the first to demonstrate the participation of a lipid molecule in glycoprotein synthesis in eukaryotes. Other groups (38,70,72) subsequently showed that the intermediate lipid moiety belongs to a family of polyisoprenol alcohols, known as dolichol. In animal tissues these compounds are usually composed of 16-21 isoprene units with 2 internal trans-double bonds. The remainder of the internal double bonds are cis-oriented and the α-isoprene unit is saturated (Fig. 6).

There are two sources of dolichol in liver, the diet and de novo synthesis (74,75). It has been determined that de novo synthesis accounts for 98% of new dolichol in the liver (75,76). The major forms of dolichol found in mammalian tissues are either the free dolichol or dolichol esterified with fatty acids (77). Furthermore, much of the cellular dolichol is distributed in fractions other than the endoplasmic reticulum (78,79), where the enzymes of oligosaccharide synthesis are predominantly located (80). It is the dolichol phosphate (Dol-P) and dolichol pyrophosphate (Dol-P-P) which serve as carriers of saccharide residues in the

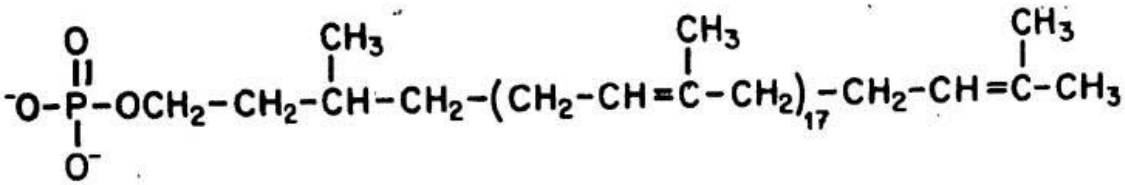

Fig. 6. Structure of dolichol phosphate. The dolichol consists of a linear chain of repeating isoprene units in which the α-isoprene unit is saturated.

assembly of N-linked oligosaccharide of glycoproteins (Fig. 6) (37,38,72,81).

Dolichol phosphate, cholesterol and ubiquinone follow a common biosynthetic pathway from acetyl CoA to farnesyl pyrophosphate. The isoprene chain is lengthened by the addition of 13-20 cis-isoprene units to farnesyl pyrophosphate which is catalyzed by the action of a long chain prenyltransferase. Synthesis is completed by the saturation of the α-isoprene unit (82). Wong and Lennarz (79) have shown that the microsomal fraction is the main site of synthesis of dolichol. As shown in Fig. 7, dolichol phosphate can be formed by two pathways, i) de novo synthesis from acetate and ii) phosphorylation of dolichol. Dolichol is phosphorylated by CTP-dependent dolichol phosphokinase (84,85). The enzyme catalyzing the reverse reaction is a dolichol phosphate phosphatase and both the enzymes are present in liver microsome fractions (86,87,88).

Synthesis of the oligosaccharide chains of N-linked glycoproteins via dolichol-linked oligosaccharide intermediates can be divided into three distinct steps: (1) assembly of the oligosaccharide core region on dolichol pyrophosphate, (2) the transfer of oligosaccharide from lipid carrier to polypeptide and (3) processing of N-linked oligosaccharide, followed by addition of terminal sugars (70,89).

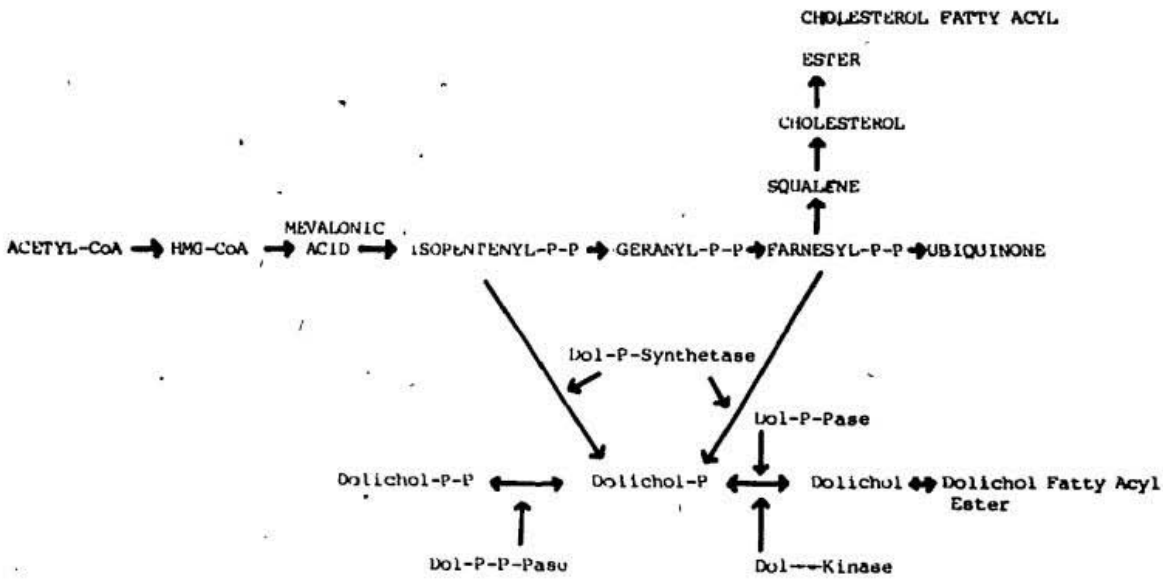

Fig. 7. Biosynthetic pathways of dolichol phosphate. Reproduced from Mookerjea et al (83).

(1) Assembly of the oligosaccharide chain on dolichol pyrophosphate.

Leloir and co-workers produced the first evidence for the synthesis of an oligosaccharide containing glucose, mannose and N-acetylglucosamine that was linked by a pyrophosphate bridge to dolichol. Oligosaccharide pyrophosphate dolichol (Fig. 8), which is formed by a series of reactions termed as 'dolichol cycle' shown in Fig. 9, has been characterized in studies with liver and other tissues.

In the first step (reaction 1) GlcNAc-1-PO_4 is transferred from UDP-GlcNAc to dolichol phosphate to form GlcNAc-P-P-Dol. In the second step (reaction 2) GlcNAc-P-P-Dol is converted to β-GlcNAc-GlcNAc-P-P-Dol by receiving a second GlcNAc molecule from UDP-GlcNAc (90,91). The addition of the first five mannose residues to this complex appears to involve direct transfer of mannose from GDP-mannose (reaction 3). Elongation of this $Man_5GlcNAc_2$-P-P-Dol occurs by transferring four mannose residues from Man-P-Dol (reaction 4). The final step (reaction 5) is the addition of three glucose residues to $Man_9GlcNAc_2$-P-P-Dol to form $Glc_3Man_9GlcNAc_2$-P-P-Dol.

Different experimental approaches have shown that a multienzyme system is involved in the dolichol cycle which spans the membranes of the rough endoplasmic reticulum, with a portion of the enzyme exposed on both cytoplasmic and luminal faces (93,94,95). According to this model, the dolichol

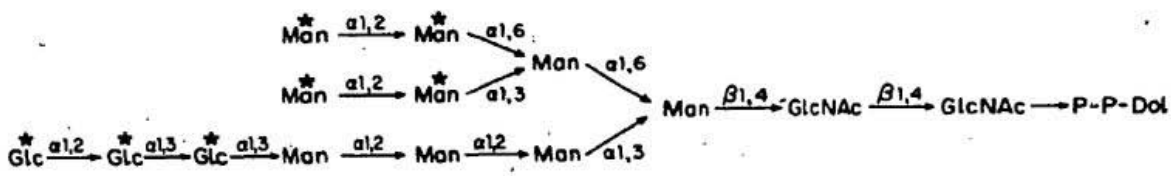

Fig. 8. Structure of glucose containing oligosaccharide linked to dolichol pyrophosphate. The sugars marked with asterisks are derived by transfer from dolichol monosaccharide donors; the others are transferred from nucleotide diphosphate sugars. Taken from Spiro and Spiro (92).

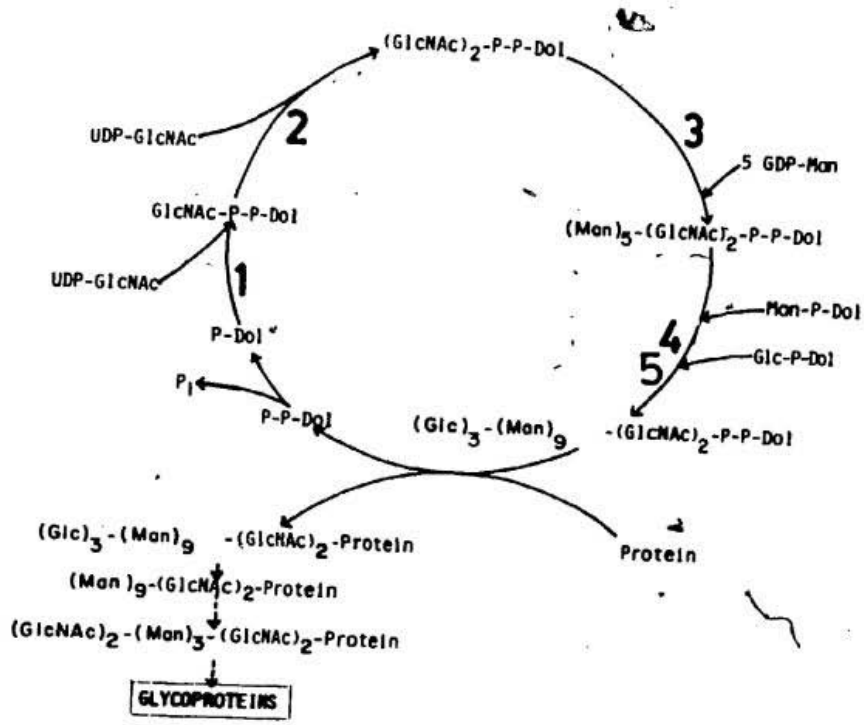

Fig. 9. The dolichol cycle which shows the assembly of lipid linked oligosaccharide. The numbers represents the different reactions in the dolichol cycle. Reproduced from Mookerjea et al (83).

pyrophosphate moiety is believed to be involved in anchoring the growing oligosaccharide chain to the membrane bound multienzyme complex.

2. The transfer of oligosaccharide from lipid carrier to polypeptide:

It is now well established that synthesis of N-linked glycoproteins involves transfer en bloc of oligosaccharide from the lipid carrier to the protein (70,72,81). There are several lines of evidence which suggest that oligosaccharide transfer occurs within the lumen of rough endoplasmic reticulum membrane (96,97). The prerequisite for glycosylation is the occurrence of an asparagine residue in the tripeptide sequence -Asn-X-Thr(Or Ser)-, where X can be any of the 20 amino acids except aspartic acid (66) or proline (98). Another additional requirement for glycosylation is the occurrence of the N-glycosylated sequence in the β-turn of polypeptide chain (99,100). Whether oligosaccharide transfer is co-translational, or post-translational or both is unclear. Studies with liver indicate that glycosylation of α_1-acid glycoprotein and fibrinogen is mainly a post-translational event (101,102). However, studies with other cell types on the glycosylation of immunoglobulins (97), lactalbumin (103), ovalbumin (104) and viral glycoproteins (96) have provided evidence that glycosylation could also be co-translational.

There is an emerging consensus that in a variety of systems the major lipid-linked oligosaccharide has the composition $Glc_3Man_9GlcNAc_2$-P-P-Dol. However, the glucose residues are not seen in the completely glycosylated N-linked proteins. The glucose residues are removed after being transferred to protein. Turco and Robbins (105) have shown that glucose-containing oligosaccharides are 8 times more active than glucose-free oligosaccharide as donors of the oligosaccharide chain to the acceptor protein. Based on this evidence, it has been suggested that glucose is required for the transfer en bloc of the oligosaccharide from lipid to the protein and this concept has been supported by Spiro et al (106). The presence of glucose residues on the oligosaccharide lipid may also serve as a recognition signal for the oligosaccharide transferring enzyme.

3. Processing of the N-linked oligosaccharide

It is now apparent that after the transfer of the oligosaccharide from the lipid carrier to protein, three glucose residues are removed to form the high mannose type, and this is followed by further processing through the action of α-mannosidase to yield variants of the high mannose type and the formation of the core of the complex type of N-linked glycoprotein. The first phase of N-linked oligosaccharide processing involves the removal of glucose residues by the action of specific glucosidases, from the

precursor oligosaccharide soon after its transfer to protein. In most cases the oligosaccharide is completely deglucosylated before any mannose residues could be removed, but some reports suggest that in a few cases one or two mannose residues are removed while glucose is still present on the oligosaccharide (107,108).

After glucose residues are removed, the protein-linked oligosaccharides contain only mannose and N-acetylglucosamine. In a variety of cells, this intermediate has the composition $Man_9GlcNAc_2$ (107,108). Variants of the high mannose type oligosaccharides of mature glycoproteins are thought to arise by removal of a variable number of α-1,2-Man residues from $Man_9GlcNAc_2$ during processing. If four of these residues are removed, it results in the formation of $Man_5GlcNAc_2$ species containing α-1,3 and α-1,6-mannose, which may be converted to the endo H-resistant, complex oligosaccharide. Further removal of two mannose residues from $Man_5GlcNAc_2$ results in the formation of the core structure $Man_3GlcNAc_2$, common to complex type N-linked glycoproteins. Addition of 'outer sugars' like N-acetylglucosamine, galactose and N-acetylneuraminic acid results in the formation of the complex type N-linked glycoprotein.

The first step in the conversion of $Man_5GlcNAc_2$ to complex oligosaccharides is catalyzed by GlcNAc transferase I, which adds a single N-acetylglucosamine residue (109) on the α-1,3-linked mannose residue of the $Man_5GlcNAc_2$ structure.

The reaction of GlcNAc transferase I is apparently essential for further processing of the α-1,3-linked and α-1,6-linked mannose residues by specific mannosidase(s) (110). The $GlcNAc(Man)_3GlcNAc_2$ structure is a substrate for GlcNAc transferase II which initiates the second antenna. The structure so formed ($GlcNAc_2Man_3GlcNAc_2$) is the substrate for GlcNAc transferases III and IV. GlcNAc transferase III can act to form the bisecting structure (111); GlcNAc transferase IV can act to catalyze the addition of GlcNAc in β-1,4-linkage to the mannose residue in α-1,3-linkage to the core to initiate the third antenna (109). The complex oligosaccharide can be completed by addition of galactose, fucose and N-acetylneuraminic acid by galactosyl, fucosyl and sialyltransferase enzymes located mainly in the Golgi complex.

1.4. Role of the nucleotide sugars

As activated glycose donors nucleotide sugars play a central role in the synthesis of oligosaccharide chains of glycoproteins. The scheme for the synthesis of nucleotide sugars is shown in Fig. 10. It is valid for different organisms and tissues including liver.

The enzymes involved in nucleotide sugar synthesis are located in the cytoplasm (112,113) with the possible exception of the sialic acid-activating enzyme found in the nuclear fraction of a variety of tissues including liver (112).

The intracellular concentrations of nucleotide sugars are under stringent control. The synthesis of the nucleotide

sugars can be regulated by controlling the first enzymatic step in the sequence of reactions involved in their synthesis. L-Glutamine:D-fructose 6-phosphate aminotransferase is the key enzyme in the formation of UDP-GlcNAc, UDP-GalNAc and CMP-NeuAc from glucose. This enzyme performs the transfer of an amino group from glutamine to fructose-6-phosphate. The resulting Schiff's base undergoes enolization to form an aldehyde center at the C-1 position and the ring is expanded from the furanose to the pyranose form (114). This enzyme is inhibited by UDP-GlcNAc. UDP-GlcNAc is formed from GlcNAc-1-P and UTP (115), GlcNAc-1-P can be derived from glucose or GlcN (glucosamine) (Fig. 10). GlcN is first acetylated and subsequently phosphorylated by a specific kinase to yield GlcNAc-6-P, which is then transformed to GlcNAc-1-P and finally converted to UDP-GlcNAc. UDP-GalNAc is formed from UDP-GlcNAc by UDP-GlcNAc 4-epimerase (116).

CMP-NeuAc is formed from UDP-GlcNAc. The first enzyme involved is UDP-GlcNAc-2-epimerase which is inhibited by CMP-NeuAc, thus exercising a negative feedback on its own synthesis.

GDP-Man is formed from glucose <u>via</u> fructose-6-P, Man-6-P and Man-1-P (Fig. 10). GDP-Man is also formed from mannose. The formation of GDP-Man from Man-1-P is catalyzed by GDP-Man pyrophosphorylase (117). GDP-Man is a precursor for the formation of GDP-Fuc which requires the formation of an intermediate, GDP-4-keto-6-deoxymannose by GDP-Man

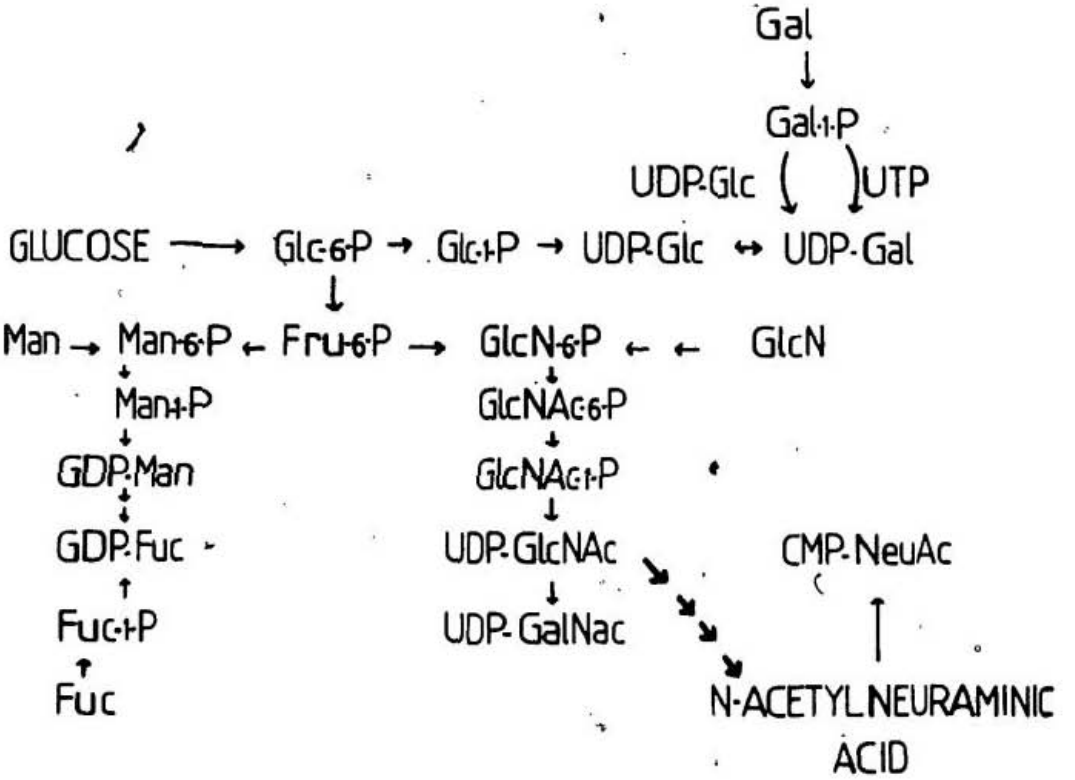

Fig. 10. Biosynthetic pathways of formation of nucleotide sugars. Abbreviations: Glc, glucose, Man, Mannose, Fru, Fructose, GlcN, glucosamine; GlcNAc, N-acetylglucosamine; Gal, Galactose; GalNAc, N-acetyl-galactosamine; Fuc, Fucose; NeuAc, N-acetylneuraminic acid; P, phosphate.

oxidoreductase (118). GDP-Fuc can also be synthesized from Fuc *via* Fuc-1-P (Fig. 10).

UDP-Gal is formed from UDP-Glc *via* epimerization by UDP-Glc-4-epimerase. Galactose can also be converted to UDP-Gal. Galactose is first phosphorylated at carbon atom 1 by galactokinase to yield to Gal-1-P, which can be converted to UDP-Gal either by hexose-1-phosphate uridylyltransferase, in a reaction involving UDP-Glc or by galactose-1-phosphate uridylyltransferase, in a reaction which involves UTP.

The intracellular levels of nucleotide sugars is also controlled by their catabolism. Nucleotide sugar pyrophosphatases which hydrolyze nucleotide sugars have been identified in various tissues (119,120,121). The specific roles of these hydrolytic enzymes are not known, but their role in regulating cellular nucleotide sugars can be predicted.

1.5. Export or secretion of glycoproteins

The biosynthesis of plasma membrane, lysosomal and secretory proteins and the process of their intracellular transport are thought to largely utilize the flow of intracellular membranes (122). According to this model these proteins are synthesized on polysomes attached to the rough endoplasmic reticulum. They are then segregated in the cisternal space of the rough endoplasmic reticulum, and are transported through the smooth surfaced elements of the endoplasmic reticulum to the Golgi apparatus where the different classes of proteins are thought to become concentrated

at specific sites, since they have been programmed for secretion or sequestration in specific cellular organelles. The "differentiated" region of the Golgi gives rise to vesicles containing lysosomal, secretory and/or membrane proteins. These vesicles eventually transfer their contents to the appropriate pre-existing cellular organelle by membrane fusion or exocytosis.

The various compartments of the endoplasmic reticulum-Golgi membrane system are thought to be functionally connected by means of a shuttle, wherein small vesicles bud off from one compartment and fuse with the next compartment. These vesicles apparently travel along well defined paths as they move through the various intracellular compartments of the endoplasmic reticulum-Golgi membrane system. Exactly how these membrane vesicles recognize the appropriate proteins is unknown. The selective assortment of lysosomal, secretory and membrane proteins must require a very sophisticated intracellular recognition system. The recognition system is presumably composed of discrete molecular entities within the structural matrix of the endoplasmic reticulum-Golgi complex which recognize specific molecular structures on the exported products. In some cases, it is assumed that it is the oligosaccharide of the glycoproteins which determines the ultimate localization of proteins. Most, if not all, lysosomal acid hydrolases are glycoproteins and there is evidence that routing of these enzymes to lysosomes requires

the specific oligosaccharide moiety (123,124).

A unique terminal sugar residue, mannose-6-P, has been identified on lysosomal enzymes. Sly and Stahl (124) have suggested that the mannose-6-P binding site is localized in a specific region in the GERL (Golgi-endoplasmic reticulum lysosome) complex and function to bind and concentrate glycoproteins with terminal mannose-6-P residues for packaging into vesicles which then deliver the glycoproteins to lysosomes (125).

1.6. Introduction to present work.

During the acute-phase response, the synthesis of certain serum proteins termed "acute-phase proteins" is significantly increased (2,9,18,126,127). Almost all acute-phase proteins contain significant amounts of N-linked carbohydrate and are synthesized via dolichol-linked intermediates (37,38) in the liver parenchymal cells. It has been shown that turpentine injection into rats causes a 6-fold increase in the level of α_1-acid glycoprotein in serum (2,126). Besides α_1-acid glycoprotein, several other acute-phase proteins are also known to increase during the acute-phase response due to the increased synthesis by the liver.

The mechanism of increased synthesis of the acute-phase proteins in response to inflammation is largely unknown. Glucocorticoids (128,129) and certain macrophage factors

(130,131), i.e. cytokines, hepatocyte stimulating factor (HSF) and interleukin-1 (IL-1) have been proposed to be involved in the induction of the acute-phase proteins.

Recent studies using adrenalectomized rats have shown a pronounced decrease in the synthesis of certain acute-phase proteins in response to turpentine induced inflammation which were restored by replacement therapy with cortisol (33,34). Glucocorticoids have also been shown to increase the synthesis of certain acute-phase proteins, eg. fibrinogen, α -macrofetoprotein and α_1-acid glycoprotein in intact and adrenalectomized rats (132,133,136,137) as well as in hepatocyte suspension culture (32). Similar results were also obtained using hepatoma tissue culture cells (133) and perfused liver (134).

Although glucocorticoids may be involved in the induction of the acute-phase proteins, current theories suggest that macrophage factors and other mediators are also required for the full expression of the elevated synthesis of these proteins. The effect of IL-1 and HSF on the increased synthesis of the acute-phase proteins has been demonstrated (131). Studies using adrenalectomized rats have shown a small increase of the acute-phase proteins after turpentine induced inflammation (132,133,135), suggesting that a second effector system, other than glucocorticoid, may also mediate the increased synthesis of the acute-phase proteins during inflammation.

Cytokine, the acute-phase mediator, increased the synthesis of α_2-macroglobulin, fibrinogen, α_1-acute-phase globulin, α_1-acid glycoprotein, haptoglobin and α_1-proteinase inhibitor in cultured rat hepatocytes. These cytokine effects on the acute-phase protein synthesis required the presence of dexamethasone (138). Regulation of monokine production during an inflammatory challenge is complex (139,140). For instance, glucocorticoids inhibit IL-1 production (140), as well as pituitary and leucocyte production of ACTH. The synthetic glucocorticoid, dexamethasone, inhibits by 75 percent production of HSF by peripheral blood monocytes (141) which suggests a feedback inhibitory loop. These data indicate a regulatory interaction between the monocytes and the pituitary-adrenal axis. Tissue injury or inflammation elicits HSF and IL-1 production, which in turn regulates the synthesis of the acute-phase proteins. These monokines also activate the pituitary-adrenal axis and increase glucocorticoid concentrations. These steroid hormones may then act on hepatocytes to enhance plasma acute-phase protein synthesis; on monocytes to block HSF and IL-1 synthesis; and on pituitary cells to inhibit ACTH release and subsequent glucocorticoid production. Langstaff et al (15) have shown that inflammation invariably results in a marked increase in serum cortisol concentration in rats.

The aims of the present studies were to demonstrate the effects of turpentine induced inflammation and of dexamethasone

on the factors involved in the synthesis of N-linked carbohydrates of glycoproteins. Increased activities of several nucleotide sugar: glycoprotein glycosyltransferases have been demonstrated in liver membranes from inflamed rats (52,53).

Since the synthesis of N-linked glycoproteins depends on a functional dolichol cycle, the effect of inflammation and dexamethasone on the synthesis of dolichol linked intermediates was studied.

In order to minimize experimental variability that occurs when using whole animals to study the effect of various hormones, in vitro experiments are often used. Therefore, a hepatocyte suspension culture system was developed and used in the present studies.

The effect of dexamethasone on the activities of mannosyl and N-acetylglucosaminyltransferases was determined in rat hepatocytes from normal and inflamed rats. Experiments were performed to synthesize and characterize the dolichol linked oligosaccharide using intact hepatocytes and/or homogenates.

Since dolichol functions as a oligosaccharide carrier during the assembly of N-linked glycoproteins, it has been suggested that alterations in the levels of dolichol and dolichol phosphate could regulate the synthesis of N-linked glycoproteins. Experiments were carried out to determine the rates of synthesis of dolichol and dolichol phosphate using mevalonate as a precursor.

Nucleotide sugar pyrophosphatases have been implicated in the regulation of nucleotide sugar levels, which in turn, are important factors in the synthesis of N-linked glycoproteins. Hence, the levels of the hepatic nucleotide sugar pools were measured and the effects of inflammation and dexamethasone on the nucleotide sugar pyrophosphatase activities were determined.

The results of the present studies have provided new information in our understanding of the role of glucocorticoids and inflammation on the control of dolichol-linked intermediates involved in the synthesis of N-linked glycoproteins.

CHAPTER II

MATERIALS AND METHODS

2.1. MATERIALS

GDP-[U-^{14}C]mannose (269 mCi/mmol); UDP-N-acetyl-D-[U-^{14}C]glucosamine (283.8 mCi/mmol); D-[1-^{14}C]mannose (48.6 mCi/mmol); (RS) - [5-^{3}H] mevalonolactone (30 Ci/mmol); CMP-[4-^{14}C]sialic acid (1.8 mCi/mmol); UDP-[U-^{14}C] galactose (47.2 mCi/mmol) were purchased from New England Nuclear Corp., Lachine, Quebec, Canada.

GDP-D-mannose, UDP-N-acetylglucosamine, UDP-glucose, dolichol phosphate, collagenase (type IV), streptomycin sulfate, Penicillin G, cycloheximide, actinomycin D, Triton X-100, were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); Waymouth's Medium MB 752/1 was from Gibco Laboratory Supplies, Grand Island, N.Y., U.S.A. and frozen horse serum was from Flow Laboratories, Melean, VA, U.S.A. The phosphate salt of dexamethasone was from Organon (Canada), Toronto, Ont. and DEAE cellulose (DE-52, microgrannular) was from Whatman, England. Sources of other chemicals were: Biogel P-4 (minus 400 mesh) from Bio-Rad Lab., Richmond, CA, 94804, U.S.A. Turpentine oil from Record Chemical Inc., Toronto, Canada. P-nitrophenyl α-D-mannopyranoside, p-nitrophenyl-α-D-maltoside, p-nitrophenyl - α -D-maltotrioside, p-nitrophenyl- α -D-maltotetraoside, p-nitrophenyl- α -D-maltopentaoside, P-nitrophenyl- α -D-maltohexaoside, P-nitrophenyl- α -D-maltoheptaoside were from Calbiochem Biochemicals, La Jolla,

CA, U.S.A.

2.2. Experimental Procedures

A. Animals and induction of inflammation

Male Sprague-Dawley rats (300-350g body wt.) were maintained on a diet of Purina Laboratory Chow and water. These rats were divided into two groups and inflammation was induced in one group by a subcutaneous injection of 0.5ml turpentine per 100g body weight into the dorsolumbar region. The rats used as controls were injected with 0.5 ml/100g body weight of sterile 0.15 M sodium chloride. After injection, rats had access to water but no food for 18 h. Hepatocytes were isolated between 10 and 11 a.m., 24 h after injection of turpentine.

B. Isolation of Hepatocytes

Fig. 11 depicts the liver perfusion system used for the isolation of hepatocytes. The buffer reservoir was a 26 cm wide glass beaker whose temperature was maintained at 37°C. A plastic disk consisting of two vertical tubes was placed in the beaker and connected to the oxygenator. The oxygenator was a glass cylinder 10 cm high and 1.5 cm wide with four openings; one inlet for gas mixture (95% O_2, 5% CO_2); one inlet for perfusate; one outlet connected to the cannula (and the liver); and one outlet for the gas and shunted perfusate (bypassing the liver). The steel cannula had a sharp tip with a movable plastic cover which was used to

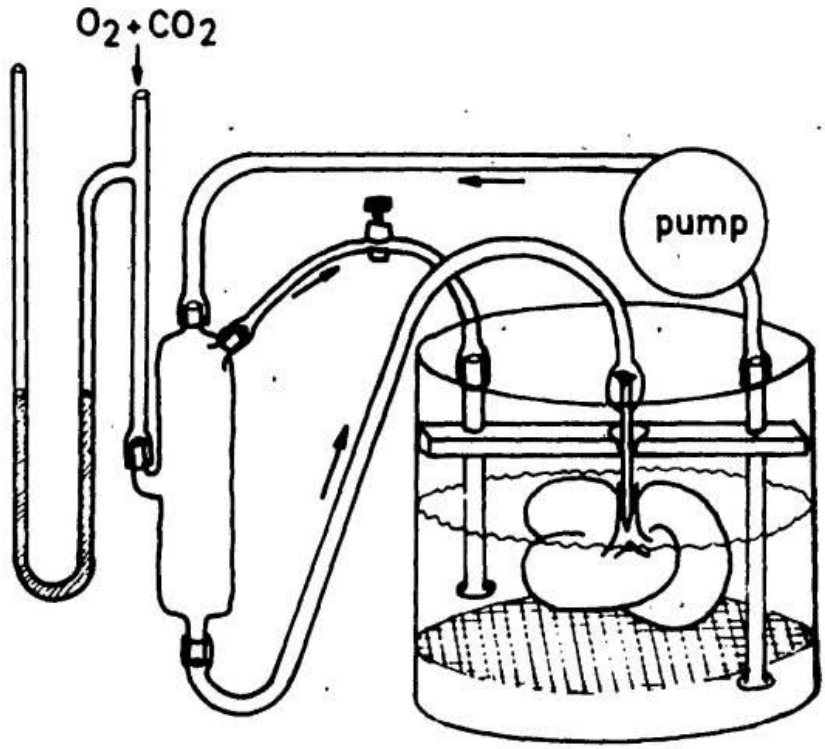

Fig. 11. Schematic diagram of the liver perfusion system.

prevent the breaking of the portal vein during the insertion of the sharp tip of the cannula. The various parts of the system were connected with appropriate sized tygon tubing.

Rats were anesthesized with ether and the peritoneal cavity was opened by a midventral incision. An oblique incision was made in the mesenteric part of the portal vein and the cannula was immediately inserted. The cannula was clamped and non-circulatory perfusion was started instantly in situ with oxygenated Hank's Buffer (142) at a flow rate of 40 ml/min. The vena cava was cut open and the liver was removed. After 4 min of perfusion with Hanks buffer, the reservoir beaker was emptied and replaced with a modified Hank's buffer containing 0.12% (w/v) collagenase and 4 mM Ca^{++}. After 15 min of circulating perfusion with modified Hanks buffer, the swollen liver was excised from the cannula and the cells were dispersed with a pair of scissors and by gentle stirring movements in Waymouth's Medium. The dispersed cells were filtered through four layers of sterile gauze to remove any remaining connective tissue and clumps of cells. The filtrate was centrifuged at 70xg for 1 min at 4°C to obtain the cells. These cells were washed three more times and finally suspended in Waymouth's Medium containing 17.5% (v/v) heat inactivated horse serum.

C. Incubation of Hepatocytes

Unless otherwise specified, a suspension of hepatocytes

($1x10^6$ cells/ml) was incubated in a 150 ml Erlenmeyer flask with Waymouth's Medium supplemented with 17.5% (v/v) heat inactivated (heated at 56°C for 30 min) horse serum in a total volume of 40 ml and flushed continuously with O_2/CO_2 (19:1) gas mixture, either in the presence or absence of dexamethasone. In all experiments the medium contained penicillin-G (60 i.u./ml) and streptomycin sulfate (0.05 mM). Cells from control or inflamed rats were incubated either in the absence or presence of a single or different dosages of dexamethasone. The cells in the absence of dexamethasone were treated with equal volume of 0.15M sodium chloride solution. The cell suspension was maintained at 37°C with gentle shaking. Dexamethasone or other additions were made 1 h after the cells were incubated, and the incubation was allowed to continue for a further 12 h or other specified periods. In some experiments [^{14}C] mannose (0.4 μCi/ml of incubation medium) was used in the incubation. For the subfractionation study the cells were washed twice with ice cold 0.25M sucrose solution after the incubation and homogenized in 0.04 M Tris-HCl (pH 7.4) containing 0.25M sucrose as described in Section D. In some experiments cycloheximide or actinomycin D were added to the incubation. These inhibitors inhibited macromolecular synthesis by 90%, but the cell viability under these conditions was maintained at 85-90%. To estimate the number of viable cells during incubation, lactate dehydrogenase activity was monitored periodically

(143). Tryptan blue-exclusion test (144) was also used to estimate viability and showed a similar high level of viability.

D. Homogenization and fractionation of hepatocytes

The cells were washed with 0.25 M sucrose solution, suspended in 10mM-Tris/HCl buffer, (pH 7.4) containing 0.15M sodium chloride and homogenized in a glass mortar with a Teflon Pestle for about ten strokes at 500 rev/min. Further disruption of the cells was done by sonication using the fine tip of a Branson Sonifer (Model S-125; Branson Instruments, Melville, Long Island, N.Y., U.S.A.) at a setting of 0.5A. Sonication was performed in a cooling bath for a total of 30 seconds with 10 seconds for each burst at 2 min intervals. The homogenate prepared in this way was used for enzyme assays. In some experiments, the homogenate was centrifuged at 105,000xg in a 50 Ti fixed-angle rotor for 60 min. The pellet obtained was resuspended in the homogenizing medium, designated as a 'total membranes preparation, and used for enzyme assays (in experiments to study nucleotide sugar pyrophosphatase, Chapter VI).

To prepare different subcellular fractions, the homogenate was centrifuged at 480xg for 10 min in Sorvall SS-34 rotor to remove the debris and nuclei. The supernatant was removed and centrifuged at 4300xg for 20 min and the pellet resuspended in the homogenizing buffer.

For the preparation of total microsomal fraction, the suspension of the disrupted cells was centrifuged at 10,000xg

for 20 min in a Sorvall SS-34 rotor. The supernatant was removed and centrifuged at 105,000xg in a Beckman L5-50 ultracentrifuge for 60 min using a 50 Ti rotor to give the total microsomal fraction. It has been shown previously, that the microsomal vesicles obtained by the sonication procedure exhibit, the same properties as vesicles obtained by other procedures (145). The marker enzyme assays for subcellular fractions were done as described by de Duve et al, (146) and protein measured by the method of Lowry et al. (147) using bovine serum albumin as standard.

E. Preparation of microsomes from rat liver

The method used for the preparation of microsomes was as described by Caccam et al (148). Rats were sacrificed by exsanguination under ether anaesthesia and the livers removed. All subsequent procedures were performed at 4°C. Weighed livers were homogenized using a Polytron homogenizer in 1.3 volumes of 0.05M Tris-HCl, pH (7.4) containing 0.25M sucrose. The homogenates were centrifuged at 20,000xg for 30 min in a Sorvall RC5 centrifuge using a type SS 34 rotor. The supernatants were decanted and recentrifuged at 145,000xg for 75 minutes using a type 40 rotor in a Beckman L5-50 ultracentrifuge. The subsequent microsomal pellets were washed and then resuspended in homogenizing buffer to a final protein concentration of 20 $mg.ml^{-1}$.

F. Enzyme Assays

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

i) Mannosyltransferase

Hepatocyte homogenates or other subcellular fractions (0.3mg protein) were incubated with GDP-[^{14}C] mannose (14 nmol) in a final volume of 80µl containing 6.5 mM $MnCl_2$, 1.5 mM EDTA, 19 mM Tris/HCl buffer, pH (7.4) and 0.03% (w/v) Triton X-100. Dolichol phosphate in chloroform/methanol solution, when included in the incubation, was first evaporated to dryness and then solubilized in the required amount of Triton X-100 by sonication in a Branson (Shelton, CT, U.S.A.) ultrasonic sonicator. Incubations were performed at 37°C either for 1 min or as stated otherwise in the text. Dolichol phosphate mannose was extracted as outlined in Fig. 12.

ii) N-acetylglucosaminyltransferase

Hepatocyte homogenate (0.3 mg protein) was incubated with UDP-(^{14}C]-N-acetylglucosamine (16 nmol) in a final volume of 310µl containing 6.5 mM Tris/HCl buffer, pH (7.4); 6.5mM $MnCl_2$; 1.5mM EDTA; Triton X-100, 0.15%(w/v) and dolichol phosphate. Dolichol phosphate was first evaporated to dryness and then solubilized in the required amount of Triton X-100 by sonication. Incubations were performed at 37°C. $(GlcNAc)_{1-2}$P-P-Dol was extracted as shown in Fig. 12.

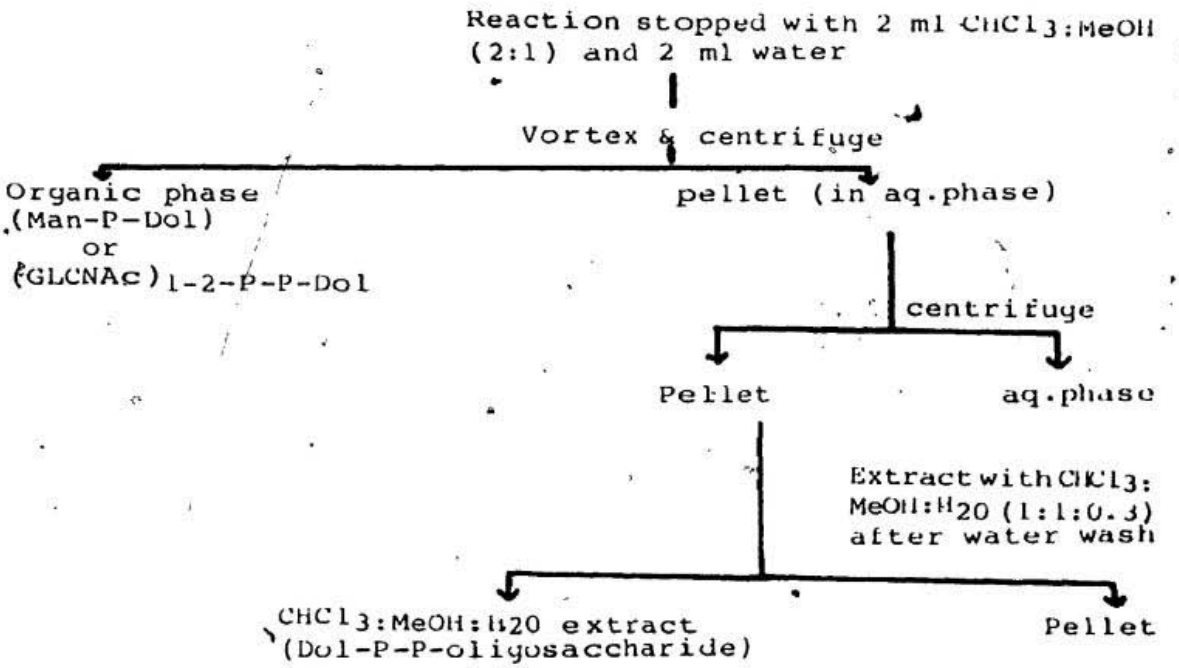

Fig. 12. Isolation of dolichol linked sugar

iii) Sialyltransferase

Enzyme assays were in a total volume of 50µl containing either serum (0.7-0.9 mg protein), liver homogenate (75-150µg protein) or liver microsomes (10-20µg protein); 6 nmol CMP-[^{14}C]sialic acid; 6.25nmol MES-buffer, pH (6.8), and 500µg asialofetuin (fetuin depleted of sialic acid). Use of asialofetuin measured mainly the synthesis of N-linked glycoprotein, although a small percentage of N-linked glycoprotein structure is also present in fetuin. Triton X-100 when included was used at a concentration of 0.5% (w/v). Incubations were done at 37°C for 60 min and terminated by the addition of 1ml of 5% trichloroacetic acid/1% phosphotungstic acid mixture, 1:1 v/v). Precipitates were filtered under suction through 2.4 cm glass fibre filters (No. 934 AH, Reeve Angel) and washed with 10 ml trichloroacetic acid/phosphotungstic acid mixture and 7 ml of ethanol/ether (1:1, v/v). The discs were dried and counted for radioactivity in a toluene based scintillation solution using a Beckman LS-9000 Scintillation Counter.

iv). Galactosyltransferase

The assay volume of 50µl contained either serum, liver homogenate or liver microsomes as above; 3 nmol UDP-[^{14}C]galactose, 6.25 nmol MES buffer, pH (6.8), and 250µg asialoagalactofetuin (fetuin depleted of sialic acid and galactose). Assays were incubated at 37°C for 60 min and the radioactivity incorporated into the product was measured

as described for sialyltransferase.

Enzyme activity in the absence of exogenous acceptor was less than 3% of that obtained in its presence.

v) Succinate:cytochrome c reductase

The activity was measured using subcellular fractions of hepatocytes in Quartz cuvettes of 1 cm light path. The final incubation mixture contained 0.05M potassium phosphate buffer, pH (7.4), 0.2mM potassium cyanide, 0.04mM oxidized cytochrome C and 0.05mg protein in a total volume of 3 ml. The reaction was started by adding 0.05M sodium succinate and the change in optical density was recorded at 550 nm. Molecular extinction coefficient (ε = 18.5 mM^{-1}) of reduced cytochrome c reductase was used for calculation of enzyme activity.

vi) NADPH-cytochrome c-reductase

The activity of NADPH-cytochrome c reductase was measured as described for succinate-cytochrome c-reductase, except that the incubation mixture contained 0.03M NADPH, instead of sodium succinate. Enzyme activity was measured as described for succinate: cytochrome c reductase.

vii) β-glucuronidase

β-Glucuronidase was assayed by measuring the liberation of phenolphthalein from phenolphthalein- β -glucuronide. Incubation in a final volume of 1 ml containing 1.25 mM phenolphthalein- β -glucuronide, 75 mM acetate buffer, pH (5.2) and subcellular fractions (0.1 mg protein) was carried

out for 15 minutes at 37°C. The reaction was stopped by adding 3 ml of a solution containing 133mM glycine, 83mM sodium bicarbonate and 67 mM sodium chloride pH (10.7) and the mixture was centrifuged for 15 min at 2,000 rpm. The supernatant was used to measure the liberation of phenolphthalein by measuring the optical density at 560 nm.

Characterization of product formed by Mannosyl and N-acetylglucosaminyltransferase

The glycolipid in the lower phase of the chloroform/methanol (2:1) extract from the mannosyltransferase assay was characterized as dolichol phosphate mannose as described previously (149). The glycolipid from the lower phase of N-acetylglucosaminyltransferase assay was dried under nitrogen and subjected to mild acid hydrolysis (150) and applied to Whatman No.1 paper. GlcNAc and N,N'-diacetylchitobiose standards were spotted on separate lanes. Descending paper chromatography was carried out in a solvent consisting of 1-butanol/pyridine/water (6:4:3, by vol). Bands corresponding to N,N'-diacetylchitobiose and GlcNAc were cut from the lanes and counted for radioactivity in toluene/omnifluor. Standards were visualized with aniline/diphenylamine reagent (151).

viii) Synthesis of oligosaccharide lipid

[^{14}C-mannose]oligosaccharide lipid was prepared by incubating hepatocyte homogenate (0.8mg protein) with GDP-

[^{14}C]mannose (0.14 nmol) and unlabelled GDP-mannose (0.23 mM) in a final volume of 310µl containing 0,5M Tris/HCl (pH 7.4); 10mM $MnCl_2$; 2mM UDP-GlcNAc, 1.2 mM UDP-Glc and 0.03% (w/v) Triton X-100. Dolichol phosphate, when included, was first evaporated to dryness and then solubilized in the required amount of Triton X-100 by sonication. Incubations were performed at 37°C for 15 min. In some experiments, the pyrophosphatase inhibitors, ATP (100mM) and dimercaptopropanol (5 mM) were used. Dimercaptopropanol was added just prior to initiation of the reaction and nitrogen was blown over the incubation mixture to prevent oxidation of dimercaptopropanol-Mn complex. The reaction was terminated by addition of 2 ml of chloroform/methanol (2:1, v/v) and 2 ml water. The chloroform phase was removed and the aqueous phase containing the pellet was extracted 2 more times. The pooled chloroform extract was discarded. The pellet was washed 3 times with 2 ml water to reduce the background count due to unreacted radioactive substrate. The pellet was extracted 3 more times with 2 ml of chloroform/methanol/water (1:1:0.3, by vol). The extracts were pooled, dried under nitrogen and stored at -20°C in a minimum volume of the same solvent for further analysis of oligosaccharides.

For oligosaccharide lipid synthesis in intact hepatocytes, the cells ($1x10^6$ cells/ml) were incubated as described previously with the exception that 0.4-0.6 µ Ci of [^{14}C]mannose/ml of incubation medium was included in the

medium. At the end of incubation, the cells were centrifuged at 70xg, for 1 min at 4°C and washed 3 times with 4 ml of ice cold Hank's buffer. The labelled cells were immediately dispersed in 4 ml chloroform/methanol (2:1, v/v) and 2 ml water. The subsequent extraction and isolation procedure was identical to that described for the isolation of oligosaccharide lipid in hepatocyte homogenate except that the volume of extractant was increased to 4 ml. The residual pellet after extraction with chloroform/methanol/water (1:1:0.3, by vol.) was digested with tissue solubilizer and counted to measure the incorporation of radioactivity in the protein fraction.

G. Purification of oligosaccharide lipid by DEAE-cellulose chromatography.

DEAE cellulose (DE-52, microgranular) was converted into the acetate form by soaking overnight in 1M acetic acid. Excess acid was removed by filtering the resin, which was then washed with water followed by methanol (2 litres). The resin was packed to a height of 7 cm in a 0.8 cm-diameter column and washed with methanol (50 ml) and then with 50 ml chloroform/methanol/water (10:10:3, by vol.). The sample was applied in chloroform/methanol/water (10:10:3, by vol). and eluted with a sequential discontinuous gradient of 0,10,25,100 mM ammonium acetate in the same solvent at a flow rate of 30 ml/h. Samples were taken for liquid

scintillation counting of radioactivity. Appropriate fractions were pooled and the solvent evaporated on a rotary evaporator under reduced pressure at 37°C. Ammonium acetate was removed during freeze drying by repeated addition of water.

H. Analysis of oligosaccharide derived from oligosaccharide-lipid.

The oligosaccharide was released from oligosaccharide-lipid by hydrolysis in 0.02N HCl containing 20% methanol for 20 min at 100°C. After cooling, the solution was neutralized with 0.1N NaOH and an equal volume of chloroform was added to partition the lipid. The upper aqueous phase was removed and freeze dried. The oligosaccharide was dissolved in 0.1M pyridine acetate buffer, pH (5.0) and applied to a 1.5 x 170 cm column of Bio-gel P-4 (minus 400 mesh) equilibrated with 0.1M pyridine acetate. A flow rate of 4 ml/h was maintained by use of a peristaltic pump and fractions (0.4 ml) were collected. Aliquots of 100-200µl were removed for determination of radioactivity. The column was calibrated using blue dextran, p-nitrophenyl β-D-mannopyranoside, p-nitrophenyl-α D-maltoside, p-nitrophenyl-α-D-maltotrioside, p-nitrophenyl-α-D-maltotetraoside, p-nitrophenyl-α-D-maltopentaoside, p-nitrophenyl-α-D-maltohexaoside, p-nitrophenyl-α-D-maltoheptaoside, and the following standards: $Man_6GlcNAc$, $Man_7GlcNAc$, $Man_8GlcNAc$, $Man_9GlcNAc$. (Kindly supplied by Dr.

Stuart Kornfeld, Dept. of Medicine, Washington University of School of Medicine, St. Louis, Missouri, U.S.A.).

I. Nucleotide Sugar Pyrophosphatase

Assay of UDP-N-acetylglucosamine pyrophosphatase activity was carried out essentially using the method of Mookerjea (152). Unless otherwise specified, each complete assay mixture contained 6.5 mM Tris/HCl buffer, pH (9.0); 6.5 mM $MnCl_2$; UDP-[^{14}C]-N-acetylglucosamine (0.4 nmol); 2.5 mM unlabelled UDP-N-acetylglucosamine; 1.6% (w/v) Triton X-100 and 80 µl 'total membrane preparation' in a total volume of 300µl. Unless otherwise specified, after 5 min of incubation at 30°C, the reaction was stopped by adding 100µl of ethanol and a drop of 5% acetic acid. Insoluble precipitates were removed by centrifugation at 2500 rpm for 10 min and the supernatant was applied as a streak on Whatman 3 MM paper. The descending chromatographic separation of the products of the reaction was performed using a solvent system containing 75 ml of ethanol and 30 ml of 1 M sodium acetate buffer, pH (3.8) (153). The segments of chromatogram corresponding to standard UDP-N-acetylglucosamine, N-acetylglucosamine phosphate and N-acetylglucosamine were cut out and counted in a toluene-based scintillation solution. GDP-mannose pyrophosphatase activity was also measured using the incubation mixture described for UDP-N-acetylglucosamine pyrophosphatase, with the exception that GDP-[^{14}C]mannose (0.14 nmol) and unlabelled

GDP-Manhose (1.3 mM) were used, instead of UDP-[^{14}C]-N-acetylglucosamine and unlabelled UDP-N-acetylglucosamine.

J. Isolation and quantitation of nucleotide sugars from hepatocytes.

Nucleotide sugars were extracted from whole cell homogenate according to the method described by Khym (154). Hepatocyte homogenates (1 ml) were mixed vigorously for 1 min with 1 ml of perchloric acid (3.5%, w/v) on a vortex mixer. The acid mixture was then added to 1 ml of 0.5 M tri-N-octylamine in Freon solution. The solution was mixed vigorously for 3 min and the aqueous phase was removed with a Pasteur pipette. The pH of the resulting mixture was between 4 and 6. The mixture was frozen immediately after extraction.

In some experiments [^{14}C]-labelled nucleotide sugars were added prior to extraction from hepatocytes to test for recoveries following high pressure liquid chromatography (HPLC). It was found that greater than 90% of the labelled nucleotide sugars was recovered from the HPLC column.

The nucleotide sugars were quantitated on an anion exchange column (75 x 7.5 mm) using a Perkin Elmer Series 4 high pressure liquid chromatograph equipped with a Perkin Elmer LC-95 UV/visible spectrophotometer detector operating at a wavelength of 254 nm. The high concentration eluant was 0.5 M KH_2PO_4, pH 4.5 (A), the low concentration eluant was 0.005 M KH_2PO_4, pH 4.0 (B). The eluant A was used with a linear gradient of

0-9% for 30 min; 9-46% for 15 min and 46-100% for 45 min with a flow rate of 1 ml/min. Peak areas were electronically integrated using a Perkin-Elmer Model LCI-100 integrator using CTP as a standard.

K. Biosynthesis and isolation of [^{3}H]dolichol and [^{3}H]dolichol phosphate.

Hepatocytes isolated from control and turpentine-inflamed rats were incubated in 150 ml Erlenmeyer flasks in Waymouth's Medium under a continuous stream of carbogen (95% O_2, 5% CO_2). The cells were pulsed with 1 mCi of (RS)-[5-^{3}H]-mevalonolactone (sp. activity 30 Ci/mmol), for 2 h prior to termination of incubation, after which the cells were transferred into centrifuge tubes and washed 3 times with ice cold Hank's buffer (pH 7.4) and extracted with 20 ml of $CHCl_3$:MeOH (2:1, by vol.) and 20 ml of water according to the procedure described by Coolbear et al (155). After centrifugation the chloroform phase was removed and the aqueous phase containing the pellets was re-extracted. The chloroform phases from these extracts were pooled together. Thin layer chromatography was performed on Silica gel F-254 (E. Marck, Germany), after evaporation of the solvent and re-dissolving the lipid in 0.5 ml chloroform, using a solvent system of chloroform/ methanol/28% ammonium hydroxide (65:35:5, by vol.). The bands corresponding to standard dolichol and dolichol phosphate were scraped off and extracted

with chloroform:methanol (2:1, by vol.) and the products were further purified by HPLC on a C-18 reverse phase column as described below (Section L).

L. Purification and quantitation of [^{3}H]-dolichol and [^{3}H]-dolichol phosphate by HPLC.

A Perkin Elmer series 4 high pressure liquid chromatograph with a C_{18} reverse phase column (75 x 4.6 mM) maintained at room temperature was used to quantitate dolichol and dolichol phosphate. The mobile phase used for dolichol was an isocratic mixture of isopropanol: methanol (1:1; v/v) at a flow rate of 1 ml/min. Dolichol phosphate was eluted with a 1:1 mixture of isopropanol and methanol containing 10 mM phosphoric acid (156,157). The variable wavelength Perkin Elmer LC-95 UV/visible spectrophotometer detector operating at a wavelength of 210 nm was used to monitor dolichol and dolichol phosphate. Fractions corresponding to authentic dolichol and dolichol phosphate were pooled, dried under nitrogen and counted for radioactivity.

M. Isolation and quantitation of dolichol and dolichol phosphate from rat liver homogenates and microsomes.

Isolation and purification of dolichol and dolichol phosphate were exactly the same as described in section L. At the time of total lipid extraction, [^{3}H]-dolichol and [^{3}H]-dolichol phosphate were added as tracers to allow corrections

to be made for dolichol and dolichol phosphate lost during extraction and purification. Recoveries of label for dolichol and dolichol phosphate after HPLC were about 80 and 60 percent respectively. Peak areas for dolichol and dolichol phosphate were electronically integrated using a Perkin-Elmer model LCI-100 integrator.

CHAPTER III

EFFECT OF INFLAMMATION ON SIALYL AND GALACTOSYLTRANSFERASE ACTIVITIES IN LIVER AND SERUM OF RATS.

3.1. Introduction

As discussed in the general introduction, certain rat serum protein components which are known as 'acute-phase proteins' are significantly increased in response to inflammatory agents such as turpentine. Jamieson et al (126) have shown that α_1-acid glycoprotein and α_2-macroglobulin, the two major acute-phase proteins are significantly increased in the serum of rats after turpentine induced inflammation. The increase in these two proteins during an inflammatory challenge is due to their increased synthesis by liver (20). In addition to increased acute phase proteins, rat liver sialyl and galactosyltransferase activities are also increased 2-3 fold, 24-48 h following onset of inflammation (163).

There are several reports that malignant diseases in humans are accompanied by elevated serum sialyl and galactosyltransferase activities (53). An elevation of their activity in the serum of cancer patients has been related to alterations in glycoprotein synthesis in tumor cells. Elevated serum and liver sialyl and galactosyltransferase activities have also been reported in rats bearing tumors (158,159). The mechanism by which these enzymes are secreted into blood is not understood, and even less is known about any functional

role that these enzymes may have. Bernacki and Kim (158) have suggested that the elevated levels of serum sialyltransferase were probably the result of tumor cell surface shedding of sialyltransferase activity into the blood stream. However, a number of recent studies have shown that the increased sialyltransferase activity observed in rats and patients with malignant cancers may be the result of changes in the normal liver cells, rather than due to the shedding of the enzyme from the tumor cells (159,160).

Although sialyl and galactosyltransferases are located primarily in the Golgi apparatus, there is some evidence that these enzymes are also present on the plasma membrane (161,162). The present studies were undertaken to investigate the effect of turpentine-induced inflammation on sialyl and galactosyltransferase activities in the liver and serum of rats when the synthesis of glycoproteins is enhanced.

3.2. RESULTS

The results of the effect of inflammation on liver homogenate, microsomal and serum sialyltransferase activities are shown in Figs. 13,14 and 15 respectively. Turpentine-induced inflammation caused a 1.5 fold increase of sialyltransferase activity in liver homogenates and microsomes. The effect was apparent 18h after turpentine treatment and showed a maximum increase 36-48h following inflammation. Sialyltransferase activity in serum started to increase at

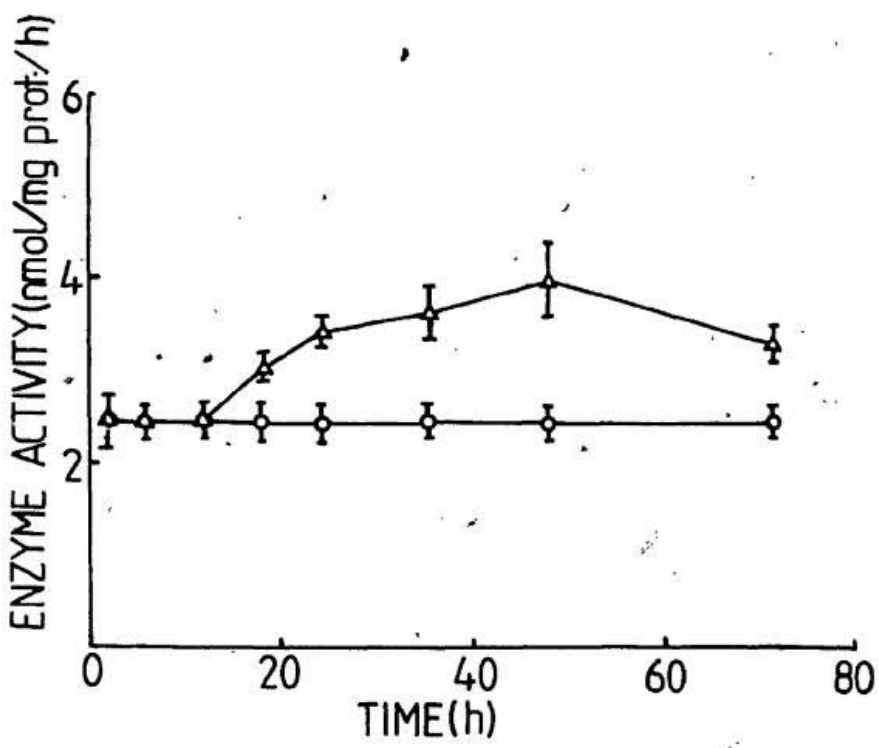

Fig. 13. Effect of inflammation on sialyltransferase activity in rat liver homogenate.

Rats were injected with turpentine or saline (0.9%) at 0 h and the animals were sacrificed at different times and the enzyme activity was measured. The results are expressed as mean ± S.D. from four separate experiments and the symbols are (o), control and (Δ), inflamed. P values starting from 18 h point are < 0.01.

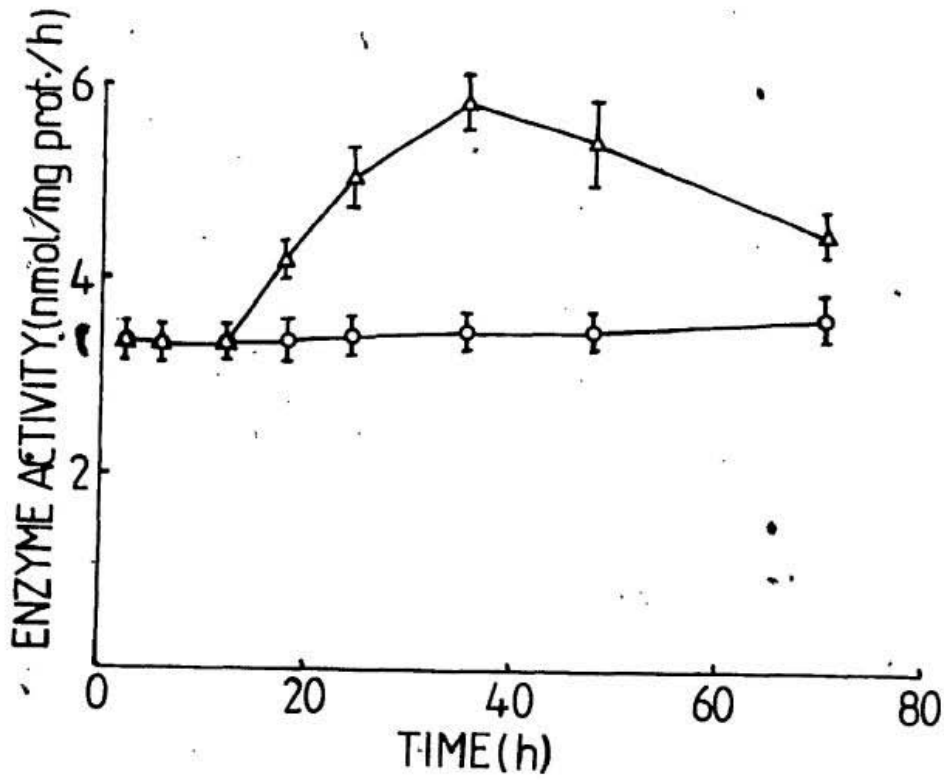

Fig. 14. Effect of inflammation on sialyltransferase activity in rat liver microsome.

Rats were injected with turpentine or saline (0.9%) at 0h and the animals were sacrificed at different times and the enzyme activity was measured. The results are expressed as mean ± S.D. from four separate experiments and the symbols are (o), control and (Δ), inflamed. P values starting from 18 h point are < 0.01.

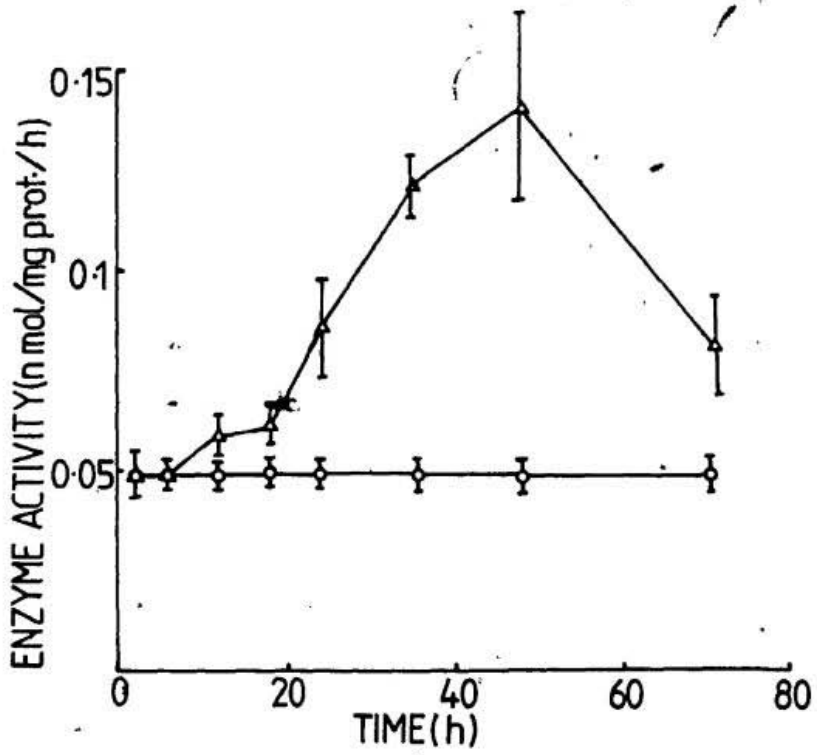

Fig. 15. Effect of inflammation on sialyltransferase activity in the rat serum.

Rats were injected with turpentine or saline (0.9%) at 0h and the animals were sacrificed at different times and the enzyme activity was measured. The results are expressed as mean ± S.D. from four separate experiments and the symbols are (o) control and (△), inflamed. P values starting from 18 h point are < 0.01.

18h and reached a maximum (4-fold increase) at 48h after injection of turpentine ($P < 0.01$).

Liver homogenate, microsomal and serum galactosyltransferase activities are shown in Figures 16-18. The activities in homogenates and microsomes started to increase 18-20h after turpentine injection and reached a maximum (2-fold) at 36-48h ($P < 0.01$). Unlike serum sialyltransferase activity, the serum galactosyltransferase activity did not increase significantly due to turpentine induced inflammation ($P = 0.05$).

Similar results were obtained by Kaplan et al (163) using liver slices from control and inflamed rats, who have shown the release of sialyl and galactosyltransferases into the medium. The maximum activity of the sialyltransferase was at 48h after inflammation.

3.3. DISCUSSION

The present studies show that the liver sialyl and galactosyltransferase activities were increased following the onset of turpentine-induced inflammation. The sialyltransferase activity was found to be increased in serum, whereas serum galactosyltransferase activity did not change significantly when compared with the control. As it has been mentioned previously, serum proteins which are increased in concentration during inflammation are termed acute-phase proteins. By this criterion sialyltransferase

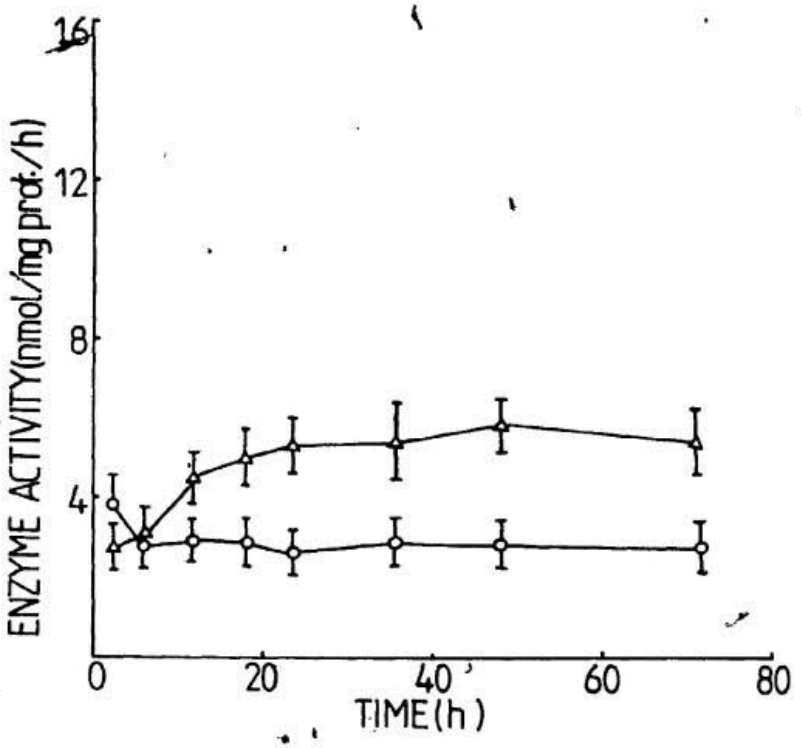

Fig. 16. Effect of inflammation on galactosyltransferase activity in rat liver homogenate.

Rats were injected with turpentine or saline (0.9%) at 0h and the animals were sacrificed at different times and the enzyme activity was measured. The results are mean ± S.D. from four separate experiments and the symbols are (O), control and (Δ), inflamed. P values starting from 12 h point are < 0.01.

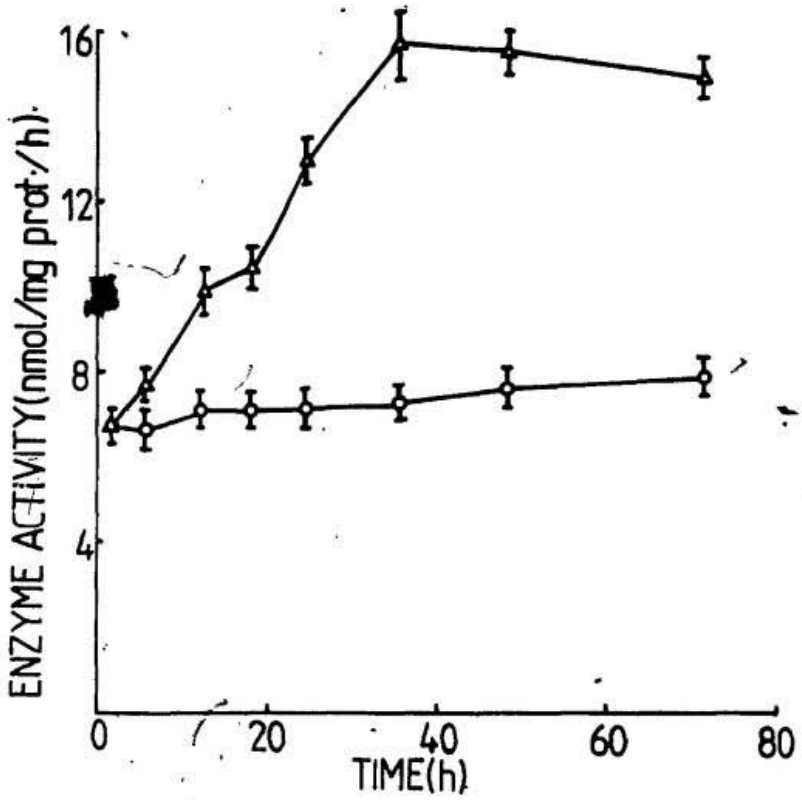

Fig. 17. Effect of inflammation on galactosyltransferase activity in rat liver microsome.

Rats were injected with turpentine or saline (0.9%) at 0h and the animals were sacrificed at different times and the enzyme activity was measured. The results are mean ± S.D. from four separate experiments and the symbols are (O), control and (△), inflamed. P values starting from 12 h point are < 0.01.

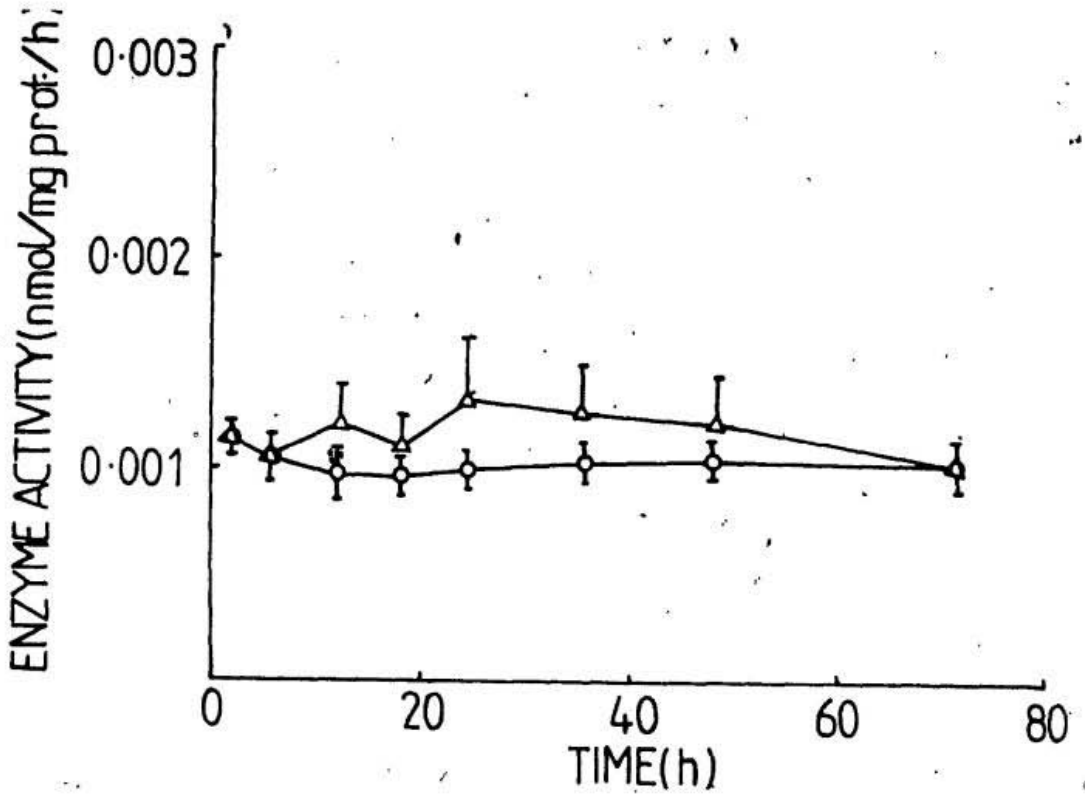

Fig. 18. Effect of inflammation on galactosyltransferase activity in the rat serum.

Rats were injected with turpentine or saline (0.9%) at 0h and the animals were sacrificed at different times and the enzyme activity was measured. The results are mean ± S.D. from four separate experiments and the symbols are (○), control and (△), inflamed. P value at 24 h point is = 0.05.

could be termed as an acute-phase protein, as suggested by Kaplan et al (163).

There is a controversy about the origin of serum sialyltransferase. Since the liver is the main source of serum glycoproteins, it has been suggested that it may also serve as a source for serum sialyltransferase (158). Hudgin and Schachter (164) compared the properties of porcine liver and serum sialyltransferase and observed that the serum activity closely resembled that of the liver enzyme. Several studies have demonstrated that serum sialyltransferase activity is elevated in patients with a variety of liver diseases (165,166) suggesting that sialyltransferase activity in serum could be secreted from the liver. However, a number of studies have also shown elevated levels of sialyltransferase in tumor tissue, suggesting that the elevation in serum sialyltransferase activity may be due to shedding of the enzyme activity from the plasma membrane of neoplastic cells (158,159).

Kaplan et al (163) have shown, using rat liver slices, that the increased serum sialyltransferase activity during inflammation originates from the liver. The enzyme in the medium of liver slice experiments and the serum enzyme were found to have similar properties. The pH optima and apparent Km values with respect to CMP-NeuAc and rat and human asialo-α_1-acid glycoprotein were very similar for the serum, liver slice and medium sialyltransferase activities.

It is not clear why turpentine-induced inflammation causes

release of sialyltransferase from liver. It has been observed that purified sialyltransferase exhibits micro-heterogeneity due to proteolytic cleavage of a polypeptide not required for catalytic activity, perhaps the polypeptide portion of the enzymes associated with the Golgi membrane. In view of the large number of changes that have been reported on inflammation (53,163), it is possible that proteolytic release of membrane-bound sialyltransferase could occur in response to inflammation. Marked increase of sialyltransferase, but not galactosyltransferase, in serum after turpentine treatment indicates that the former enzyme is not as tightly embedded in the Golgi membrane. This may cause a preferential release of sialyltransferase from the liver in inflammatory conditions. Evidence of a differential arrangement of these two enzymes on the lipid bilayer of Golgi membranes, has been presented (167). The changes in serum enzyme activity probably reflect the state of integration of these enzymes in membranous structures. It is conceivable that exclusive increased release of sialyltransferase into serum of turpentine-treated rats represent a specific change of the membrane organization of this enzyme in Golgi apparatus.

The significance of the increased level of sialyltransferase in serum under inflammatory conditions is not known. The liver enzyme is responsible for catalyzing the addition of sialic acid to the terminal position of N-linked complex type oligosaccharides, but the involvement

of the serum enzyme in the glycosylation of serum glycoproteins seems unlikely, because of the absence of asialo-protein acceptors as well as nucleotide sugars in the blood. The serum sialyltransferase increased on inflammation may be regarded as an acute-phase protein.

The increased serum sialyltransferase activity may result from translocation of Golgi membrane to the cell surface membrane, and sialyltransferase is, thereby, shed into extracellular space. Thus sialyltransferase may be merely a byproduct of membrane turnover without having any extracellular metabolic role. If sialyltransferase proves to be elevated in serum in all other types of inflammation, it may be possible to use measurement of this serum enzyme as a marker for acute inflammatory conditions.

Increases in liver homogenate glycosyltransferases have been ascribed to Golgi membrane proliferation in inflamed rats (52). The overall increase in the synthesis of acute-phase glycoproteins and their oligosaccharide moieties appears to be related to increased glycosyltransferase activities in the endoplasmic reticular and Golgi membranes. It has been shown previously in our laboratory that dolichol phosphokinase activity is increased within 12h after turpentine injection (168) resulting in greater amounts of dolichol phosphate in the microsomes. Further studies showed that dolichol phosphokinase activity is localized in the rough endoplasmic reticular membrane fraction (168). However,

studies by Lombart et al (52) show that the ultrastructure of rough and smooth endoplasmic reticular membranes is normal even 24 h after turpentine injection. Therefore, a generalized proliferation of Golgi membrane, as observed 24h after turpentine injection, cannot adequately explain the elevation of dolichol phosphokinase activity at a much earlier time and at different sites. Furthermore, elevated mannose and glucose incorporation into Man-P-Dol and Glc-P-Dol in rough and smooth endoplasmic reticular fractions within 24h after inflammation (168) suggest that events of dolichol-linked glycosylation of the oligosaccharide in the core region of the glycoproteins may be regulated by a mechanism other than Golgi proliferation. In view of the above discussion it appeared logical to extend further work primarily related to the dolichol linked mechanism of glycoprotein synthesis in inflammation and other conditions.

CHAPTER IV

EFFECT OF INFLAMMATION AND DEXAMETHASONE ON MAN-P-DOL AND $(GlcNAc)_{1-2}$-P-P-DOL BIOSYNTHESIS IN RAT LIVER MICROSOMES AND HEPATOCYTES.

4.1. Introduction

It has been mentioned earlier, that initiation of the precursor oligosaccharide results in the formation of GlcNAc-P-P-Dol and its conversion to $GlcNAc_2$-P-P-Dol. Both the first and second GlcNAc residues are donated by UDP-GlcNAc. The complete oligosaccharide lipid is formed by sequential addition of mannose residues to $(GlcNAc)_2$-P-P-Dol. The innermost four or five mannose residues are transferred directly from GDP-mannose. The last four mannose residues are transferred from Man-P-Dol. At the present time, the mechanisms which regulate the chain lengths of the oligosaccharide lipids are not clearly understood. Kean (169) has reported that Man-P-Dol is an allosteric activator of N-acetyl-glucosaminyltransferase which catalyzes the biosynthesis of GlcNAc-P-P-Dol. Dolichol phosphate glucose (Glc-P-Dol), while not an activator of the reaction, acted as a negative modifier of the stimulation by Man-P-Dol, by acting as a competitive inhibitor of the stimulation. Chapman et al (170) have reported that class E thy-1-negative mutant mouse lymphoma cells, which are unable to synthesize Man-P-Dol, produce a lipid- linked oligosaccharide having sugar chains much smaller in size. Thus, Man-P-Dol may

function to modulate both the rate at which saccharide units are assembled through the pathway due to its stimulation of GlcNAc-lipid synthesis, as well as their sizes by functioning as a substrate.

Dolichol phosphate (Dol-P) acts as the "lipid carrier" in the assembly of pyrophosphate-linked oligosaccharides, and also as an acceptor for the synthesis of the sugar donors Man-P-Dol and Glc-P-Dol. Dolichol phosphate appears to be synthesized predominantly in endoplasmic reticular membrane. However, most of the cellular dolichol is found in fractions other than endoplasmic reticulum (157), where the enzymes of oligosaccharide lipid synthesis are predominantly located (80). Thus, the active involvement of the pool sizes of dolichol and dolichol phosphate in different cell compartments in protein glycosylation is unknown. Keller et al (171) have suggested that de novo dolichol phosphate synthesis is regulated in the pathway, at the level of dolichol phosphate synthetase. Dolichol phosphate can also be synthesized in liver from free dolichol by the CTP-dependent reaction (85). The enzymes that dephosphorylate dolichol phosphate or convert dolichol pyrophosphate to dolichol-phosphate have been detected in cell free preparations.

The biosynthesis of membrane and soluble glycoprotein has been studied during estrogen induced differentiation of the chick oviduct by De Rosa and Lucas (172). They have concluded, on the basis of indirect evidence, that the

intracellular level of dolichol phosphate is increased in oviduct membranes as a result of estrogen treatment.

Induction of phosphorylation of dolichol during embryonic development of the sea urchin has been studied by Rossignol et al (173). Gastrulation of sea urchin embryos requires the synthesis of N-linked glycoproteins and is preceded by synthesis of dolichol and dolichol phosphate. By using membrane preparations from embryos at various stages of development, these authors showed that dolichol kinase activity was increased several fold over the time course of development. Carson and Lennarz (174) used compactin, a potent inhibitor of polyisoprenoid biosynthesis, to establish a link between dolichol phosphate biosynthesis and gastrulation. The results suggested that there was an impairment of protein glycosylation concurrent with the inhibition of dolichol phosphate synthesis by compactin.

As mentioned previously in the general introduction, synthesis of glycoproteins is increased during inflammation and on dexamethasone treatment. Thus, experiments were performed using rat liver microsomes and hepatocytes from control and inflamed rats to determine the effect of inflammation and dexamethasone on the formation of Man-P-Dol, $(GlcNAc)_{1-2}$-P-P-Dol dolichol and dolichol phosphate.

4.2. Results

A. Effect of inflammation on the formation of Man-P-Dol

from GDP-[^{14}C] mannose in rat liver microsomes.

Microsomes were prepared from control rats and rats 24 h after turpentine injection and the formation of Man-P-Dol was measured as a function of incubation time (Fig. 19). The reaction rate levelled off after 7 min of incubation. The rate of Man-P-Dol synthesis was higher in microsomes obtained from inflamed rats compared to controls.

Fig. 20 shows the formation of Man-P-Dol as a function of GDP-[^{14}C] mannose concentration. Microsomes were prepared 24 h after injection of turpentine and incubated with 0-10 nmol of GDP-[^{14}C] mannose. At all concentrations of GDP-[^{14}C] mannose used, the production of Man-P-Dol in the microsomes from inflamed rats was at least 2 fold higher compared to controls.

The effect of inflammation on Man-P-Dol production over a 72 h period was studied (Fig. 21). Increase in the levels of Man-P-Dol was observed as early as 6 h after induction of inflammation and the maximum production was attained at 36h ($P < 0.01$). However, the rate of synthesis decreased after 36h.

B. Effect of increasing amounts of exogenous dolichol phosphate on the formation of Man-P-Dol from GDP-[^{14}C] mannose in microsomes.

Microsomes prepared from 24 h inflamed and control rats were incubated with 0-20 nmol of dolichol phosphate and the

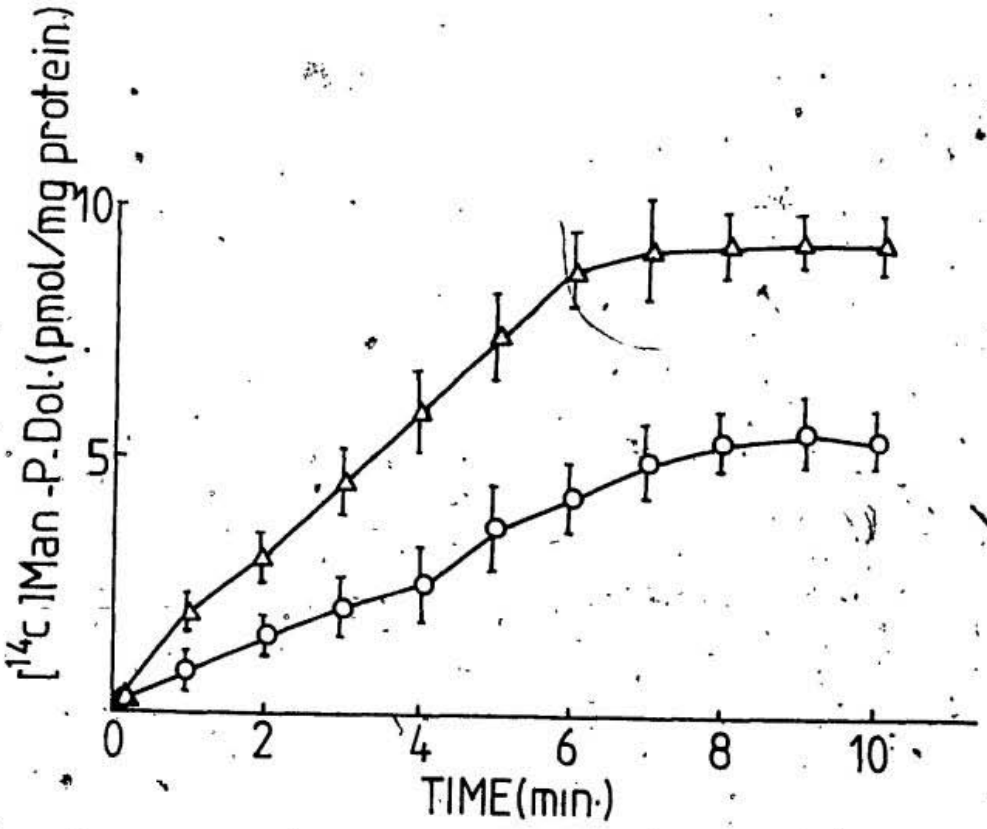

Fig. 19. Formation of [^{14}C] Man-P-Dol as a function of time.

Microsomes were prepared from (0), control and (△), 24h inflamed rats and incubated with GDP-[^{14}C] mannose. At the times indicated, the incubation mixtures were extracted and the radioactivity in [^{14}C]Man-P-Dol was measured. Results are mean ± S.D. from three separate experiments.

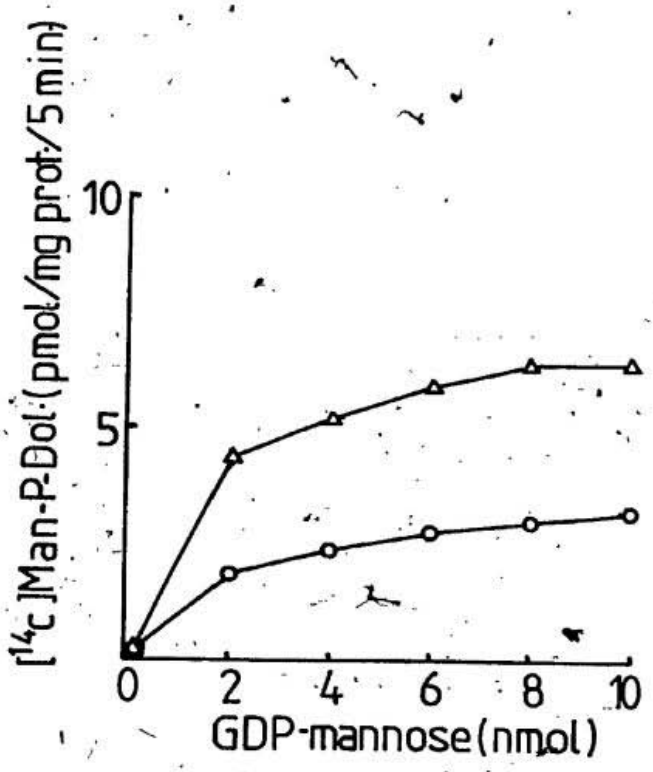

Fig. 20. Incorporation of [^{14}C] mannose into Man-P-Dol as a function of GDP-[^{14}C] mannose concentration.

(o) Control and (Δ) inflamed liver microsomes prepared from rats 24h after injection of saline and turpentine respectively, were incubated for 5 min with 0-10 nmol of GDP-[^{14}C] mannose and incubation mixtures were extracted to measure the radioactivity in Man-P-Dol. Results are mean values from three separate experiments.

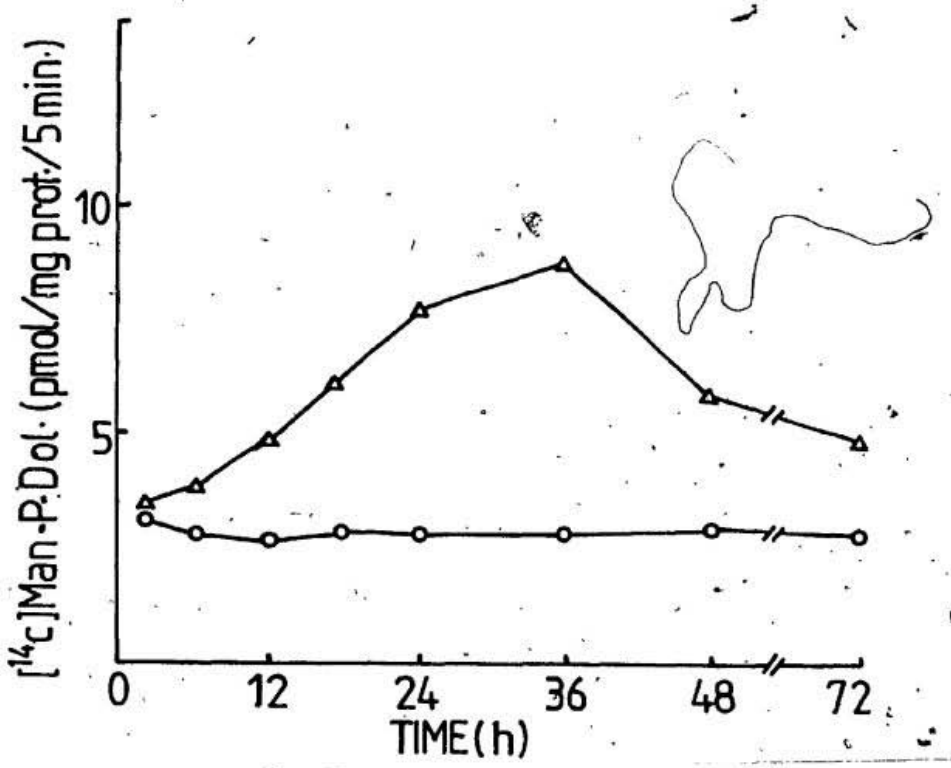

Fig. 21. The progression of Man-P-Dol production as function of time of inflammation.

Microsomes were prepared at the times indicated after injection of turpentine and incubated with 6 nmol of GDP-[^{14}C] mannose for 5 min and incubation mixtures were extracted for Man-P-Dol. Symbols are (0), control and (Δ), inflamed. Mean values of three experiments are presented.

resulting incorporation of [^{14}C] mannose from GDP-[^{14}C] mannose into Man-P-Dol was calculated as the ratio of activity in inflamed microsomes to that in controls (Fig. 22). With no addition of dolichol phosphate the ratio obtained was 2.2. In presence of 3.3 nmol of dolichol phosphate, the ratio decreased to 1.7 and as the amount of dolichol phosphate was further increased, the ratio approached unity.

C. Increase of dolichol and dolichol phosphate concentration during inflammation.

The dolichol and dolichol phosphate concentrations of rat liver were increased during inflammation (Table 3). At 24 h after turpentine treatment, the dolichol and dolichol phosphate concentrations in rat liver homogenate were increased about 6 and 2-fold respectively. There was also an increase of dolichol and dolichol phosphate concentrations in microsomes.

Coolbear and Mookerjea (168) had previously attempted to measure total concentrations of dolichol phosphate by testing the ability of lipid extracts from the microsomes of turpentine treated animals to stimulate the dolichol phosphate dependent mannosyltransferase. The enzyme was stimulated 30% by the lipid extract from control microsomes and 50% by lipid extract from microsomes taken 24-36 h after turpentine administration. Increased transfer of mannose was attributed to increased dolichol phosphate concentrations, although the actual

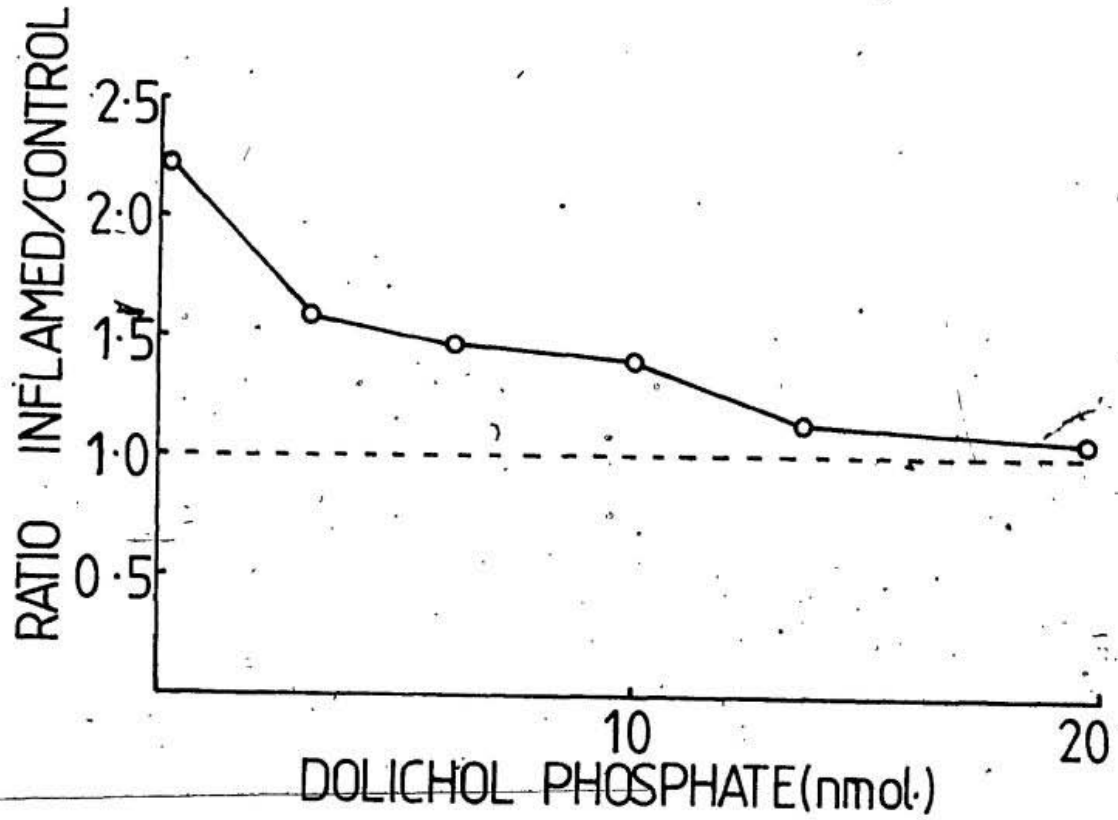

Fig. 22. Effect of increasing amount of exogenous dolichol phosphate on incorporation of [^{14}C] mannose from GDP [^{14}C] mannose into Man-P-Dol.

Microsomes prepared from control and inflamed rats were incubated with 0-20 nmol of dolichol phosphate and 6 nmol of GDP-[^{14}C] mannose for 5 min and incubation mixtures were extracted for Man-P-Dol. Results are presented as the ratio of inflamed to control activities.

Table 3. Effect of turpentine-induced inflammation on dolichol and dolichol phosphate concentrations in rat liver homogenates and microsomes. Results are presented as mean ± S.D. from 4 separate experiments.

	Homogenates		Microsomes	
Treatment	dolichol (μg/g wet tissue)	dolichol phosphate (μg/g wet tissue)	dolichol (μg/mg protein)	dolichol phosphate (μg/mg protein)
Control	29±5	3.7±0.6	0.12±0.04	0.06±0.02
12h inflamed	148±9*	5.7±0.4*	0.3 ±0.06*	0.12±0.02*
24h inflamed	155±13*	6.6±0.4*	0.4 ±0.07*	0.14±0.03*

*significantly different from control ($P < 0.01$).

amounts of dolichol phosphate were not measured. The present results (Table 3) indicate that dolichol phosphate concentrations increase dramatically during inflammation and this increase was detected within 12 h ($P < 0.01$).

D. Effect of inflammation and dexamethasone on the incorporation of [^{14}C] Man and [^{14}C] GlcNAc into Man-P-Dol and $(GLcNAc)_{1-2}$-P-P-Dol from respective labelled nucleotide sugars in hepatocyte homogenates obtained from control and inflamed rats.

Fig. 23 shows the time course of the transfer of [^{14}C] mannose from GDP-[^{14}C] mannose into Man-P-Dol in hepatocyte homogenates. Hepatocytes from control and inflamed rats were incubated with 1μM dexamethasone for 12 h. The rate of Man-P-Dol formation was in general higher when exogenous dolichol phosphate was used (Fig. 23B). The reaction rates increased rapidly within 1-2 minutes. The rate of Man-P-Dol formation in the hepatocytes from inflamed rats was higher than in hepatocytes from control rats. Dexamethasone (1μM) increased the rate of Man-P-Dol formation in hepatocytes obtained from both control and inflamed rats (Fig. 23A and B).

Fig. 24 shows the N-acetylglucosaminyltransferase activity in hepatocyte homogenates obtained from control and inflamed rats. The pattern of increase of activity on inflammation and dexamethasone treatment was similar to that

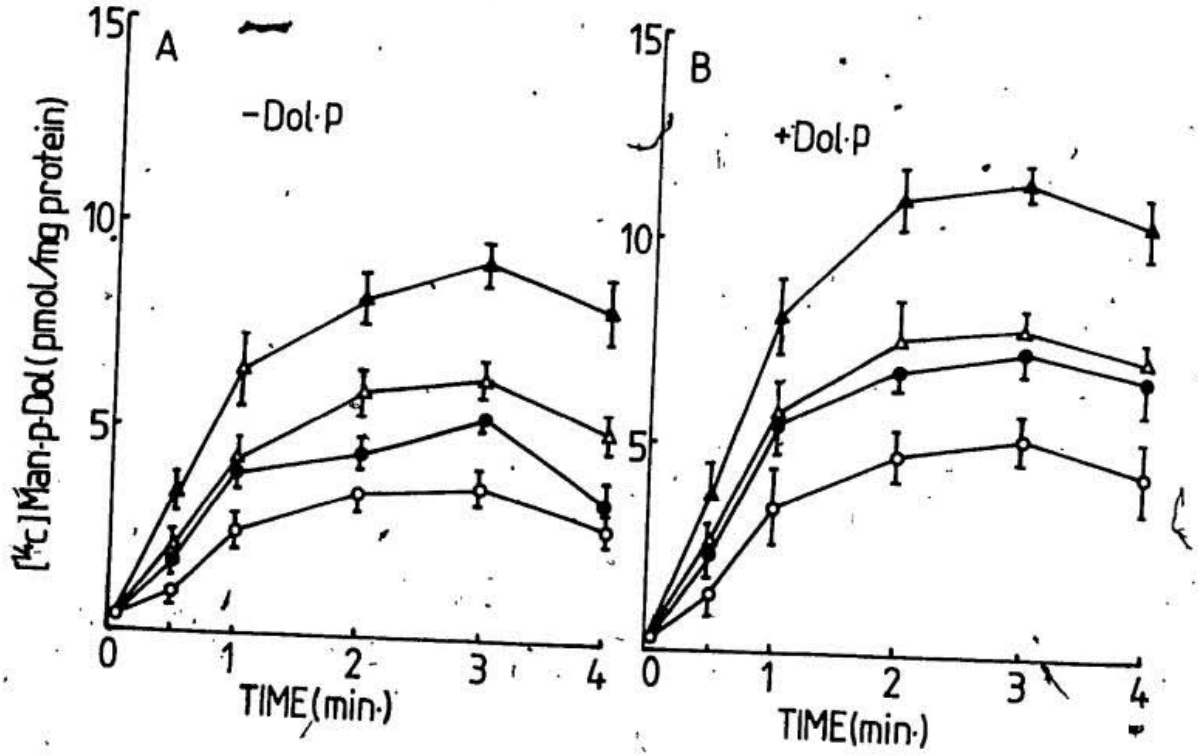

Fig. 23. Incorporation of [^{14}C] Man from GDP-[^{14}C] mannose into Man-P-Dol in hepatocyte homogenates.

Hepatocytes isolated from control and 24h turpentine inflamed rats were incubated with 1 μM dexamethasone in Waymouth's medium for 12h. The enzyme activity was measured in the cell homogenates. ○, control; ●, dexamethasone; △, inflamed; ▲, inflamed and dexamethasone. A, in absence of exogenous Dol-P; B, in presence of Dol-P (7.5 nmol). Results are mean ± S.D. from 3-5 different experiments. In Fig. 23A, P values between control vs inflamed groups (I) are < 0.01 at times 1/2 to 4 min; between control vs dexamethasone groups (II) are < 0.01 at times 1/2 to 3 min and between inflamed vs inflamed dexamethasone groups (III) are < 0.01 at times 1/2 to 4 min. Correspondingly in Fig. 23B the P values for I, II and III are < 0.01.

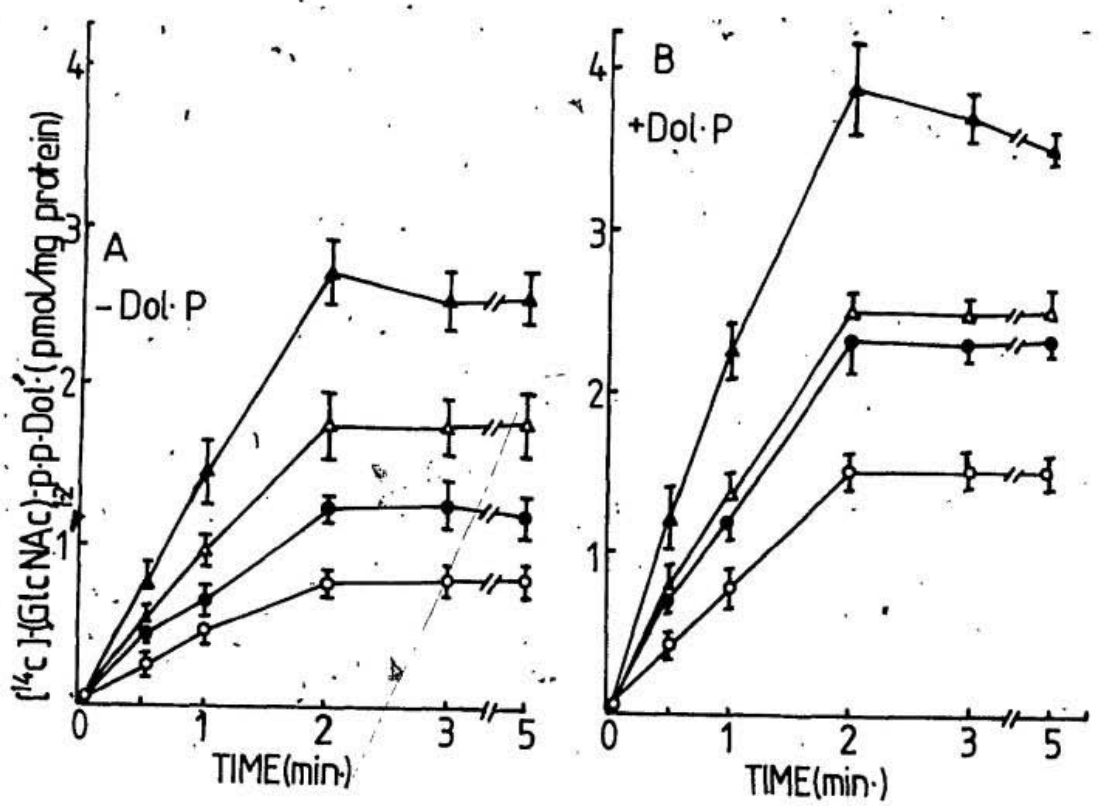

Fig. 24. Incorporation of [^{14}C] GlcNAc from UDP-[^{14}C] GlcNAc into $(GlcNAc)_{1-2}$-P-P-Dol in hepatocyte homogenates.

Hepatocytes from control and inflamed rats were incubated either in the absence or presence of dexamethasone (1μM) in Waymouth's medium for 12h. Activity was measured in the cell homogenates. O, control; ●, dexamethasone; Δ, inflamed; ▲, inflamed and dexamethasone. A, in absence of exogenous Dol-P.; B, in presence of Dol-P (7.5 nmol). Results are mean ± S.D. from 3-4 different experiments. P values between control vs inflamed groups are < 0.01; between control vs dexamethasone groups are < 0.01 and between inflamed vs inflamed + dexamethasone groups are < 0.01.

observed for mannosyltransferase activity.

Fig. 25 shows the rate of Man-P-Dol formation as a function of protein concentration in the assay. The homogenates from inflamed rat hepatocytes incorporated more label into Man-P-Dol ($P < 0.01$). Treatment of hepatocytes with dexamethasone caused a further increase of Man-P-Dol synthesis in both control and inflamed groups ($P < 0.01$). Exogenous dolichol phosphate caused an increase of Man-P-Dol production in all experiments.

Fig. 26 shows the results of Man-P-Dol formation in a 1 min assay for mannosyltransferase. Dexamethasone at a dose of 0.1-10μM increased the rate of Man-P-Dol formation in hepatocytes from both control and inflamed rats ($P < 0.01$). However, at 100μM dexamethasone, the Man-P-Dol formation was decreased in both groups.

The Man-P-Dol formation in hepatocytes of control and inflamed rats was maintained at a steady level for about 12h (Fig. 27). The homogenates from inflamed rat hepatocytes incorporated more label into Man-P-Dol compared to control ($P < 0.01$). Addition of dexamethasone to the hepatocytes resulted in an increase of Man-P-Dol formation when the assays were done either in the presence or absence of exogenous dolichol phosphate ($P < 0.01$).

Table 4 shows the effect of 1μM dexamethasone on Man-P-Dol formation in the homogenates of hepatocytes from control and inflamed rats. Dexamethasone caused a significant

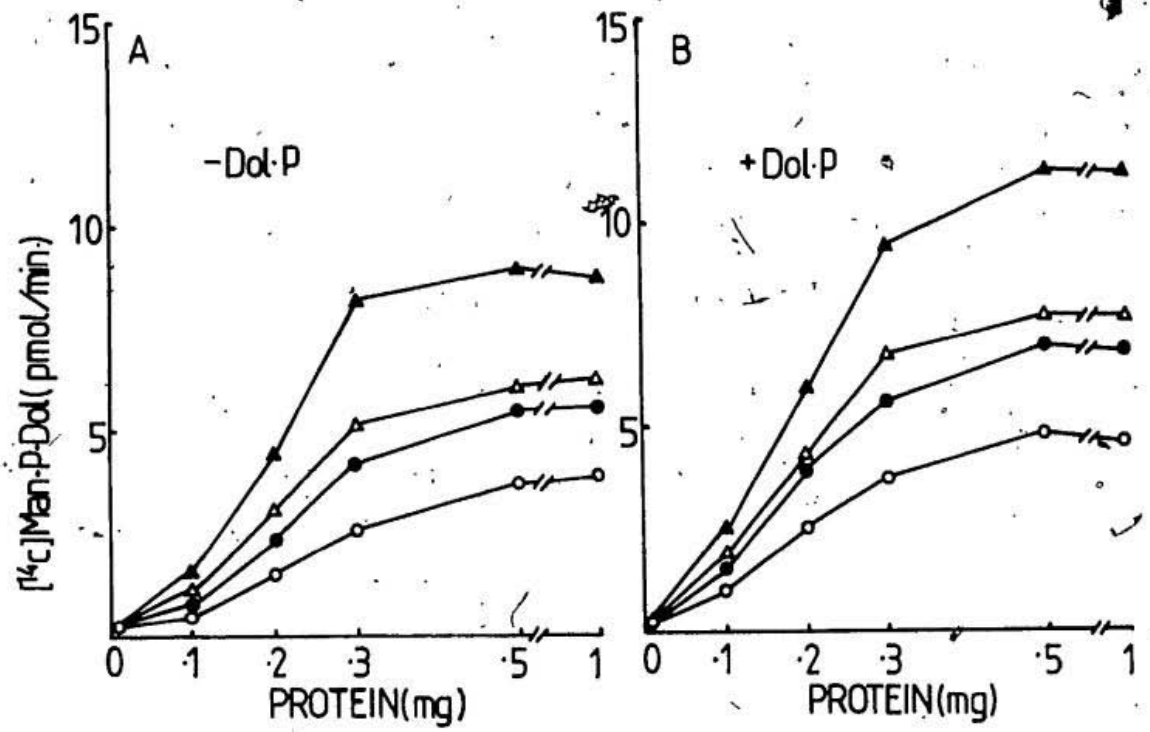

Fig. 25. Incorporation of [^{14}C] Man from GDP-[^{14}C] mannose into Man-P-Dol in hepatocyte homogenates as a function of amount of homogenate protein.

Hepatocytes isolated from control and 24 h inflamed rats were incubated either in the absence or presence of dexamethasone (1μM) in Waymouth's medium for 12h. Incubation for enzyme activity was carried out for 1 min at 37°C. O, Control; ●, dexamethasone; Δ, inflamed; ▲, inflamed and dexamethasone. A, in absence of exogenous Dol-P., B, in presence of exogenous Dol-P (7.5 nmol). Results are mean from three different experiments.

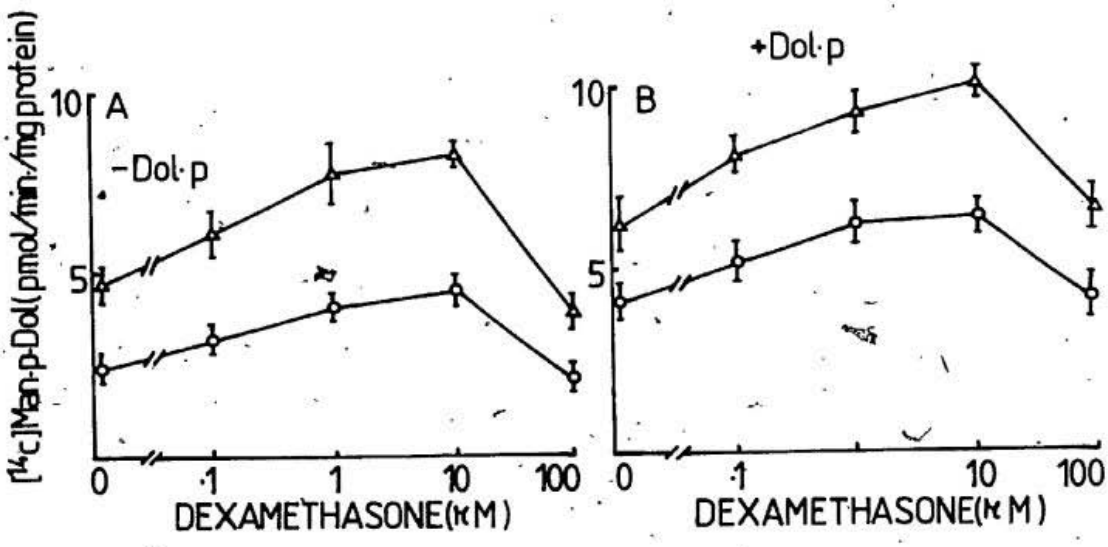

Fig. 26. Effects of various concentrations of dexamethasone on the incorporation of [^{14}C] Man from GDP-[^{14}C] mannose into Man-P-Dol in hepatocyte homogenates. Hepatocytes from (○), control and (△), inflamed rats were incubated with 0.1 - 100μ M dexamethasone in 'Waymouth's medium for 12h. Incubation for enzyme activity was carried out for 1 min at 37°C. A, absence of exogenous Dol-P; B, presence of Dol-P (7.5 nmol). Results are mean ± S.D. from 3-5 different experiments.

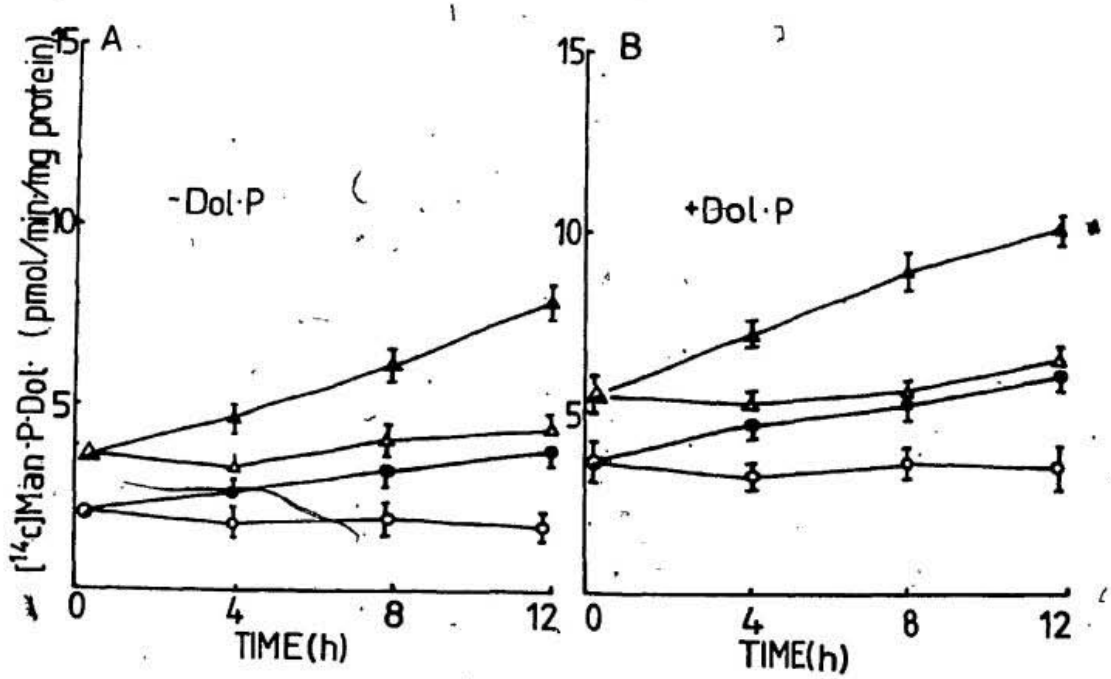

Fig. 27. Effect of dexamethasone on the incorporation of [^{14}C] mannose from GDP-[^{14}C] mannose into Man-P-Dol in hepatocyte homogenates.

Hepatocytes from control and inflamed rats were incubated in the presence of dexamethasone (1μM), for different periods of time and enzyme activity was measured in the cell homogenates. ○, control, ● , dexamethasone; △ , inflamed; ▲ , inflamed and dexamethasone. A, absence of exogenous Dol-P; B, presence of Dol-P (7.5 nmol). Results are mean ± S.D. from 4-6 different experiments.

Table 4. Effect of dexamethasone on the synthesis of Man-P-Dol by rat hepatocytes.

Hepatocytes isolated from control and inflamed rats were incubated in Waymouth's medium for 12h either in the presence (1μM) or in the absence of dexamethasone. Enzyme activity was measured in homogenates. Each value represents the mean ± S.D. for six different experiments.

Treatment	Man-P-Dol Synthesis	
	without dolichol phosphate (pmol/min/mg protein)	with dolichol phosphate (pmol/min/mg protein)
Saline control (0.9% NaCl)	2.26±0.36	3.21±0.27
Dexamethasone	3.4 ±0.32*	5.06±0.5*
Inflamed	4.13±0.42*	5.28±0.62*
Inflamed and dexamethasone	6.47±0.39+	8.16±0.65+

*Significantly different from control ($p < 0.01$).

+Significantly different from inflamed ($p < 0.01$)

increase (1.5-2 fold; $P < 0.01$) of Man-P-Dol formation in both control and inflamed rat hepatocytes, when the assays were performed with or without exogenous dolichol phosphate. Results in Table 4 show that Man-P-Dol formation is about 2-fold greater in the hepatocytes from inflamed rats. Variations of endogenous nucleotide sugar pools might affect the rate of synthesis of Man-P-Dol. It has been shown in this thesis (Chapter VI) that endogenous pool sizes of GDP-Man, UDP-Glc and UDP-GlcNAc are not altered in inflammation and dexamethasone treatment. The increased rate of incorporation of [^{14}C]-Mannose into Man-P-Dol in experimental groups probably represented the true increased rate of synthesis.

E. Effect of increasing amount of exogenous dolichol phosphate on the formation of Man-P-Dol from GDP-[^{14}C] mannose by hepatocyte homogenates.

The increase of Man-P-Dol formation observed in inflamed and dexamethasone treated hepatocytes could be explained either on the basis of an increased level of endogenous dolichol phosphate or increased mannosyltransferase(s) activities. In these conditions, if the increase of Man-P-Dol is due to an increase of endogenous level of dolichol phosphate with no alteration in the enzyme level, then the level of dolichol phosphate should be the limiting factor for the formation of Man-P-Dol. Use of saturating amounts of exogenous dolichol

phosphate into the assay should result in no difference of Man-P-Dol formation between inflamed, dexamethasone treated and control groups. If the results are presented as ratios of inflamed to control or dexamethasone treated to untreated, the ratios should approach unity. On the other hand, if the levels of Man-P-Dol formation are due to increased level of mannosyltransferase enzyme, the hepatocyte homogenate from inflamed rats or dexamethasone treated cells should continue to synthesize more Man-P-Dol with exogenous dolichol phosphate compared to their controls. In this case, if the results are presented as ratios of inflamed to control or dexamethasonetreated to untreated, the ratios should not approach unity. To test these possibilities, the Man-P-Dol formation assays were performed in the presence of different amount of exogenous dolichol phosphate. The rates of Man-P-Dol formation with saturating amounts of dolichol phosphate (30 nmol) (Table 5) indicated no difference in the level of activity between control and inflamed preparations. In contrast, mannosyltransferase(s) activity was greater in the presence of exogenous dolichol phosphate in dexamethasone treated cells, and the higher level of mannosyltransferase activity was maintained even in the presence of 30 nmol dolichol phosphate.

The results obtained from experiments with increasing amounts of dolichol phosphate as presented in Table 5 were replotted as the ratios of Man-P-Dol formation in homogenate

Table 5. Effect of exogenous dolichol phosphate on the incorporation of [^{14}C] mannose from GDP-[^{14}C] mannose into Man-P-Dol in hepatocyte homogenates.

Hepatocytes from control and inflamed rats were incubated in Waymouth's medium for 12 h either in the absence or presence of dexamethasone (1μM). Enzyme activity was measured in the homogenate in the presence of increasing amounts of dolichol phosphate (0-30 nmol). Results are mean ± S.D. from 3-5 different experiments.

Man-P-Dol formation in hepatocytes

(Pmol/min/mg protein)

Amounts of dolichol phosphate (nmol)	Control A	Inflamed B	Ratio B/A	Control A	Dexamethasone C	Ratio C/A	Inflamed B	Inflamed + Dexamethasone D	Ratio D/B
—	2±0.3	3.5±0.5	1.75	2±0.3	3.1±0.3	1.55	3.5±0.5	6.0±0.5	1.71
7.5	3.8±0.6	5.7±0.6	1.5	3.8±0.6	5.32±0.4	1.4	5.7±0.6	8.9±0.8	1.56
15	5.4±0.4	7.0±0.7	1.29	5.4±0.4	8.1±0.6	1.5	7.0±0.7	12.0±0.9	1.71
22.5	7.5±0.2	9.0±0.7	1.2	7.5±0.2	12.0±0.7	1.6	9.0±0.7	15.5±1	1.72
30	9±0.4	10.1±1	1.1	9±0.4	13.5±0.9	1.5	10.1±1	16.6±1.1	1.64

from inflamed to saline controls, and dexamethasone treated cell homogenates to untreated hepatocyte homogenates (Fig. 28). With no addition of dolichol phosphate, the ratio of enzyme activity in inflamed to control was 1.8. On incubation with 7.5 nmol dolichol phosphate, the ratio decreased to 1.5. As the amount of dolichol phosphate was further increased, the ratio approached close to unity.

In contrast the ratios of enzyme activity of inflamed + dexamethasone to inflamed, and control + dexamethasone to control were 1.7 and 1.5 respectively when the assays contained no exogenous dolichol phosphate. In the presence of increasing amounts of dolichol phosphate, the above ratios remained high and did not decrease nor approach unity, like the ratios between inflamed and control groups without any dexamethasone treatment.

F. Effect of inflammation and dexamethasone on the synthesis of [^{3}H]-dolichol and [^{3}H]-dolichol phosphate from [^{3}H] mevalonate.

Carson and Lennarz (174,175), have demonstrated that in sea urchin embryos, a rise in dolichol phosphate level occurs that correlates with enhanced glycoprotein synthesis. Moreover, compactin, a specific inhibitor of dolichol synthesis was able to decrease glycoprotein synthesis by 50%. Since the intracellular concentration of dolichol phosphate regulates

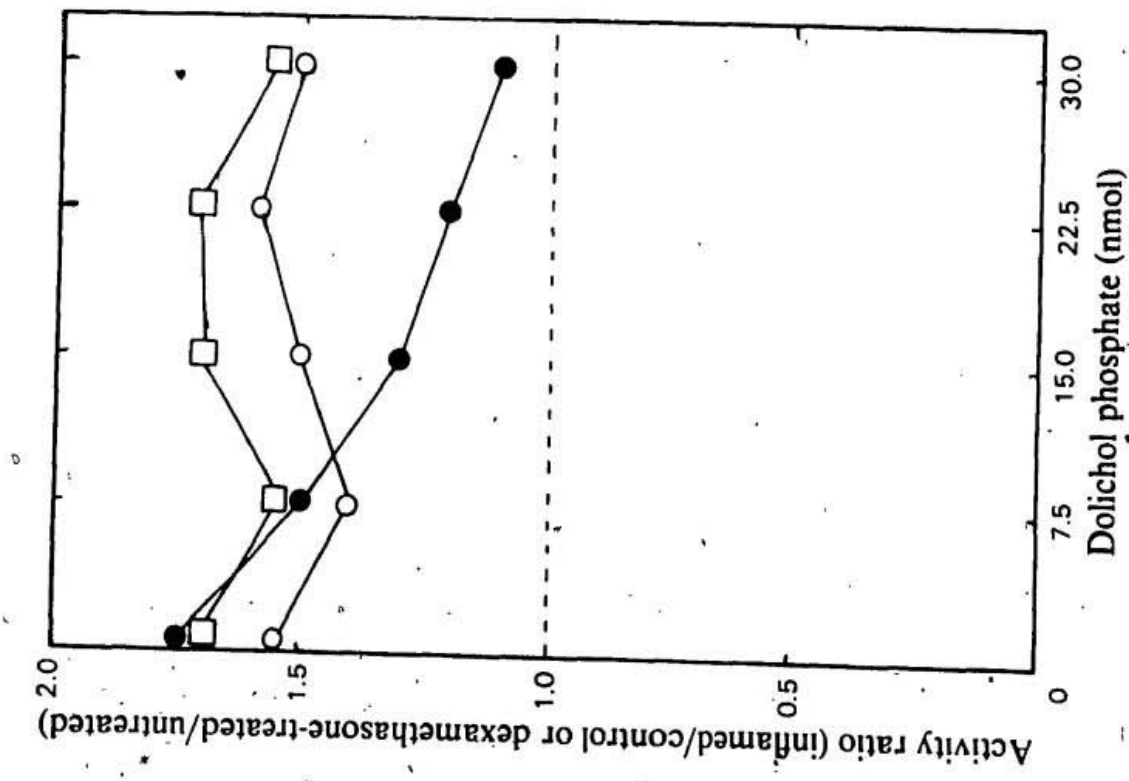

Fig. 28. Effect of exogenous dolichol phosphate on the incorporation of [^{14}C] Man from GDP-[^{14}C] mannose into Man-P-Dol in hepatocyte homogenates.

Experimental details are as in Table 5. Results are presented as ratios of activities between inflamed rat to controls in the absence of dexamethasone (●), Control cells in presence to in absence of dexamethasone (0), and inflamed-rat cells in presence to in absence of dexamethasone (□).

the synthesis of Man-P-Dol (176,177), it was interesting to examine the synthesis of dolichol and dolichol phosphate in cells using [^{3}H] mevalonate as a precursor. This was particularly important since glucocorticoids induce the activity of hydroxymethylglutaryl CoA reductase in Hela cells (178) and this enzyme is implicated in the regulation of dolichol synthesis (179).

Hepatocytes were incubated with [^{3}H] mevalonate, and the formation of [^{3}H]-dolichol and [^{3}H]-dolichol phosphate was measured as described in Material and Methods. The results in Figs. 29 and 30 show that the rates of synthesis of [^{3}H]-dolichol and [^{3}H]-dolichol phosphate were about 2-fold higher in hepatocytes from inflamed rats compared to controls ($P < 0.01$). Addition of dexamethasone (1μM) to the cells increased the synthesis of these two lipids by 20-30% after 12 h incubation of hepatocytes from control and inflamed rats.

G. Effect of actinomycin D and cycloheximide on the increase of mannosyl and N-acetylglucosaminyltransferases during inflammation and dexamethasone treatment.

During the increased demand for glycoprotein synthesis, cells can increase their glycosylation capacity by several possible mechanisms. These include (a) increasing formation of dolichol and dolichol phosphate, (b) increasing the amount of nascent chains of proteins available for glycosylation

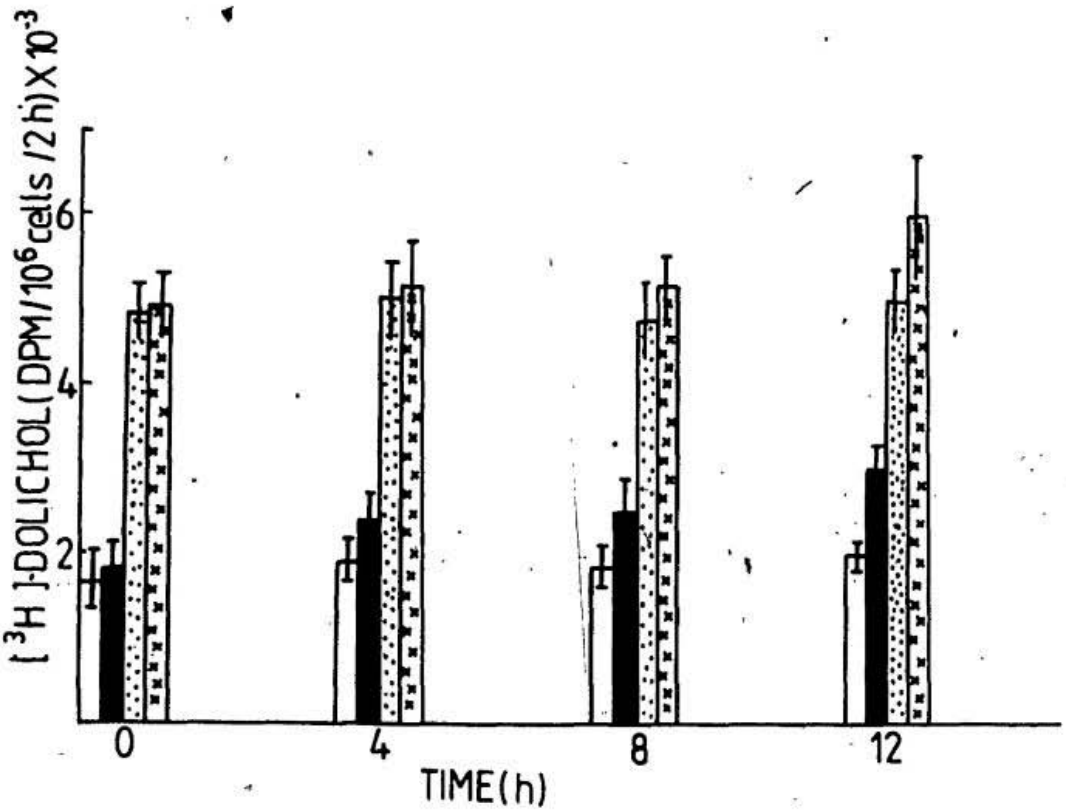

Fig. 29. Effect of dexamethasone on the incorporation of [^{3}H]-mevalonate into [^{3}H]-dolichol in hepatocytes.
Hepatocytes from control and inflamed rats were incubated either in the absence or presence of dexamethasone (1μM). The cells were pulsed with [^{3}H]mevalonate for 2h before the termination of incubation. [^{3}H]-dolichol was isolated as described in 'Materials and Methods' section. Results are mean $\pm$ S.D. from 3-5 different experiments. □, Control; ■, Dexamethasone; ⊡, inflamed; ⊞, inflamed and dexamethasone.

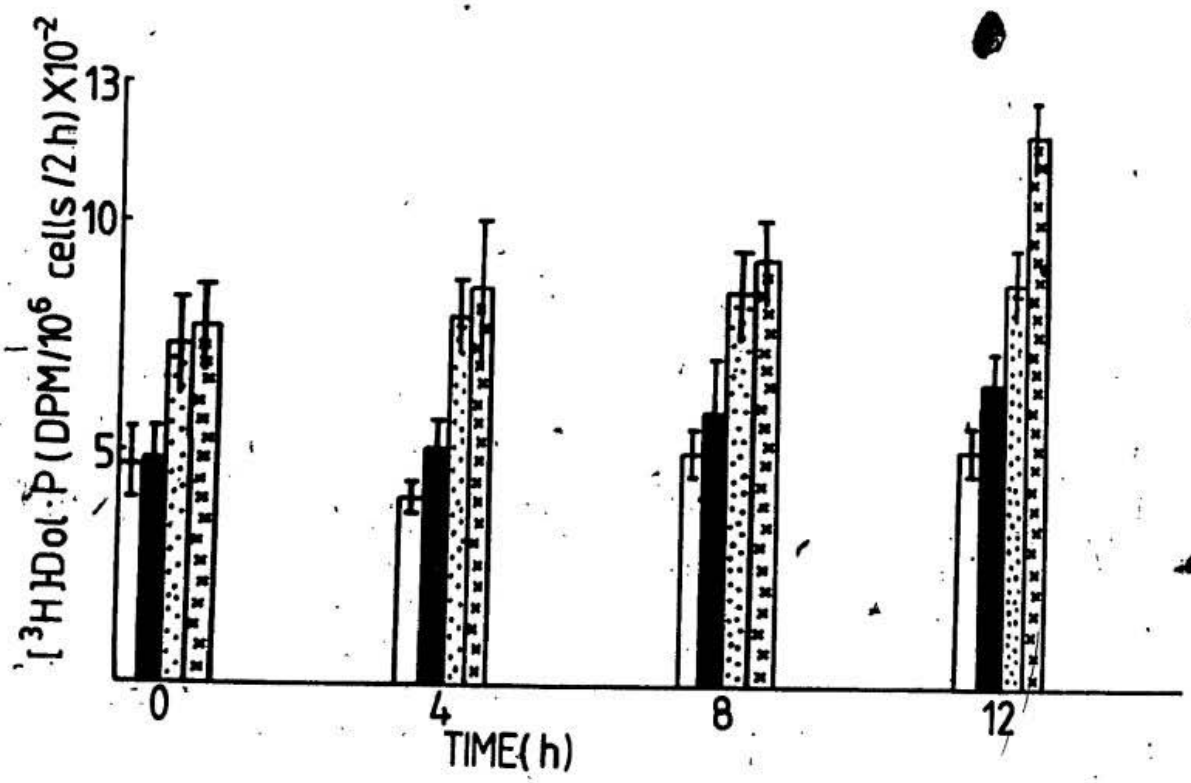

Fig. 30. Effect of dexamethasone on the incorporation of [^{3}H] mevalonate into [^{3}H]-dolichol phosphate in hepatocytes.

Hepatocytes from control and inflamed rats were incubated either in the absence or presence of dexamethasone (1 μM). The cells were pulsed with [^{3}H]-mevalonate for 2 h before the termination of incubation. [^{3}H]-dolichol phosphate was isolated as described in "Materials and Methods" section. Results are mean ± S.D. from 3-5 different experiments.

□, control; ■, dexamethasone; ⊡, Inflamed; ▦, Inflamed and dexamethasone.

and (c) increasing the number of glycosyltransferases (171,176,177,180). It has been mentioned previously in the introduction, that inflammation and dexamethasone-induced increase in acute-phase glycoproteins was the result of de novo synthesis of proteins. Hence, it was of interest to study the effect of an RNA synthesis inhibitor (actinomycin D) and a protein translation inhibitor (cycloheximide) on the glycosyltransferases induced by inflammation and dexamethasone treatment.

The effects of actinomycin D on the inflammation and dexamethasone-induced increase of mannosyl and N-acetyl glucosaminyltransferase are shown in Table 6 and 7. Administration of actinomycin D together with dexamethasone to the incubation medium completely suppressed the dexamethasone induced increase of mannosyl and N-acetylglucosaminyltransferase activities. When actinomycin D was added 4 and 8 h after addition of dexamethasone, only a partial increase of activities was observed for both the enzymes. Actinomycin D failed to block the inflammation induced increase of enzyme activities when it was added to hepatocytes from inflamed rats (Table 7).

The effects of cycloheximide on the mannosyl and N-acetylglucosaminyltransferase activities induced by inflammation and dexamethasone treatment are shown in Table 8 and 9. Cycloheximide completely blocked the dexamethasone induced increase of enzyme activities either in hepatocytes from

Table 6. Effects of dexamethasone and actinomycin D on the formation of Man-P-Dol and $(GlcNAc)_{1-2}$-P-P-Dol in hepatocytes obtained from control rats.

Hepatocytes were cultured for 12h. Addition of dexamethasone (1μM) and actinomycin D (1μg/ml) were at t=0h unless otherwise indicated. Enzyme activities were measured in the homogenate either in the presence or absence of exogenous dolichol phosphate. Results are mean ± S.D. from 3-4 different experiments.

Treatment	Synthesis of dolichol-linked Intermediates			
	[^{14}C] Man-P-Dol		[^{14}C] $(GlcNAc)_{1-2}$-P-P-Dol	
	-Dol-P (pmol/min/mg protein)	+Dol-P (pmol/min/mg protein)	-Dol-P (pmol/min/mg protein)	+Dol-P (pmol/min/mg protein)
No addition	2.7±0.09	4.5±0.25	0.45±0.04	0.95±.26
Dexamethasone alone	4.3±1.2*	6.9±1.75*	0.8 ±0.07*	1.72±0.5*
Dexa.+Actinomycin D	2.4 ±0.07	3.5±0.67	0.4 ±0.03	0.8 ±0.25
Actinomycin D alone	2.4 ±0.05	3.4±0.32	0.3 ±0.02	0.8 ±0.32
Dexa.+Act. D at t=4h	3.4 ±0.5+	5.0±1.1+	0.56±0.05+	1.2 ±0.45+
Dexa.+Act.D. at t=8h	3.7 ±0.6*	5.5±1.48*	0.64±0.05*	1.5 ±0.56*

, +, significantly different from control ($p < 0.01$; + $p < 0.05$).

Table 7. Effects of dexamethasone and actinomycin D on the formation of Man-P-Dol and $(GlcNAc)_{1-2}$-P-P-Dol activities in hepatocytes obtained from inflamed rats. The experimental details are as in Table 6.

Treatment	Synthesis of dolichol-linked Intermediates			
	[^{14}C] Man-P-Dol		[^{14}C] $(GlcNAc)_{1-2}$-P-P-Dol	
	-Dol.P (pmol/min/mg protein)	+Dol-P (pmol/min/mg protein)	-Dol-P (pmol/min/mg protein)	+Dol-P (pmol/min/mg protein)
No addition	5.3±0.95	7.3±1.0	0.9 ±0.1	1.6±0.2
Dexamethasone alone	8.4±1.3*	12.8±1.5*	1.5 ±0.17*	2.8±0.5*
Dexa.+Actinomycin D	5.2±1.2	6.7±0.63	0.83±0.03	1.5±0.23
Actinomycin D alone	4.8±0.6	6.7±0.3	0.79±0.1	1.4±0.23
Dexa.+Act.D. at t=4h	6.4±0.7+	9.0±0.7+	1.1 ±0.4+	1.9±0.3+
Dexa.+Act.D. at t=8h	7.1±1.0*	10.1±1.0*	1.2 ±0.1*	2.3±0.5*

, +, significantly different from inflamed ($p < 0.01$; + $p < 0.05$).

Table 8. Effects of dexamethasone and cycloheximide on the formation of Man-P-Dol and $(GlcNAc)_{1-2}$-P-P-Dol in hepatocytes obtained from control rats.

Hepatocytes were cultured for 12h. Addition of dexamethasone (1μM) and cycloheximide (100μg/ml) were at t=0h unless otherwise indicated. Enzyme activities were measured in the homogenate either in the presence or absence of exogenous dolichol phosphate. Results are mean ± S.D. from 3-4 different experiments.

Treatment	Synthesis of dolichol-linked Intermediates			
	[^{14}C] Man-P-Dol		[^{14}C] $(GlcNAc)_{1-2}$-P-P-Dol	
	-Dol-P (pmol/min/mg protein)	+Dol-P (pmol/min/mg protein)	-Dol-P (pmol/min/mg protein)	+Dol-P (pmol/min/mg protein)
No addition	2.9±0.7	4.1±0.4	0.5±0.1	0.9±0.02
Dexamethasone alone	4.4±0.5*	6.5±0.85*	0.7±0.2*	1.4±0.04*
Dexa.+Cycloheximide	2.6±0.4	3.6±0.2	0.4±0.15	0.75±0.04
Cyclohemixide alone	2.4±0.3	3.4±0.4	0.4±0.1	0.6±0.03
Dexa.+Cycloheximide at t=4h	2.5±0.3	3.5±0.2	0.4±0.1	0.7±0.05
Dexa.+Cycloheximide at t=8h	2.6±.4	3.6±0.6	0.4±0.1	0.8±0.06

*significantly different from control, ($p < 0.01$).

Table 9. Effects of dexamethasone and cycloheximide on the formation of Man-P-Dol and $(GlcNAc)_{1-2}$-P-P-Dol in hepatocytes obtained from inflamed rats. The experimental details are as Table 8.

Treatment	Synthesis of dolichol-linked Intermediates			
	[^{14}C] Man-P-Dol		[^{14}C] $(GlcNAc)_{1-2}$-P-P-Dol	
	-dol-P (pmol/min/mg protein)	+Dol-P (pmol/min/mg protein)	-Dol-P (pmol/min/mg protein)	+Dol-P (pmol/min/mg protein)
No addition	5.2±1.2	6.8±0.3	0.8±0.03	1.4±0.3
Dexamethasone alone	7.8±1.2*	12.8±1.0*	1.3±0.02*	2.7±0.7*
Dexa.+cycloheximide	3.5±0.6	4.9±0.4	0.67±0.06	1.2±0.4
Cycloheximide alone	3.6±0.1	5.0±0.5	0.6±0.05	1.1±0.1
Dexa.+cycloheximide at t=4h	3.8±0.1	5.4±0.6	0.6±0.04	1.2±0.2
Dexa.+cycloheximide at t=8h	4.0±0.1	5.6±0.5	0.7±0.08	1.1±0.1

*Significantly different from inflamed ($p < 0.01$).

controls or from inflamed rats. No increase of activities was observed when cycloheximide was added even 4 and 8 h after the addition of dexamethasone.

H. Studies on the incorporation of [^{14}C] mannose into Man-P-Dol in intact hepatocytes.

In order to assess if the increased formation of Man-P-Dol, as observed in earlier experiments, with hepatocyte homogenates also occurs in intact hepatocytes, experiments were performed in which incorporation of [^{14}C] mannose from [^{14}C] mannose into Man-P-Dol was measured. The hepatocytes from control and inflamed rats were incubated with dexamethasone, and [^{14}C] mannose incorporation studies were performed as described in "Materials and Methods".

Fig. 31 shows the formation of Man-P-Dol as a function of incubation time. The formation of Man-P-Dol increased over the period of incubation. Hepatocytes from inflamed rats incorporated [^{14}C] mannose at a rate about 2-fold higher compared to controls ($P < 0.01$). Dexamethasone (1μM) also caused an increase of this incorporation in hepatocytes obtained from both control and inflamed rats, which was statistically significant (Table 10).

I. Subcellular distribution of mannosyltransferase

Table 11 shows the results of marker enzymes of various subcellular fractions from a single representative experiment

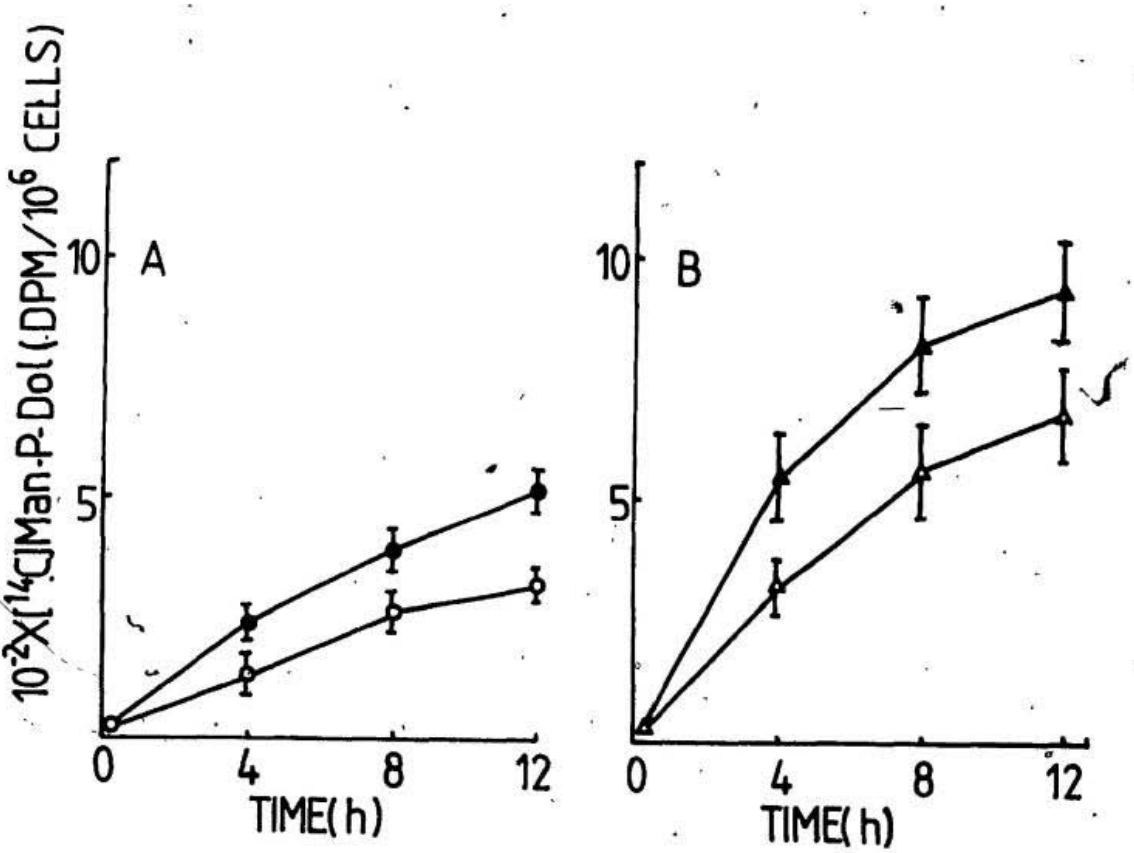

Fig. 31. Effect of dexamethasone on the incorporation of [^{14}C] mannose from [^{14}C] mannose into Man-P-Dol in intact hepatocytes.

Hepatocytes isolated from control and inflamed rats were incubated either in the absence or presence of dexamethasone (1 μM). The cells were labelled with 0.4 μCi of [^{14}C] mannose/ml of the incubation medium throughout the entire period of incubation. Man-P-Dol was extracted as described in 'Materials and Methods'. Panels (A) and (B) are experiments from control and inflamed rats respectively. Solid symbols are dexamethasone treated; open symbols untreated cells. Results are mean ± S.D. from triplicate experiments.

Table 10. Effect of dexamethasone on the incorporation of [^{14}C] mannose into Man-P-Dol in rat hepatocytes.

Hepatocytes isolated from control and inflamed rats were incubated for 12h either in presence (1μM) or absence of dexamethasone. The cells were pulsed with 0.4μCi of [^{14}C] mannose/ml of incubation medium for 6h before the termination of incubation. The incorporation of radioactivity into Man-P-Dol was determined as described in the Materials and Methods sections. Values are given as means ± S.D. for five experiments. (*Significantly different from control ($p < 0.01$); $^{+}$ significantly different from inflamed ($P < 0.01$).

Treatment	Incorporation of [^{14}C] mannose into Man-P-Dol (DPM/10^6 cells)
Saline	324±50
Dexamethasone	497±69*
Inflamed	594±187*
Inflamed and dexamethasone	849±207^{+}

with hepatocytes obtained from control and turpentine treated rats. These activities were not changed due to either inflammation or dexamethasone treatment. Mannosyltransferase activity was enriched in microsomal fractions (60% of total activity is recovered in this fraction) in all the experiments (Table 11). Both the specific activity and the total enzyme activity were increased in the microsomal fraction as a result of either inflammation and dexamethasone treatment.

4.3. DISCUSSION

Increased formation of Man-P-Dol and $(GLcNAc)_{1-2}$-P-P-Dol was observed in microsomes and hepatocyte homogenates obtained from inflamed rats. In a variety of inflammatory and pathological conditions, elevated glycosyltransferase activities have been previously observed. These pathological conditions include: various liver diseases (165,166), partial hepatectomy (181) and neoplastic diseases (182,183). Increased activity of several nucleotide sugar: glycoprotein glycosyltransferases have also been demonstrated in liver membranes from inflamed rats (52,53).

It has been shown by various workers (18,35) that there is enhanced glycoprotein biosynthesis during inflammation. Our studies have shown increases in the formation of Man-P-Dol and $(GlcNAc)_{1-2}$-P-P-Dol, the intermediates for oligosaccharide lipid synthesis at a time of elevated glycoprotein biosynthesis. This observation is consistent

Table 11. Subcellular localization of mannosyltransferase and other marker enzymes. Values in parentheses indicate the total activity.

Treatment	Subcellular fraction	Enzyme Activity			
		Succinate:cytochrome c reductase (μ mol of cytochrome c reduced/min per mg protein)	NADPH:cyto chrome c reductase (μ mol of cytochrome c reduced/min per mg protein)	β-Glucuronidase (μ mol of glucuronic acid released/min per mg protein)	Mannosyltransferase (pmol of product/min per mg protein)
Control	Homogenate	0.028 (3.25)	0.07 (8.7)	0.013 (1.62)	2.05 (258)
	Debris (480 g pellet)	0.0015 (0.01)	0.002 (0.02)	0.002 (0.02)	0.9 (9)
	Mitochondria (4300 g pellet)	0.18 (2.61)	0.015 (0.2)	0.07 (0.93)	1.2 (16.8)
	Microsomes (105000 g pellet)	0.006 (0.18)	0.24 (7.2)	0.006 (0.18)	4.95 (148)
Dexamethasone	Homogenate	0.032 (4.03)	0.072 (9.0)	0.012 (1.5)	3.1 (390)
	Debris (480 g pellet)	0.005 (0.05)	0.003 (0.03)	0.001 (0.01)	1.1 (11)
	Mitochondria (4300 g pellet)	0.22 (3.02)	0.01 (0.14)	0.06 (0.84)	1.5 (20)
	Microsomes (105000 g pellet)	0.007 (0.21)	0.23 (6.9)	0.006 (0.18)	7.2 (216)
Inflamed	Homogenate	0.033 (4.15)	0.074 (9.25)	0.015 (1.87)	4.01 (505)
	Debris (480 g pellet)	0.0018 (0.02)	0.0035 (0.035)	0.002 (0.02)	1.1 (11)
	Mitochondria (4300 g pellet)	0.22 (3.02)	0.1 (1.37)	0.1 (1.37)	1.6 (22)
	Microsomes (105000 g pellet)	0.008 (0.24)	0.25 (7.5)	0.007 (0.21)	9.5 (285)
Inflamed and dexamethasone	Homogenate	0.031 (3.9)	0.071 (8.8)	0.013 (1.62)	5.69 (717)
	Debris (480g pellet)	0.0019 (0.02)	0.001 (0.01)	0.001 (0.01)	1.2 (12)
	Mitochondria (4300 g pellet)	0.2 (2.75)	0.08 (1.12)	0.08 (1.12)	1.7 (23.5)
	Microsome (105000 g pellet)	0.008 (0.24)	0.23 (6.9)	0.006 (0.18)	13.3 (399)

with the enhanced glycoprotein synthesis in inflammation as previously observed (35).

Dexamethasone, a potent synthetic glucocorticoid has been found to influence the biosynthesis of glycoproteins (133). Dexamethasone, when added to hepatocytes obtained from either control or inflamed rats, caused an increase of mannosyl and N-acetylglucosaminyltransferase activities. It is believed that glucocorticoids, in common with other steroid hormones, exert their effects via interaction with nuclear DNA (184). It is generally accepted that steroids enter into the cell by diffusion and after binding to specific cytoplasmic receptors, migrate to the nucleus. A number of authors have suggested that the uptake of glucocorticoids by rat liver cells involves a carrier-mediated, energy dependent process (185,186). It is generally accepted that glucocorticoid has to enter into the cell to produce its physiological effects.

Glucocorticoid hormones appear to produce many of their physiological effects through modulating the activity of specific enzymes or proteins in target cells, most often as a result of an increase in their rates of synthesis. In addition to the synthesis of acute-phase proteins, glucocorticoids enhance gluconeogenesis, glycogen deposition, amino acid conversion to CO_2 and urea production in liver. An increase of mRNA synthesis occurs within 10 minutes after glucocorticoid administration, indicating some of these effects may result from direct action of the glucocorticoids

on liver.

Studies in this thesis have examined the effect of dexamethasone on hepatocytes from control and 24 h inflamed rats. It has been shown that 1μM dexamethasone saturates the glucocorticoid receptors in hepatocytes (187). In hepatocytes from both control and inflamed rats, dexamethasone at doses of 0.1-10μM increased the rate of Man-P-Dol formation. However, a higher dose of dexamethasone (100μM) decreased the Man-P-Dol formation in both control and inflamed conditions.

The observed increase in Man-P-Dol formation may be interpreted on the basis of the elevated levels of either endogenous dolichol phosphate or the relevant enzymes. Our results from the experiments using various concentrations of exogenous dolichol phosphate in the assays would tend to support the former interpretation when the results from inflamed rats were compared against the controls (Figs. 22 and 28). The formation of Man-P-Dol was 2 fold higher in microsomes or hepatocytes from inflamed rats compared to controls. When increasing amounts of exogenous dolichol phosphate were used in the assays, the increase in the ratio of mannosyltransferase (or Man-P-Dol formation) activity resulting from inflammation approached towards unity. The ratio decreased to unity at 20 nmol and 30 nmol of exogenous dolichol phosphate in microsomes and hepatocyte homogenates respectively.

In contrast, the increase of enzyme activity resulting

from dexamethasone treatment compared to untreated hepatocytes was unchanged, even when the assay mixture contained up to 30 nmol of exogenous dolichol phosphate. The response of mannosyltransferase activity to exogenous dolichol phosphate is greater in the homogenates from dexamethasone treated cells. The increase in activity cannot be abolished with increasing amount of exogenous dolichol phosphate which suggests that the increase in dolichol phosphate may not be of major significance in the augmentation of Man-P-Dol formation, in the case of the experiments with dexamethasone treatment.

It was, therefore, concluded from the results with microsomes and hepatocytes from inflamed rats that the increase in mannosyltransferase was due to an increase in the endogenous concentration of the dolichol phosphate in the liver and there was no true increase of enzyme activity. It is worthy to note that Hanford et al (177) have found dolichol phosphate levels to be about three fold higher in membranes from actively myelinating pig brain white matter than in preparations from nonmyelinating adult brains. A study of the level of Man-P-Dol synthesis in membranes from the oviducts of chicks before and after estrogen induced differentiation indicates that the primary factor in enhanced synthesis is an increase in the level of dolichol phosphate rather than mannosyltransferase(s) (176).

In this thesis, direct evidence for the increase of

dolichol and dolichol phosphate level during inflammation has been provided by measuring dolichol and dolichol phosphate in homogenates and microsomes obtained from inflamed rats. Similar increase of dolichol and dolichol phosphate during inflammation was earlier shown by Rip et al (188).

Additional direct evidence for the increase of dolichol and dolichol phosphate during inflammation has also been provided by incubating the cells with [^{3}H] mevalonate. A similar increase in dolichol and dolichol phosphate synthesis from labelled acetate has been observed in developing sea urchin embryo, where a significant amounts of N-linked glycoprotein synthesis is required for the maturation of the embryo (174). Rossignol et al (189) had shown, using isoprenoid precursors of dolichol chain, that dolichol synthesis precedes dolichol phosphate synthesis. Dolichol is phosphorylated by a CTP-dependent dolichol kinase in the endoplasmic reticular membrane. The increase of dolichol kinase activity was found in liver microsomal membrane from inflamed rats (168). Similarly, a dolichol kinase activity was found to be present in sea urchin embryo, and its activity increased as the embryo proceeds towards maturation (173). Together, these results indicate that both de novo synthesis of the polyisoprenoid chain of dolichol and its phosphorylation by dolichol kinase play a role in the increase in N-linked glycoprotein synthesis in inflammation and in some other cases like developing sea urchin embryos. Adair and Keller

(76) have shown that de novo synthesis is responsible for maintaining the hepatic pool of dolichol and dolichol phosphate. For a 10 gm liver, an in vivo rate of synthesis of 1.7-2.6 nmol/day of dolichol phosphate has been calculated. By dolichol feeding studies, the dietary contribution to the liver dolichol pool was less than 0.046 nmol per 30 h. A salvage pathway is another alternative way, under different physiological conditions, to control the hepatic dolichol level.

In contrast, the results presented in this thesis with dexamethasone-treated hepatocytes, show a lack of any effect of increased amounts of exogenous dolichol phosphate added to the assay on the ratio of Man-P-Dol formation in dexamethasone treated to untreated hepatocytes. This suggests that dexamethasone treatment of hepatocytes probably resulted in a true increase of the mannosyltransferase. A similar increase of glycosyltransferase levels: galactosyl- and sialyltransferases was observed in endometrial tissues of ovariectomized rats after estrogen administration (190). A study by Ramachandran et al (191) has shown that dexamethasone causes an increase in the formation of Man-P-Dol in Hela cells. The response of mannosyltransferase activity to exogenous dolichol phosphate was greater in the homogenates from dexamethasone treated Hela cells, suggesting that dexamethasone affects mannosyltransferase in an independent manner not involving the increase in the concentration of cellular dolichol phosphate. This conclusion was substantiated

by the result presented in this thesis that dexamethasone was found to have little effect on the synthesis of dolichol and dolichol phosphate from [^{3}H]-mevalonate.

It seems that inflammation induced leucocyte-derived regulatory factors (cytokines, IL-1, HSF) and hormones from endocrine tissues might be involved in the control of dolichol-linked saccharide synthesis in vivo. Leukocytes derived factors regulate the acute phase response including the synthesis of a acute phase protein and pituitary-adrenal axis is also activated during the acute phase response. It has been shown that HSF and IL-1 were potent stimuli for ACTH release in AtT-20 pituitary tumor cells (141). This communication between the immune and neuroendocrine systems probably explain the synergistic mode of action of glucocorticoid hormones and HSF to induce the hepatic synthesis of acute phase proteins (135).

We have examined the relationship of Man-P-Dol and $(GlcNAc)_{1-2}$-P-P-Dol synthesis to nascent peptide synthesis by incubating cells with protein synthesis inhibitors. Actinomycin D blocked the dexamethasone induced response of Man-P-Dol and $(GlcNAc)_{1-2}$-P-P-Dol synthesis when it was added together with dexamethasone in control hepatocytes. But the response was partly maintained when actinomycin D was added 4 and 8 h after dexamethasone stimulation. Actinomycin D also failed to block the inflammation induced increase of Man-P-Dol and $(GlcNAc)_{1-2}$-P-P-Dol synthesis,

when it was added together with dexamethasone in hepatocytes from inflamed rats.

Cycloheximide, a potent inhibitor of protein translation, inhibited the inflammation or dexamethasone induced increase of mannosyl and N-acetylglucosaminyltransferase activities. The mechanism by which these inhibitors affect glycosylation is not known.

One obvious explanation for this inhibition is that these inhibitors cause an extremely rapid accumulation of GDP-mannose synthesized predominantly from endogenous stores of unlabelled mannose. This would result in a greatly lowered specific radioactivity of labeled GDP-mannose in the homogenate, which would be manifested by an apparent inhibition of saccharide lipid synthesis.

Another possible explanation is that these inhibitors could alter the dolichol phosphate or enzyme(s) levels in the cells which is revealed by a decrease in the saccharide lipid synthesis.

The specific activity of mannosyltransferase activity was found to be highest in microsomes, although somewhat very little activity was present in mitochondrial and debris fraction respectively (Table 11). The cross contamination of the cell fractions and the consequent appearance of microsomal enzymes in non microsomal fractions are not unexpected. There was a small decrease of recovery of mannosyltransferase activity in microsomal fraction in all

three experimental groups. This may be due to translocation of enzyme into cytosol fraction. Since our conclusions are based on the specific activity of the enzyme, this apparent translocation of the enzyme will not affect the conclusions. In fact, in most of our studies, enzyme changes noted in whole homogenates, which included the total enzyme activity of the cell. It has been mentioned previously that dolichol phosphate is the major intermediate in N-linked glycoprotein biosynthesis. Dolichol phosphate was found to be present in highest concentration in rough endoplasmic reticular membranes (188). Since dolichol phosphate has major involvement in N-glycosylation, the presence of mannosyltransferase activity in microsomal fraction to initiate glycoprotein synthesis seems plausible.

CHAPTER V

EFFECT OF INFLAMMATION AND DEXAMETHASONE ON THE BIOSYNTHESIS OF DOLICHOL-LINKED OLIGOSACCHARIDES AND GLYCOPROTEINS IN HEPATOCYTES.

5.1. INTRODUCTION

It has been mentioned previously that the precursor oligosaccharide-lipid is assembled in the membrane of the endoplasmic reticulum from the nucleotide sugars: UDP-GlcNAc, GDP-Man and UDP-Glc. The first sugar added to dolichol phosphate is GlcNAc-P with the formation of GlcNAc-P-P-Dol. Then six more sugars are added directly from the nucleotide sugars to yield the key intermediate $Man_5GlcNAc_2$-P-P-Dol, to which seven outer sugars are added *via* the dolichol phosphate linked intermediates, Man-P-Dol and Glc-P-Dol. These two compounds (Man-P-Dol and Glc-P-Dol) are made from dolichol phosphate and the respective nucleotide sugars. Upon completion, the $Glc_3Man_9GlcNAc_2$ oligosaccharide can be transferred from its lipid carrier to growing polypeptide chains in the membrane of the endoplasmic reticulum.

It is apparent that the biosynthesis of this oligosaccharide lipid involves many different enzymes. Although all of the enzymes involved in oligosaccharide lipid synthesis (38,95) have been well characterized, little is known about the ways by which cells may regulate the activity of these enzymes. Each enzyme may have an important regulatory role

in the biosynthesis of the lipid linked oligosaccharide intermediates.

It is well established that dolichol phosphate acts as the "lipid carrier" in the assembly of oligosaccharide lipid and for the synthesis of Man-P-Dol and Glc-P-Dol. Dolichol phosphate may itself have a role in controlling oligosaccharide lipid synthesis. Supplementation of exogenous dolichol phosphate to mouse LM cells, by preincubation of the cells for 1 h with dolichol phosphate, resulted in a 300% increase of oligosaccharide lipid synthesis when compared to non-supplemented cells (192). Carson and Lennarz (175) have demonstrated that during embryonic development of the sea urchin, a rise in dolichol phosphate levels occurs that correlates with enhanced glycoprotein synthesis. Other investigators have postulated that the synthesis of the lipid linked oligosaccharide may be controlled by translation of the nascent peptide itself (192,193). This was based on the finding that the administration of inhibitors of protein translation caused a decreased incorporation of radiolabelled sugars into lipid linked oligosaccharides.

Regulation of the pathway may also occur by the modulation of the specific glycosyltransferases which transfer sugar units onto dolichol phosphate. Activities of various glycosyltransferases may be controlled by allosteric mechanisms. Kean (194) has reported that GDP-mannose stimulated the incorporation of radiolabelled N-acetylglucosamine residues

from UDP-GlcNAC into $(GlcNAc)_{1-2}$-P-P-Dol. The stimulating effect of GDP-mannose has also been seen for the transfer of the terminal glucose residue to the high mannose lipid-linked oligosaccharide (195). It is possible that the presence of this nucleotide sugar is important for the metabolic regulation of lipid-linked oligosaccharide biosynthesis. If nucleotide sugars are important for this pathway, then mechanisms that limit the availability of these molecules may also play a role in the regulation.

Synthesis of glycoproteins in liver has been shown to be increased during inflammation and in dexamethasone treatment. This might cause an alteration in the assembly of oligosaccharide lipid. Hence, experiments were carried out to study the effect of inflammation and dexamethasone on the incorporation of radiolabelled sugars into oligosaccharide lipid and proteins in rat hepatocytes. The oligosaccharide lipid intermediates formed during this treatment were characterized by gel filtration chromatography.

5.2. RESULTS

A. Effect of inflammation and dexamethasone on the incorporation of [^{14}C] mannose from GDP-[^{14}C] mannose into oligosaccharide lipid in hepatocyte homogenates.

The rate of formation of oligosaccharide in hepatocyte homogenates was linear up to 20 minutes of incubation and 1 mg of homogenate protein (results not shown). Fig. 32 shows

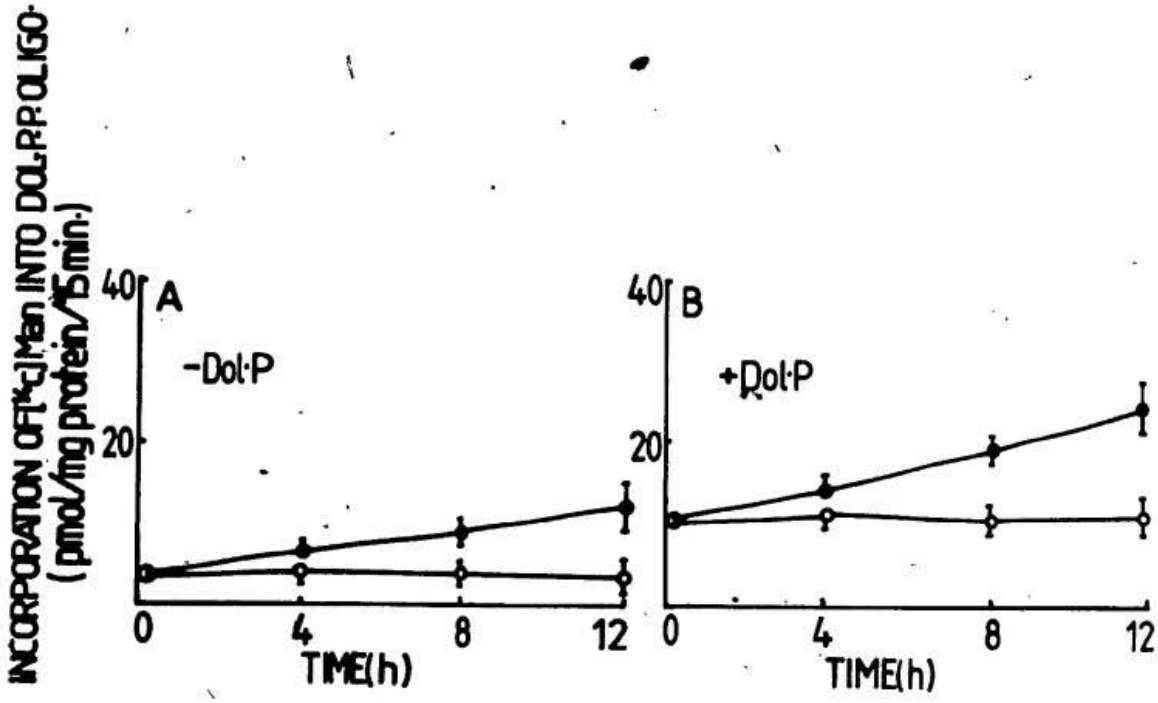

Fig. 32. Effect of dexamethasone on the rate of oligosaccharide lipid synthesis by hepatocyte homogenates from control rats.

Hepatocytes isolated from control rats, were incubated for 12 h in Waymouth's medium either in the presence (1 μM) or absence of dexamethasone. The enzyme activity was measured in homogenates in the absence (A) and in presence (B) (7.5 nmol) of exogenous dolichol phosphate. Solid symbols, dexamethasone treated cells; open symbols, untreated cells. The results are mean ± S.D. from three experiments.

the incorporation of [^{14}C] mannose into oligosaccharide lipid as a function of time of incubation. Hepatocytes were prepared from control rats and incubated for 12 h either in the absence or presence of dexamethasone (1μM). Homogenates prepared from these hepatocytes were incubated with GDP-[^{14}C] mannose in the presence (7.5 nmol) or absence of dolichol phosphate. The incubation mixture was extracted for the [Man-^{14}C]oligosaccharide lipid. Dexamethasone treatment resulted in an increase of the formation of [man-^{14}C] oligosaccharide lipid by about 3-fold either in the presence or absence of dolichol phosphate ($P < 0.01$).

Fig. 33 shows the incorporation of [^{14}C] mannose into oligosaccharide lipid in hepatocytes obtained from inflamed rat. Hepatocytes were incubated either in the absence or presence of dexamethasone (1μM) for 12 h. Homogenates prepared from these hepatocytes were incubated with GDP-[^{14}C] mannose in the presence or absence of exogenous dolichol phosphate and the incubation mixtures were extracted for [man-^{14}C] oligosaccharide lipid. In hepatocytes from inflamed rats the formation of oligosaccharide lipid was 1.5-2 fold higher compared to controls (Fig. 32 and 33; $P < 0.01$). Addition of dexamethasone to hepatocytes from inflamed rat resulted in a further increase of the formation of [man-^{14}C] oligosaccharide lipid by about 3-fold ($P < 0.01$).

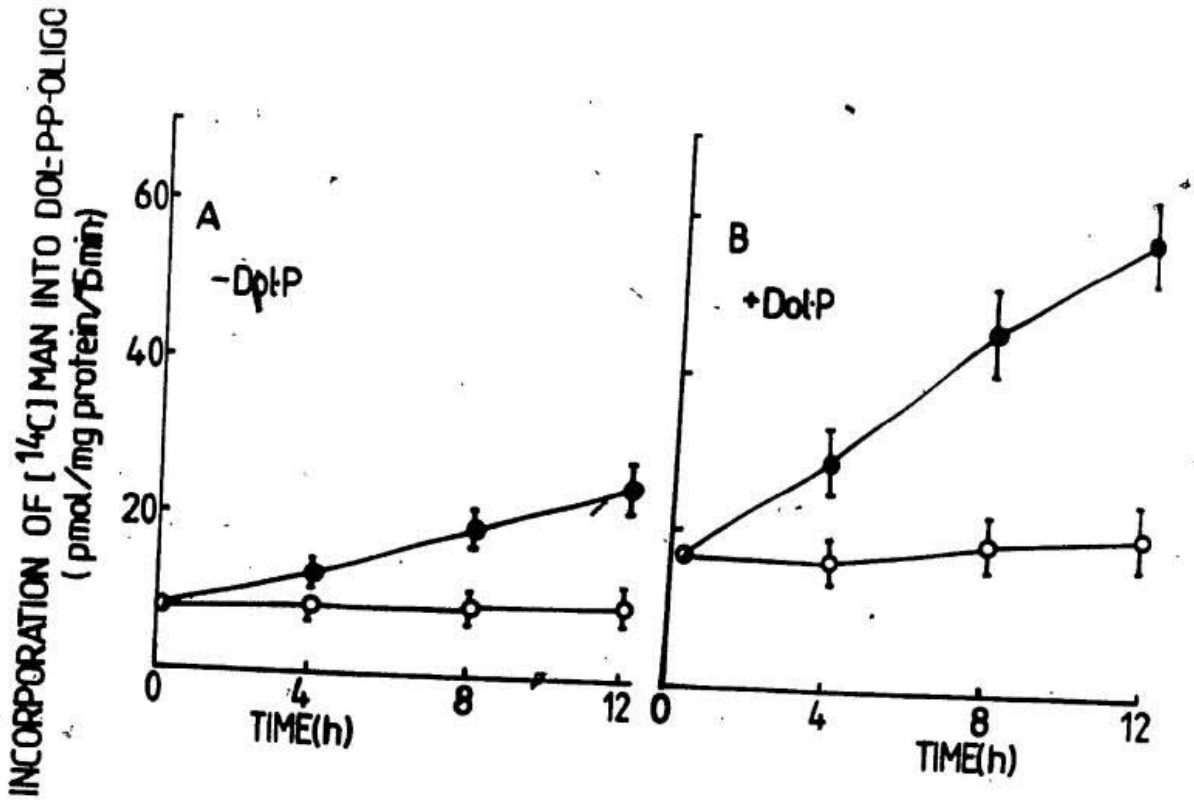

Fig. 33. Effect of dexamethasone on the rate of oligosaccharide lipid synthesis by hepatocyte homogenates from inflamed rats.

Hepatocytes isolated from inflamed rats, were incubated for 12 h in Waymouth's medium either in the presence or absence of dexamethasone. The enzyme activity was measured in homogenates in the absence (A), and in presence (B), (7.5 nmol) of exogenous dolichol phosphate. Solid symbols, dexamethasone treated cells; open symbols, untreated cells. The results are mean ± S.D. from three experiments.

B. Effect of increasing amounts of exogenous dolichol phosphate on [man-^{14}C] oligosaccharide lipid synthesis in hepatocyte homogenates.

Since the addition of dexamethasone to hepatocytes produced a greater increase of [man-^{14}C] oligosaccharide lipid formation with exogenous dolichol phosphate, the effect of increasing concentrations of dolichol phosphate (0-37.5 nmol) on the synthesis of [man-^{14}C] oligosaccharide was studied. The formation of oligosaccharide lipid increased in control, inflamed and dexamethasone treated-cell homogenates in assays containing increasing concentration of dolichol phosphate (Table 12). Dexamethasone-treated cells had a higher rate of oligosaccharide lipid formation at concentrations up to 30 nmol of dolichol phosphate, but a further increase of dolichol phosphate decreased the rate. Results in Fig. 34 show the ratios of [man-^{14}C]oligosaccharide lipid formation between the dexamethasone-treated groups over untreated groups as a function of increasing amounts of dolichol phosphate. The dexamethasone treatment of the cells from either control or inflamed rats maintained a higher rate of oligosaccharide lipid formation in the presence of increasing concentrations of dolichol phosphate. In contrast, the increase in the ratio of oligosaccharide lipid formation between hepatocytes from inflamed over control rats achieved a value close to unity as the amount of dolichol phosphate in the assay was increased (Fig. 34).

Table 12. Effect of exogenous dolichol phosphate on the incorporation of [^{14}C] mannose into oligosaccharide lipid.

Hepatocytes prepared from control and inflamed rats were incubated for 12 h in Waymouth's medium either in presence (1μM) or absence of dexamethasone. Enzyme activity was measured in the homogenates in the presence of increasing quantities of dolichol phosphate (0-37.5 nmol). The results are mean ± S.D. from three separate experiments.

[Man-^{14}C] oligosaccharide formation in hepatocytes
(Pmol/mg protein/5 min)

Amounts of dolichol phosphate (nmol)	Control A	Inflamed B	Ratio B/A	Control A	Dexamethasone C	Ratio C/A	Inflamed B	Inflamed + Dexamethasone D	Ratio D/B
-	5.5±0.5	9.9±1.2	1.8	5.5±0.5	12.4±1.4	2.3	9.9±1.2	25.4±2.2	2.5
7.5	10±1	16.5±2.1	1.65	10±1	24±3	2.4	16.5±2.1	43±5	2.6
15	14±1.8	22.4±1.8	1.6	14±1.8	35±5	2.5	22.4±1.8	58±11	2.6
22.5	19±3	26.4±3.2	1.5	19±3	50±7	2.6	26.4±3.2	72±13	2.7
30	28±4.3	35±5	1.25	28±4.3	78±6	2.8	35±5	98±14	2.8
37.5	26±5	30±5	1.1	26±5	60±6	2.3	30±5	91±18	2.8

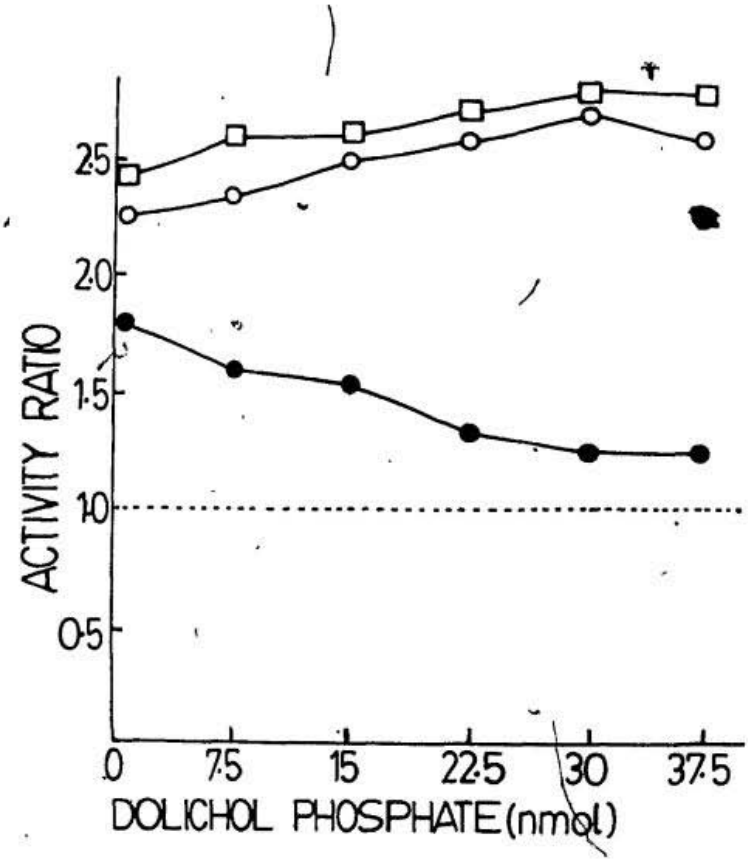

Fig. 34. Effects of exogenous dolichol phosphate on the incorporation of [^{14}C] mannose into oligosaccharide lipid.

Hepatocytes prepared from control and inflamed rats were incubated in Waymouth's medium for 12 h either in the presence (1μM) or in absence of dexamethasone. Enzyme activity was measured in the homogenates in the presence of increasing quantities of dolichol phosphate. Results are presented as ratios of activities between inflamed and control in the absence of dexamethasone (●), Control cells in the presence and in the absence of dexamethasone (○) and inflamed cells in the presence and in the absence of dexamethasone (□).

c. Effects of actinomycin D and cycloheximide on the oligosaccharide lipid synthesis induced by inflammation and dexamethasone treatment.

Several laboratories (107,193,196) have reported that inhibition of protein synthesis leads to inhibition of the incorporation of [^{14}C] mannose into oligosaccharide lipid. It therefore seems likely that the mechanism of this inhibitory effect is related to regulation for N-linked glycosylation, since the various drugs employed inhibit protein synthesis by different modes of action (107,193,196). It was suggested previously that an increase of acute-phase glycoprotein synthesis by inflammation and dexamethasone treatment was the result of de novo protein synthesis. It was therefore important to study the effect of an RNA synthesis inhibitor (actinomycin D) and a protein translation inhibitor (cycloheximide) on the induced increase of oligosaccharide lipid formation. Tables 13 and 14 show the effect of actinomycin D on the increase of oligosaccharide lipid synthesis by inflammation and dexamethasone treatment in hepatocyte homogenates. Administration of actinomycin D with dexamethasone at 0 h to hepatocytes obtained from control and inflamed rats completely suppressed the dexamethasone effect on oligosaccharide lipid synthesis. When actinomycin D was added 4 and 8h after the addition of dexamethasone, only a partial increase in oligosaccharide lipid synthesis was observed in both types of hepatocytes.

Table 13. Effect of actinomycin D on the dexamethasone induced increase of oligosaccharide lipid synthesis in hepatocytes obtained from control rats.

Hepatocytes were cultured for 12h. Addition of dexamethasone (1μM) and actinomycin D (1μg/ml) were at t = 0 h unless otherwise indicated. Enzyme activities were measured in the homogenate with or without exogenous dolichol phosphate. Results are expressed as mean ± S.D. from 3-4 different experiments. *, +, significantly different from control (*$p < 0.01$; + $p < 0.05$).

Treatment	[man-^{14}C]oligosaccharide lipid (pmol/mg protein/15 min)	
	-Dol-P	+Dol-P
No addition	4.4±0.8	8.8±1.1
Dexamethasone alone	10.5±2.5*	22.0±2.0*
Dexa.+actinomycin D	3.8±0.3	7.9±0.8
Actinomycin D alone	4.1±0.5	7.6±0.7
Dexa.+actinomycin D at t=4h	5.4±0.1+	9.7±1.1+
Dexa.+actinomycin D at t=8h	8.0±1.6*	15.6±0.7*

Table 14. Effect of actinomycin D on dexamethasone and inflammation-induced increases of oligosaccharide lipid formation in hepatocytes obtained from inflamed rats. The experimental details are as in Table 13. *, significantly different from inflamed (*, $P < 0.01$).

	[Man-^{14}C]-oligosaccharide lipid (pmol/mg protein/15 min)	
Treatment	-Dol-P	+Dol-P
No addition	8.4±0.8	14.5±0.2
Dexamethasone alone	20.6±0.9*	41.7±5.2*
Dexa.+actinomycin D	7.4±0.4	13.5±2.9
Actinomycin D alone	7.8±0.9	12.6±0.4
Dexa.+actinomycin D at t=4h	13.3±2.1*	21.2±3.2*
Dexa.+actinomycin D at t=8h	15.7±1.7*	26.3±4.3*

Actinomycin D failed to block the inflammation induced increase of oligosaccharide lipid synthesis when it was added to the hepatocytes obtained from inflamed rats.

Tables 15 and 16 show the effect of cycloheximide on the inflammation and dexamethasone-induced increase of oligosaccharide lipid synthesis. Cycloheximide completely blocked the dexamethasone induced-increase of oligosaccharide lipid formation in hepatocytes either from control or inflamed rats. When cycloheximide was added 8 h after dexamethasone, no increase of oligosaccharide lipid formation was observed. Cycloheximide also partially blocked the inflammation induced increase of oligosaccharide lipid synthesis when it was added to hepatocytes from inflamed rats.

D. Studies on the incorporation of [^{14}C] mannose into [man-^{14}C] oligosaccharide lipids and glycoproteins in intact hepatocytes from control and inflamed rats.

Hepatocytes isolated from control and inflamed rats were incubated with [^{14}C] mannose either in the presence or absence of dexamethasone, and the incorporation of [^{14}C] mannose into oligosaccharide lipid and glycoprotein was monitored for 12 h. Fig. 35 shows the results of incorporation of [^{14}C] mannose into oligosaccharide lipid. Inflammation increased the incorporation of [^{14}C] mannose into oligosaccharide lipid by about 1.5 fold. However, in both control and inflamed hepatocytes, the incorporation of

Table 15. Effect of cycloheximide on the dexamethasone induced increase of oligosaccharide lipid synthesis in hepatocytes obtained from control rats.

Hepatocytes from control rat were cultured for 12h. Addition of dexamethasone (1 μM) and cycloheximide (100μg/ml) were at t = oh unless otherwise indicated. Enzyme activities were measured in the homogenate with or without exogenous dolichol phosphate. Results are expressed as mean ± S.D. from 3-4 different experiments. *Significantly different from control ($p < 0.01$).

	[Man-^{14}C] oligosaccharide lipid (pmol/mg protein/15 min)	
Treatment	-Dol-P	+Dol-P
No addition	5.0±0.3	9.4±0.4
Dexamethasone alone	12.4±0.7*	24.6±2.5*
Dexa.+ cycloheximide	4.3±0.1	8.0±1.0
Cycloheximide alone	4.0±0.6	8.4±1.3
Dexa. +/cycloheximide at t=4h	4.5±0.1	10.6±1.8
Dexa. + cycloheximide at t=8h	4.6±0.2	8.2±1.3

Table 16. Effect of cycloheximide on the inflammation and dexamethasone induced increase of oligosaccharide lipid synthesis in hepatocytes obtained from inflamed rats.

Hepatocytes were isolated from inflamed rats and cultured for 12 h. The experimental details are as in Table 15. *Significantly different from inflamed ($p < 0.01$).

	[Man-^{14}C]oligosaccharide lipid (pmol/mg protein/15 min)	
Treatment	-Dol-P	+ Dol-P
No addition	9.0±1.9	15.6±1.8
Dexamethasone alone	21.8±1.9*	45.2±4.5*
Dexa. + cycloheximide	6.6±1.4	10.9±1.8
Cycloheximide alone	6.2±0.8	10.8±2.0
Dexa. + cycloheximide at t=4h	6.6±0.7	10.7±2.2
Dexa. + cycloheximide at t=8h	7.2±0.3	11.3±0.9

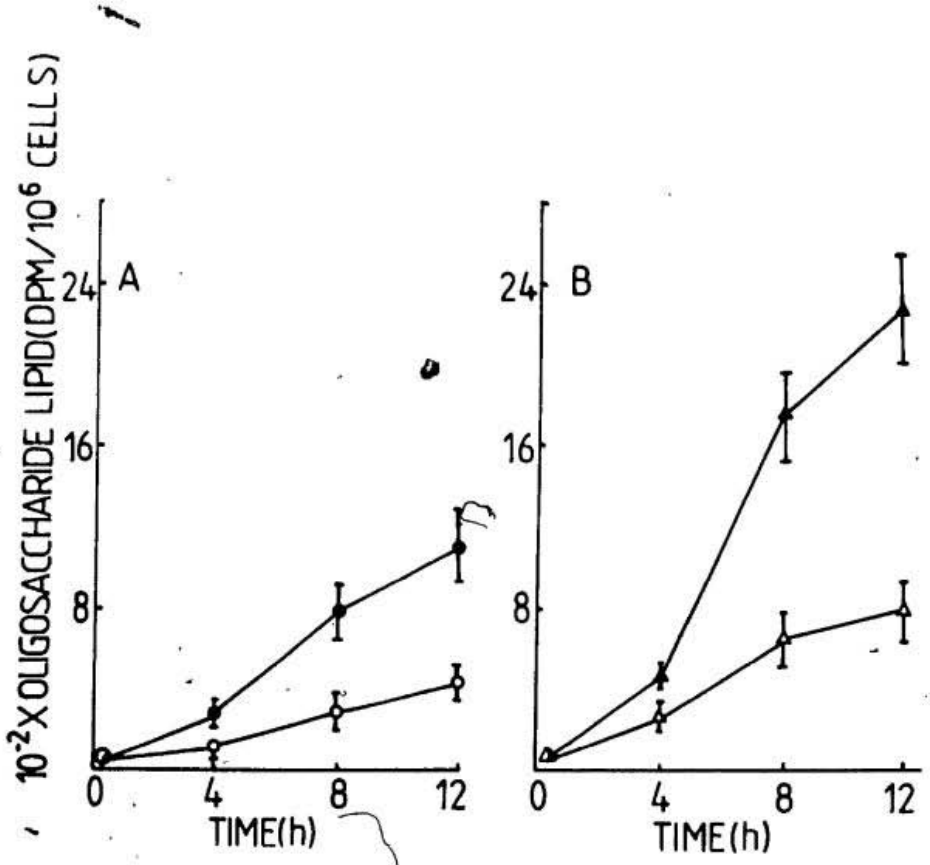

Fig. 35. Effect of dexamethasone on [^{14}C]-mannose incorporation into oligosaccharide lipid in intact hepatocytes.

Hepatocytes (20×10^6 cells) isolated from control and inflamed rats were incubated with [^{14}C] mannose (0.4μ Ci/ml of incubation medium) either in the presence (1$^{\mu}$ M) or absence of dexamethasone. Panels, (A) and (B) are cells from control and inflamed rats respectively. Solid symbols, dexamethasone treated cells; open symbols, untreated cells. Results are mean ± S.D. of three experiments.

[^{14}C] mannose into oligosaccharide lipid was increased about 3 fold by dexamethasone treatment. Fig. 36 show the incorporation of [^{14}C] mannose into cellular protein. Inflammation increased the incorporation of [^{14}C] mannose into cellular protein about 1.5 fold. A similar increase of incorporation into cellular protein was observed in hepatocytes from control and inflamed rats by dexamethasone. The stimulation of [^{14}C] mannose incorporation into oligosaccharide lipids and glycoproteins was statistically significant (Table 17).

E. Effect of cell density on the [^{14}C] mannose incorporation into Man-P-Dol, [Man-^{14}C] oligosaccharide lipid and proteins in intact hepatocytes.

To elucidate whether the incorporation of [^{14}C] mannose into Man-P-Dol, oligosaccharide lipid and glycoprotein depended upon the cell density, hepatocytes from control and inflamed rats at various cell densities were incubated with [^{14}C] mannose either in the absence or presence of dexamethasone. Table 18 shows the effect of various cell densities on the incorporation of [^{14}C] mannose into Man-P-Dol, oligosaccharide lipid and glycoproteins. The highest incorporation of [^{14}C]mannose into these products was observed when the cell density of 0.41×10^6 cells/ml was used in the incubation medium. However, the effects in different experimental groups remained unchanged up to

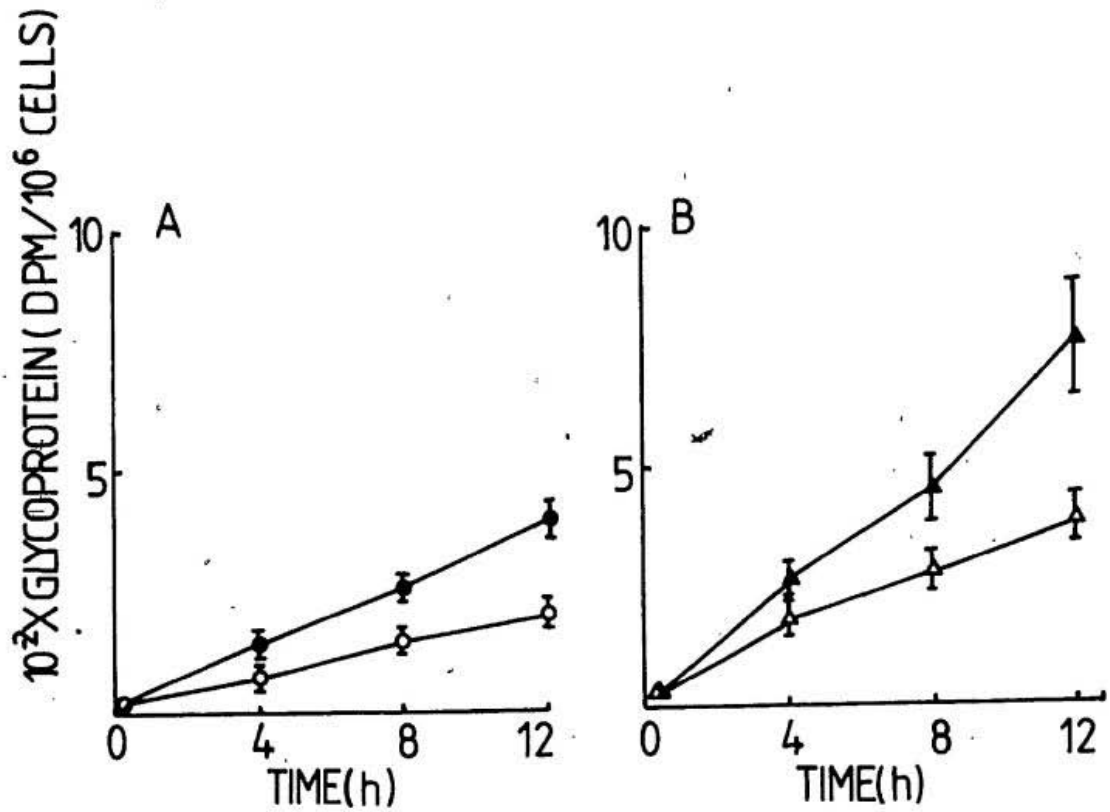

Fig. 36. Effect of dexamethasone on [^{14}C] mannose incorporation into cellular protein in intact hepatocytes.

Hepatocytes ($20x10^6$ cells) isolated from control (A) and inflamed (B) rats were incubated with [^{14}C] mannose (0.4μ Ci/ml of incubation medium) either in the presence (Solid symbol) (1κM) or absence of dexamethasone (Open symbol). The samples were withdrawn at appropriate times and incorporation of [^{14}C] mannose into cellular proteins was measured as described in 'Materials and Methods' section.

Table 17. Effect of dexamethasone on the incorporation of [^{14}C] mannose into oligosaccharide lipid and cellular proteins.

Hepatocytes ($20x10^6$ cells) isolated from control and inflamed rats were incubated with 0.4 µCi of [^{14}C] mannose/ml of incubation for 12h either in the absence or presence of dexamethasone (1 µM). The incorporation of radioactivity into oligosaccharide lipid and glycoprotein were determined as described in 'Materials and Methods' sections. Values are mean ± S.D. from five different experiments. *Significantly different from control ($P < 0.01$).

	Incorporation (d.p.m.)/10^6 cells) into:	
Treatment	Oligosaccharide lipid	N-linked glycoprotein
Control	417±77	215±45
Dexamethasone	1098±308*	383±109*
Inflamed	687±187*	363±85*
Inflamed and dexamethasone	1787±205*	642±244*

Table 18. Effect of cell density and dexamethasone on[^{14}C]mannose incorporation into Man-P-Dol, oligosaccharide lipid and proteins.

Hepatocytes isolated from control and turpentine-inflamed rats were incubated for 12h either in the presence or absence of dexamethasone. The cells were incubated in Waymouth's medium supplemented with 17.5% (v/v) heat-inactivated horse serum in a total volume of 20 ml and were labelled with 0.4μCi of [^{14}C]mannose/ml of incubation medium for 6h before the termination of incubation. The incorporation of radioactivity into different fractions was measured as described in the Materials and Methods section. Results given are means ± S.D. for triplicate experiments.

Treatment	10^{-6} x Cell density (cells/ml)	Incorporation (d.p.m.) into: Man-P-Dol	Oligosaccharide lipid	N-linked glycoprotein
Saline	3.2	5835±1020	6448±1077	3553±216
	1.6	8312±993	10178±906	4861±314
	0.82	4843±1087	5412±782	2678±512
	0.41	2665±787	2912±502	1373±233
Dexamethasone	3.2	6776±1371	7408±1573	3857±60
	1.6	11556±1336	25814±4650	6483±339
	0.82	7021±1693	13741±2646	4681±549
	0.41	4451±710	7375±1935	2414±748
Inflamed	3.2	6566±1121	7002±991	4192±229
	1.6	12384±1033	16223±1312	7162±329
	0.82	7361±983	7213±929	3282±292
	0.41	4310±1310	4252±739	2205±414
Inflamed and dexamethasone	3.2	6823±1094	7521±989	4524±253
	1.6	17929±898	38354±1409	9626±409
	0.82	9213±772	16343±1021	4322±339
	0.41	5949±1013	11926±1125	3476±173

$1.6x10^6$ cell/ml.

F. Analysis of oligosaccharide lipid complexes

Results in Figs. 32,33 and 35 show that there are differences in the amount of oligosaccharide lipid formation when the hepatocytes, either from control or inflamed rats, were treated with dexamethasone. However, the oligosaccharide lipid preparation contained all the oligosaccharide intermediates of the dolichol cycle. In order to determine if inflammation and dexamethasone treatment caused a change in the distribution of the various intermediates, the oligosaccharides were separated on the basis of their sizes. This was performed by mild acid hydrolysis of the oligosaccharide lipid complexes to remove the lipid, followed by fractionation of the oligosaccharides on Bio gel P-4 column.

Cell-free oligosaccharide lipid preparations were analyzed by gel filtration chromatography and the results are shown in Fig. 37. The oligosaccharide profiles from four different preparations were very similar, and consisted of three peaks. Results show no preferential increase of any particular peak. The amount of radioactivity under each peak was proportionately increased by inflammation and dexamethasone treatment.

[Man^{14}C] oligosaccharide lipid was prepared by incubating the cells with [^{14}C] mannose. Gel filtration chromatography profiles of these oligosaccharides are shown in Fig. 38. The

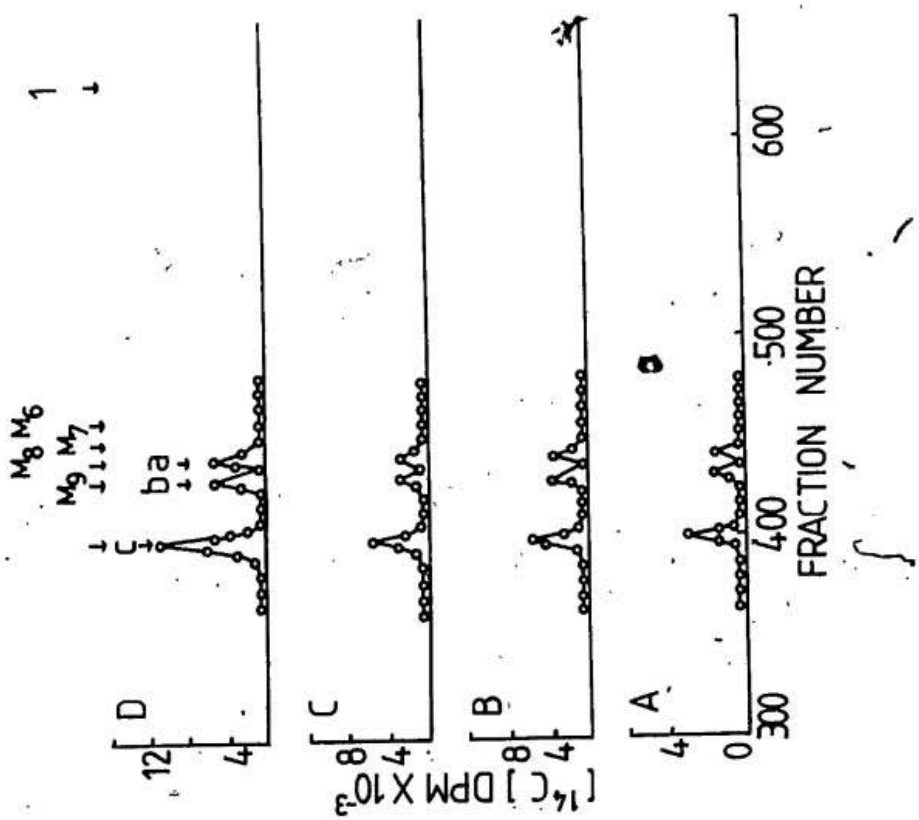

Fig. 37. Chromatography of oligosaccharide derived from oligosaccharide lipid complex isolated from hepatocyte homogenates.

After mild acid hydrolysis of the lipid linked oligosaccharides, the free oligosaccharides were fractionated using bio gel P-4 column as described in 'Materials and Methods' sections. Panel A, control; Panel B, dexamethasone treated control cells; Panel, C, inflamed; Panel D, dexamethasone treated cells from inflamed rats. Arrows indicate the elution position of standards: M_6, $Man_6GlcNAc_1$; M_7, $Man_7GlcNAc_1$, M_8, $Man_8GlcNAc_1$; M_9, $Man_9GlcNAc_1$, 1, Mannose.

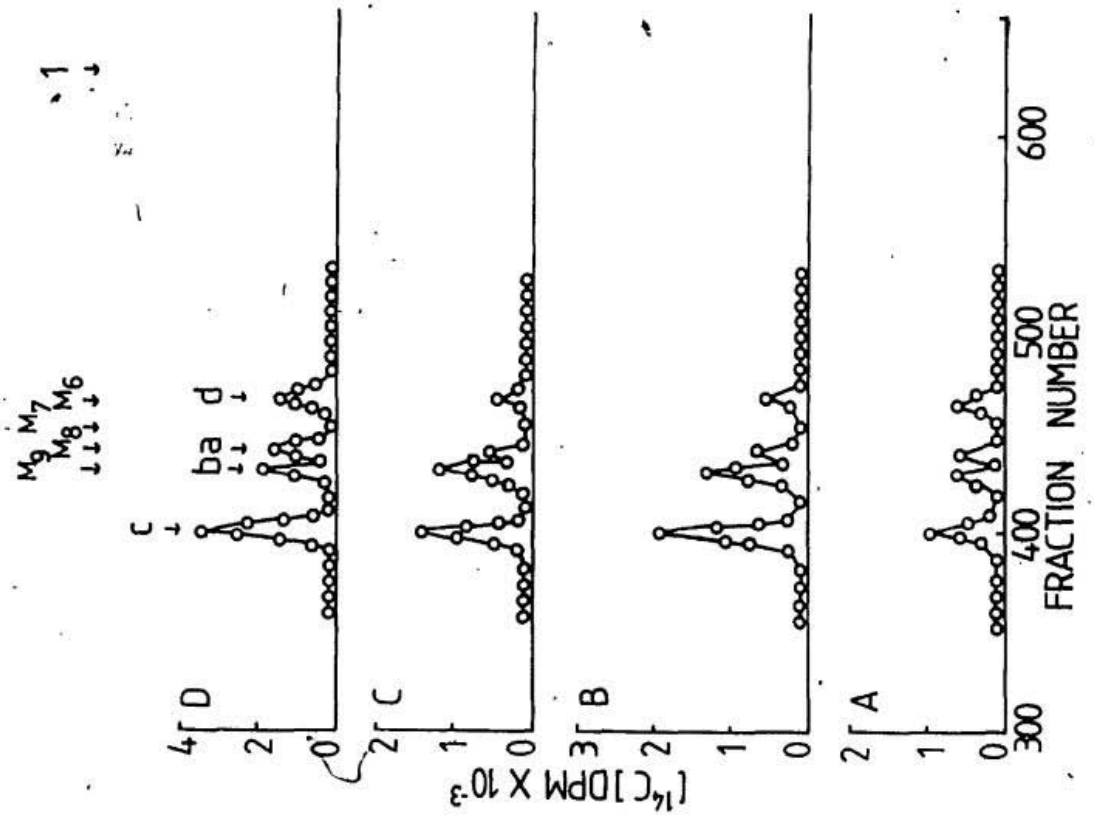

Fig. 38. Chromatography of oligosaccharides derived from oligosaccharide lipid complex prepared by incubation of hepatocytes with [^{14}C] mannose.
Isolated oligosaccharide, after being, hydrolyzed by acid were fractionated in bio gel P-4 column according to the procedure as described in 'Materials and Methods' sections. Panel, A, control; Panel B, dexamethasone treated control cells; Panel C, inflamed; Panel D, dexamethasone treated cell from inflamed rats. Arrows indicate the elution position of standards: M_6, $Man_6GlcNAc_1$; M_7, $Man_7GlcNAc_1$; M_8, $Man_8GlcNAc_1$; $Man_9GlcNAc_1$, 1, Mannose.

oligosaccharide profiles of the four different preparations consisted of four peaks. This result does not indicate a preferential increase of any particular peak, instead the amount of radioactivity under each peak was increased by inflammation and dexamethasone treatment.

5.3. DISCUSSION

Hepatocytes prepared from control and inflamed rats were incubated either in the presence or absence of dexamethasone (1 μM). The homogenates prepared from the hepatocytes were used to prepare oligosaccharide lipid. The formation of oligosaccharide lipid was about 2-fold higher in inflamed rats compared to controls. Addition of dexamethasone to the incubation caused a 3-fold increase of oligosaccharide lipid formation in hepatocytes from either control or inflamed rats. Similar results were obtained when the cells were incubated with [^{14}C] mannose in the presence of dexamethasone. It was shown in Chapter IV that dexamethasone caused a 1.5 to 2-fold increase in the formation of Man-P-Dol and $(GlcNAc)_{1-2}$-P-P-Dol in hepatocytes obtained from both control and inflamed rats. The results showing a lesser degree of stimulation of $(GlcNAc)_{1-2}$-P-P-Dol compared to oligosaccharide lipid formation by dexamethasone, suggest that dexamethasone may activate some step(s) in the elongation of sugar chains from $(GlcNAc)_{1-2}$-P-P-Dol to oligosaccharide lipid. The increased stimulation of oligosaccharide lipid

formation may not be merely due to the increased specific radioactivity of GDP-man, UDP-GlcNAc or dolichol phosphate, since such a change must be reflected initially in the incorporation of radioactive labels into Man-P-Dol or $(GlcNAc)_{1-2}$-P-P-Dol.

We have examined the effect of exogenous dolichol phosphate on oligosaccharide lipid synthesis in hepatocytes from control and inflamed rats treated with dexamethasone. Exogenous dolichol phosphate has been found to increase the oligosaccharide lipid synthesis in the hepatocytes.

The increase observed in oligosaccharide lipid synthesis, either in inflamed hepatocytes or after treatment with dexamethasone, may be interpretated on the basis of elevated levels of either endogenous dolichol phosphate or the relevant enzyme(s) involved in oligosaccharide lipid synthesis. It has been shown previously (Chapter IV) that the increased formation of Man-P-Dol is due to elevated levels of endogenous dolichol phosphate in inflammation, whereas in dexamethasone treatment, the increase is due to higher levels of mannosyltransferase activity (149). The results presented in this thesis using exogenous dolichol phosphate support a similar postulate that the increase observed in oligosaccharide lipid synthesis during inflammation is probably due to higher levels of endogenous dolichol phosphate. In the presence of low amounts of dolichol phosphate (7.5 nmol), the formation of oligosaccharide lipid was increased in

control, inflamed and dexamethasone treated hepatocytes. When increasing amounts of dolichol phosphate were used in the assay, the ratio of oligosaccharide lipid synthesis between inflamed and control hepatocytes decreased towards unity. This indicated that the enzyme(s) levels in inflamed hepatocytes were probably not altered compared to controls, the increase seen being due to higher amounts of endogenous dolichol phosphate formation in inflammation (149). On the other hand, when increasing amounts of dolichol phosphate were used in the assay, the ratio of oligosaccharide lipid synthesis between dexamethasone treated and untreated hepatocytes did not decline. This result suggests that treatment of the hepatocytes with dexamethasone probably resulted in a true increase of enzyme(s) involved in the assembly of oligosaccharide lipids. This conclusion is similar to the one previously made for Man-P-Dol synthesis during inflammation and dexamethasone treatments (Chapter IV).

We also examined the effect of actinomycin D and cycloheximide on the increase of oligosaccharide lipid synthesis in inflammation and dexamethasone treatment. Actinomycin D blocked the dexamethasone induced increase of oligosaccharide lipid synthesis, when it was added simultaneously with dexamethasone. It failed to suppress the increase when the addition was delayed for 4 and 8 h after addition of dexamethasone. Actinomycin D also failed to block the increase of oligosaccharide lipid synthesis

induced by inflammation. Cycloheximide, on the other hand inhibited the dexamethasone induced effect completely. It also partially inhibited the inflammation induced effect. Studies in several laboratories (107,192,193) have suggested that the observed block in oligosaccharide lipid synthesis by protein synthesis inhibitors might result from a deficiency in dolichol phosphate, since this compound would not be regenerated by transfer of the oligosaccharide chain to protein in the absence of newly synthesized glycosylatable proteins. This might also explain our observations on the cycloheximide effect on oligosaccharide lipid synthesis.

Furthermore, Grant and Lennarz (192) have shown that the synthesis of mannose labelled oligosaccharide lipids was markedly inhibited in the presence of cycloheximide in mouse LM cells. They tested the hypothesis that in LM cells, limitation of dolichol phosphate synthesis was the reason for inhibition of oligosaccharide lipid synthesis by cycloheximide, and concluded that this was not the case. They preincubated the cells with dolichol phosphate and then oligosaccharide lipid synthesis was measured in the absence or presence of cycloheximide. Oligosaccharide lipid synthesis was markedly (300%) stimulated by preincubation with dolichol phosphate, indicating that as in other systems (174,175), dolichol phosphate is the limiting factor for the synthesis of oligosaccharide lipids <u>in vivo</u>. More importantly in relation to the cycloheximide effect, when cells were

preincubated with dolichol phosphate and then treated with cycloheximide, the same marked inhibition was observed as in cells not pre-exposed to dolichol phosphate. Thus although LM cells apparently can utilize exogenous dolichol phosphate from the culture medium for enhanced oligosaccharide lipid synthesis, this enhanced availability of dolichol phosphate cannot relieve the inhibition caused by cycloheximide. Our studies show that, cycloheximide treated cell homogenates can utilize exogenous dolichol phosphate and increase the oligosaccharide lipid synthesis, but could not relieve the inhibition caused by cycloheximide.

Gel filtration elution profiles of oligosaccharides isolated from control, inflamed and dexamethasone-treated hepatocyte homogenates incubated with GPD-[^{14}C]-mannose were found to be similar. In each case only three peaks a,b, and c were obtained. These peaks were identified on the basis of a comparison with elution positions of homologous series of tritium labelled high mannose oligosaccharide standards. Peaks a and b have been identified to have the composition of man_7-$GlcNAc_2$ and $man_8GlcNAc_2$ respectively. The largest oligosaccharide peak c was not identified.

Gel filtration elution profiles of oligosaccharides isolated from control, inflamed and dexamethasone treated intact hepatocytes incubated with [^{14}C] mannose were also similar. In this case, however, four peaks were eluted and peaks a,b, and d were identified to have the composition of $man_7GlcNAc_2$, $man_8GlcNAc_2$ and $man_5GlcNAc_2$ respectively.

CHAPTER VI

EFFECT OF INFLAMMATION AND DEXAMETHASONE ON GDP-MANNOSE AND UDP-GlcNAc PYROPHOSPHATASE ACTIVITIES IN RAT HEPATOCYTES.

6.1. Introduction

"Acute-phase proteins" in serum, contain significant amounts of N-linked oligosaccharide, which are synthesized via the dolichol pathway in rat liver parenchymal cells, The synthesis of these proteins is increased during inflammation and dexamethasone treatment. In the previous chapters (IV and V), it has been reported that incorporation of [^{14}C] mannose and [^{14}C]-N-acetylglucosamine to Man-P-Dol, $(GlcNAc)_{1-2}$-P-P-Dol, oligosaccharide lipid and N-linked glycoproteins was increased in cultured hepatocytes from inflamed rats. Dexamethasone, added to the hepatocytes obtained from control and inflamed rats, also increased the synthesis of the above products.

Nucleotide sugars are essential as sugar donors in the synthesis of dolichol linked intermediates involved in N-linked glycoprotein synthesis. Nucleotide sugar pyrophosphatases, which hydrolyze nucleotide sugars, have been implicated in the regulation of the metabolism of nucleotide sugars in relation to glycoprotein synthesis. In bacteria, the action of a nucleotide sugar pyrophosphatase has been suggested to play a role in the regulation of intracellular concentrations of various nucleotide sugars

(197). Kean and Bighouse (198) have characterized a cytidine 5'-monophospho sialic acid hydrolase from rat liver, and have speculated that it may play a role in regulating the concentration of CMP-sialic acid within the cell.

It has been reported that nucleotide sugar pyrophosphatase and phosphodiesterase present in various tissues might have a shared activity against certain phosphodiester bonds (199,200). A recent study by Rousseau et al indicated that a similar phosphodiesterase activity had been induced by various glucocorticoids in a rat hepatoma cell line (201). This observation indicates that a specific increase in nucleotide sugar pyrophosphatase activity might be induced by the administration of dexamethasone. Inflammation also increases the non-specific hydrolytic activity of phosphodiester bonds in different tissue systems (202). Nucleotide-sugar pyrophosphatase activities have been shown to be affected in a variety of physiological and pathological conditions (203).

Glycosyltransferase reactions have been found to be affected by the presence of highly active nucleotide sugar pyrophosphatases. The focus of the studies in this chapter has been to determine if the nucleotide sugar pyrophosphatase activity is affected during inflammation and dexamethasone treatment. The present study was also undertaken to investigate whether the rate of formation of the Man-P-Dol, $(GlcNAc)_{1-2}$-P-Dol and oligosaccharide lipid undergo changes in the presence of nucleotide pyrophosphatase inhibitors. The

level of nucleotide sugars was also determined from control, and dexamethasone treated hepatocytes and in hepatocytes from inflamed rats.

6.2. Results

A. Increase of nucleotide sugar pyrophosphatase activity by dexamethasone.

Hepatocytes isolated from control rats were incubated in the presence of 1μM dexamethasone for 12 h, since this concentration of dexamethasone is known to saturate the glucocorticoid receptor and evoke maximal effect on tyrosine aminotransferase in rat liver cells (187). Fig. 39 and 40 show the effect of dexamethasone on GDP-mannose pyrophosphatase and UDP-GlcNAc pyrophosphatase activities as a function of time and enzyme protein concentration, respectively. The products of GDP-mannose and UDP-GlcNAc pyrophosphatase reactions were Man-1-P and GlcNAc-1-P, respectively. The initial rate of Man-1-P formation was 42 and 71 nmol of [^{14}C]Man-1-P/min/mg protein for control and dexamethasone treated 'total membranes preparation' of hepatocytes, respectively ($P < 0.01$). The initial rate of GlcNAc-1-P formation was similarly 32 and 59 nmol of [$_{14}$C] GlcNAc-1-P/min/mg protein ($P < 0.01$).

Fig. 41 shows the effect of dexamethasone on GDP-mannose and UDP-GlcNAc pyrophosphatase activities in hepatocytes incubated for 0-12 h. The results show that the level of

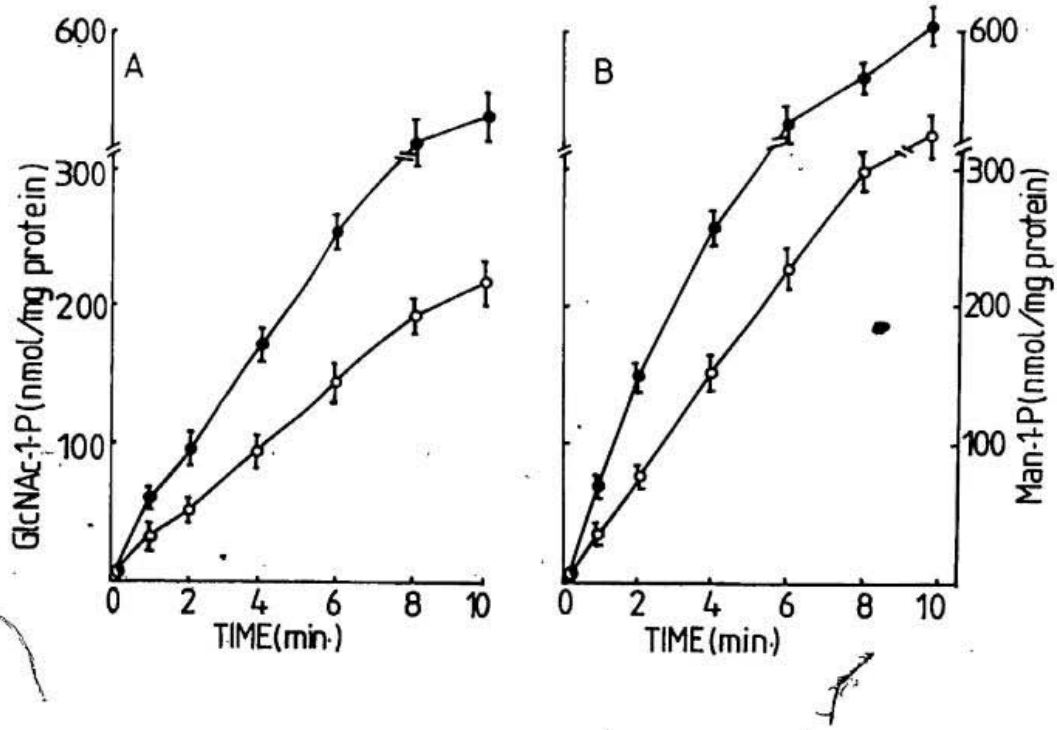

Fig. 39. Effect of dexamethasone on nucleotide sugar pyrophosphatase activities as a function of assay time.

Hepatocytes isolated from control rats and cultured for 12h in presence of dexamethasone (1 μ M). 'Total membranes preparation' was prepared as described in 'Materials and Methods' section and used for the enzyme assay. O, control; ●, dexamethasone treated. A, UDP-GlcNAc pyrophosphatase; B, GDP-Man pyrophosphatase. Results are mean ± S.D. from 3-6 different experiments.

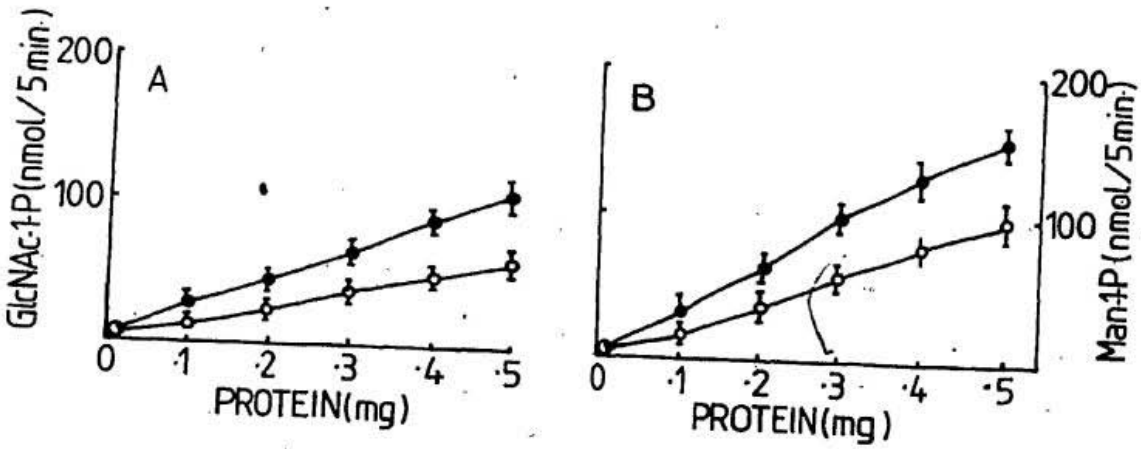

Fig. 40. Effect of dexamethasone on nucleotide sugar pyrophosphatase activities as a function of protein concentration in the assay.

Hepatocytes isolated from control rats and cultured for 12 h in the presence of dexamethasone (1 μM). 'Total membranes preparation' was prepared as described in 'Materials and Methods' section and used for the enzyme assay. ○ control; ●, dexamethasone treated. A, UDP-GlcNAc pyrophosphatase; B, GDP-Man pyrophosphatase. Results are mean ± S.D. from 3-6 different experiments.

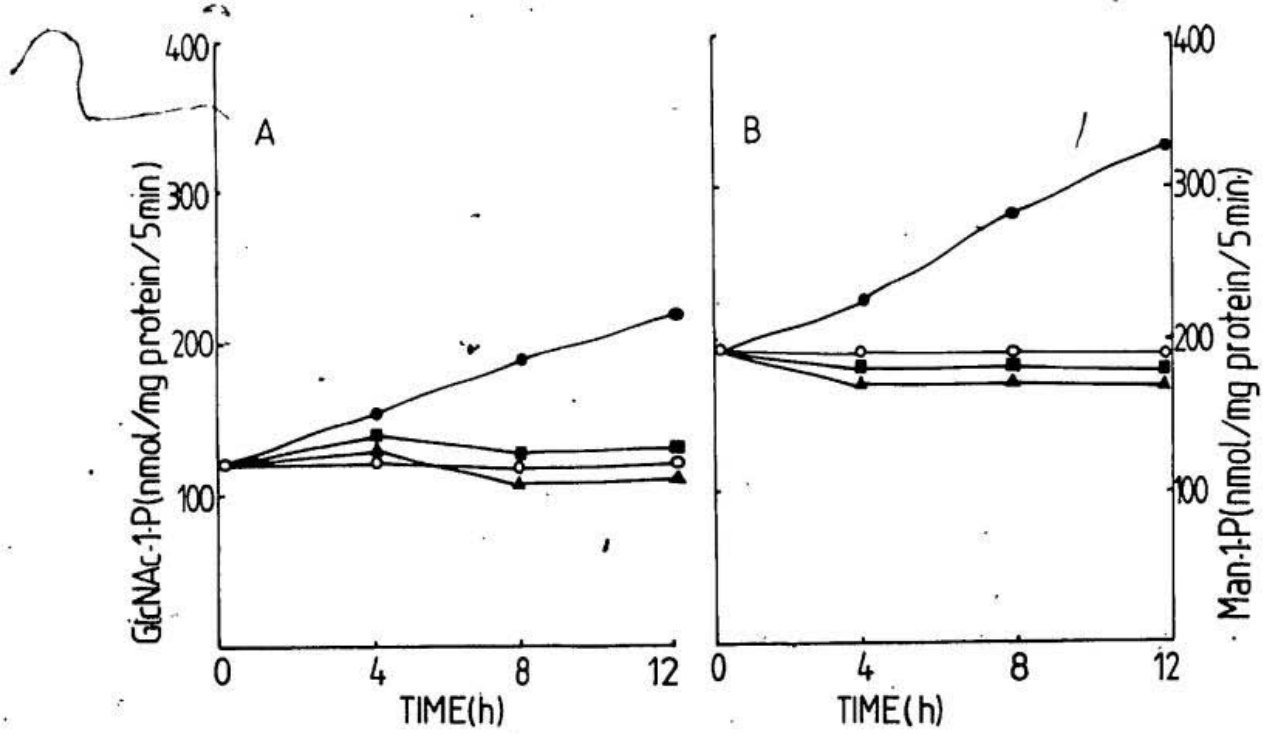

Fig. 41. Effect of dexamethasone on nucleotide sugar pyrophosphatase activities for 12 h in hepatocyte homogenates from control rats.

Hepatocytes isolated from control rats were incubated with 1μM dexamethasone over a period of 12h. 'Total membranes preparation' was prepared following procedure as described in 'Materials and Methods' section and was used for the enzyme assay. A, UDP-GlcNAc pyrophosphatase, B, GDP-Man pyrophosphatase. ○, control; ●, presence of dexamethasone; △, dexa. + actinomycin D; ■, dexa + cycloheximide. The results are mean from triplicate determinations from three different experiments.

UDP-N-acetyl glucosamine pyrophosphatase activity was increased about 2 fold (137 nmol/mg protein/5 min to 223 nmol/mg protein/5 min) over a 12 h period of incubation in dexamethasone treated cells ($P < 0.01$). Similarly GDP-mannose pyrophosphatase activity was increased from 196 nmol/mg protein/5 min to 327 nmol/mg protein/5 min by dexamethasone treatment ($P < 0.01$). The initial increases of enzymatic activities occurred as early as 8 hour of incubation and continued up to 12 h. The results in Fig. 41 also show that the increase of GDP-mannose and UDP-GlcNAc pyrophosphatase activities were completely suppressed when actinomycin D or cycloheximide were added in combination with dexamethasone.

Dexamethasone also increased the GDP-mannose and UDP-GlcNAc pyrophosphatase activities in hepatocytes obtained from inflamed rats. However, no increase of activities was observed in inflamed rat hepatocytes compared with controls (Fig. 42).

B. Mechanism of dexamethasone effect on the increase of nucleotide sugar pyrophosphatase activities.

The possibility that the increase of nucleotide sugar pyrophosphatase activity was due to a direct action of dexamethasone upon the enzyme itself was investigated. The direct addition of dexamethasone to the assay did not affect the enzymatic activities (results not shown), suggesting that stimulation is not due to a direct effect of dexamethasone

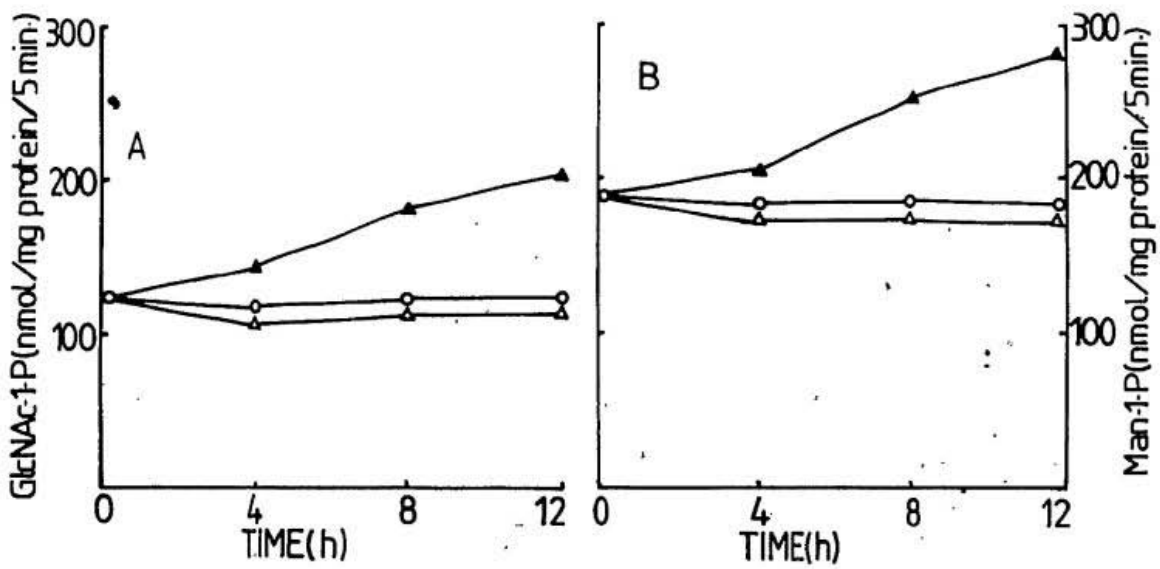

Fig. 42. Effect of dexamethasone on nucleotide sugar pyrophosphatase activities for 12h in hepatocyte homogenates from inflamed rats.

Hepatocytes isolated from inflamed rats were cultured in the presence of dexamethasone (1μ M) over a period of 12h. 'Total membranes preparation' was prepared following procedure as described in 'Materials and Methods' and was used for the enzyme assay. A, UDP-GlcNAc pyrophosphatase; B, GDP-Man pyrophosphatase. ○, control, △, inflamed; ▲, inflamed + dexamethasone. The results are mean from 2-3 different experiments.

on the enzyme. As shown in Fig. 41, the enzyme activity increased as early as 8 h by dexamethasone. A lag of 8 h for the increase of pyrophosphatase activity is consistent with the fact that the stimulation of the specific activity of the enzyme is probably due to the synthesis of new enzymes. To investigate this possibility, inhibitors of macromolecular synthesis i.e., actinomycin D and cycloheximide were used in the hepatocyte cultures. If the mechanism of induction involved increased gene transcription and subsequent translation into protein, then inhibitors of RNA transcription and protein translation should prevent the induction of enzymatic activity. As shown in Fig. 41, induction of pyrophosphatase activities was greatly inhibited in the presence of actinomycin D or cycloheximide. When actinomycin D was added at 0 h, the stimulatory effect of dexamethasone on GDP-mannose pyrophosphatase activity was abolished (Table 19). But, addition of actinomycin D, 8 h after the addition of dexamethasone resulted in an escape from the inhibitory effect of transcription inhibitor. At this time actinomycin D only inhibited 11% of the activity due to dexamethasone. Similarly, the effect of addition of cycloheximide at 8 h of incubation of hepatocytes in presence of dexamethasone showed an escape from inhibition by the translation inhibitor. At this time cycloheximide only inhibited 18% of the activity due to dexamethasone. Addition of actinomycin D and cycloheximide at different times of incubation of the

Table 19. Effect of actinomycin D and cycloheximide on dexamethasone induced stimulation of nucleotide sugar pyrophosphatase activity in hepatocyte culture. Results are mean ± S.D. from three different experiments.

Treatment	GDP-mannnose pyrophosphatase (nmol/mg protein/ 5 min)± SD	% of control	Inhibition (%) from dexamethasone stimulation	UDP-GlcNAC pyrophosphatase (nmol/mg protein/ 5 min)± SD	% of control	Inhibition (%) from dexamethasone stimulation
Saline	195±6	0	-	135±14	0	-
Dexamethasone alone	326±5	167	0	222±18	164	0
Dexamethasone + actinomycin D	174±18	89	47	129±10	95	42
Dexamethasone + actinomycin D at t=4h	231±15	118	29	157±21	116	29
Dexamethasone + actinomycin D at t=8h	292±14	149	11	186±17	138	16
Dexamethasone + cycloheximide	180±14	92	45	118±21	87	47
Dexamethasone + cycloheximide at t=4h	214±17	110	34	169±23	125	30
Dexamethasone + cycloheximide at t=8h	267±18	137	18	181±9	134	18

Hepatocytes were isolated from control rats and cultured for 12h. Addition of dexamethasone (1μM) and actinomycin D (1μ g/ml); cycloheximide (100μg/ml) were added t=0h unless otherwise indicated. Control values are set at 100 when the effects of dexamethasone are compared against control. Dexamethasone stimulated activities are set at 100 when the effect of inhibitors are compared.

hepatocytes in the presence of dexamethasone also showed a similar effect on UDP-GLcNAc, pyrophosphatase activity (Table 19).

The other possibility, that dexamethasone induces a rapidly turning over factor, which in turn enhances the catalytic activity of nucleotide sugar pyrophosphatase activity or an inhibitor of enzymatic activity, present in control cells being lost after treatment with dexamethasone, was examined by appropriate mixing experiments. The increased activity in mixtures of cell homogenates from control and dexamethasone treated cells was strictly additive (Fig. 43). This ruled out the possibility that dexamethasone promotes the appearance of an soluble activator or removal of an soluble inhibitor present in control cells.

We also examined whether dexamethasone increased the V_{max} of the nucleotide sugar pyrophosphatases or affinity for the substrate. The apparent Km of the enzyme was similar in dexamethasone treated and control cells (Table 20). However the V_{max} of the enzymes were increased for both UDP-GlcNAc and GDP-mannose by dexamethasone treatment (Table 20).

C. Glycosyltransferase activities in the presence and absence of nucleotide sugar pyrophosphatase inhibitors.

The study of glycosyltransferase reactions in a variety of animal tissues has shown the presence of a highly active nucleotide sugar pyrophosphatase, which may affect the

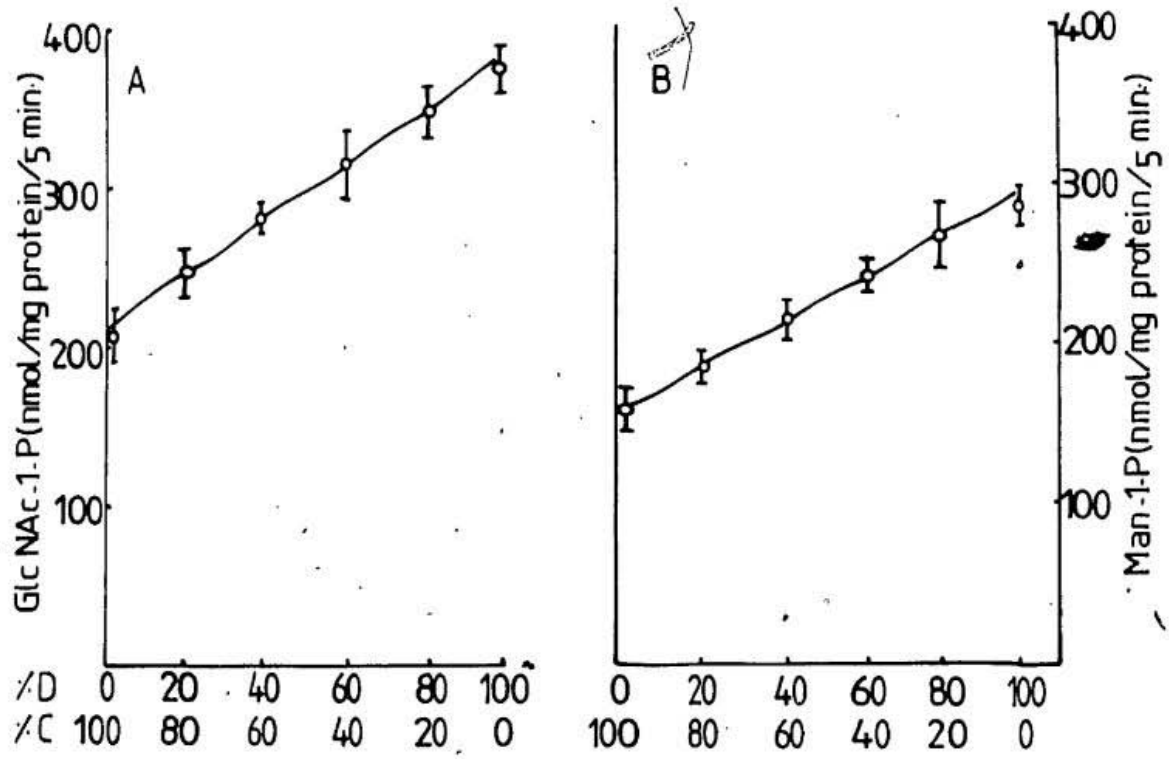

Fig. 43. Nucleotide sugar pyrophosphatase activity in mixtures of homogenate from control (C) and dexamethasone-treated (D) hepatocyte.

Both C and D cell homogenates were brought to the same protein concentration. A, UDP-GlcNAc pyrophosphatase and B, GDP-mannose pyrophosphatase activity. Results are mean ± S.D. from three different experiments.

Table 20. Kinetic parameters of nucleotide sugar pyrophosphatase activity in control and dexamethasone treated hepatocytes. Results are mean values from three different experiments.

	Control		Dexamethasone	
	GDP-Man Pyro-phosphatase	UDP-GlcNAc pyro-phosphatase	GDP-Man pyro-phosphatase	UDP-GlcNAcpyro-phosphatase
Apparent Km, mM	0.43	0.47	0.43	0.47
Vmax, nmol/min/mg protein	0.07	0.12	0.1	0.22
pH for maximal activity	9-10	9-10	9-10	9-10

The cells were incubated either in the absence or presence of 1μM Dexamethasone. Enzyme assays were done as described in "experimental procedure". To establish pH optima, 100 mM acetate (pH 4-5); 100 mM phosphate (pH 6-8) and 100 mM glycine-NaOH (pH 9-10.6) buffer systems were used.

glycosyltransferase assays. The methods most often utilized to control such degradation have involved the addition of a wide variety of nucleotide mono-, di-, and triphosphates to serve as competitive inhibitors (204,205). However, at a concentration adequate to spare sugar nucleotide substrates, the added nucleotide is often inhibitory to the glycosyltransferase reactions (206). It was reported that sugar nucleotide degradation by Zn^{2+}-requiring nucleotide pyrophosphatase is effectively inhibited by the addition of the chelator 2,3-dimercaptopropan-1-ol, along with low concentrations of nucleotides (207). As dexamethasone increases the nucleotide sugar pyrophosphatase activities, as well as N-acetylglucosaminyl and mannosyltransferase activities, it was of interest to study the effect of pyrophosphatase inhibitor on the dexamethasone induced increase of glycosyltransferases.

i) Effect of nucleotide sugar pyrophosphatase inhibitors, dimercaptopropanol and ATP, on N-acetylglucosaminyltransferase activity:

The effect of dimercaptopropanol and ATP on the incorporation of [^{14}C] GlcNAc into $(GlcNAc)_{1-2}$-P-P-Dol in hepatocyte membrane preparations is shown in Fig. 44. Dexamethasone increases the N-acetylglucosaminyltransferase activity compared to controls (Fig. 44), either in the presence or absence of nucleotide pyrophosphatase inhibitors,

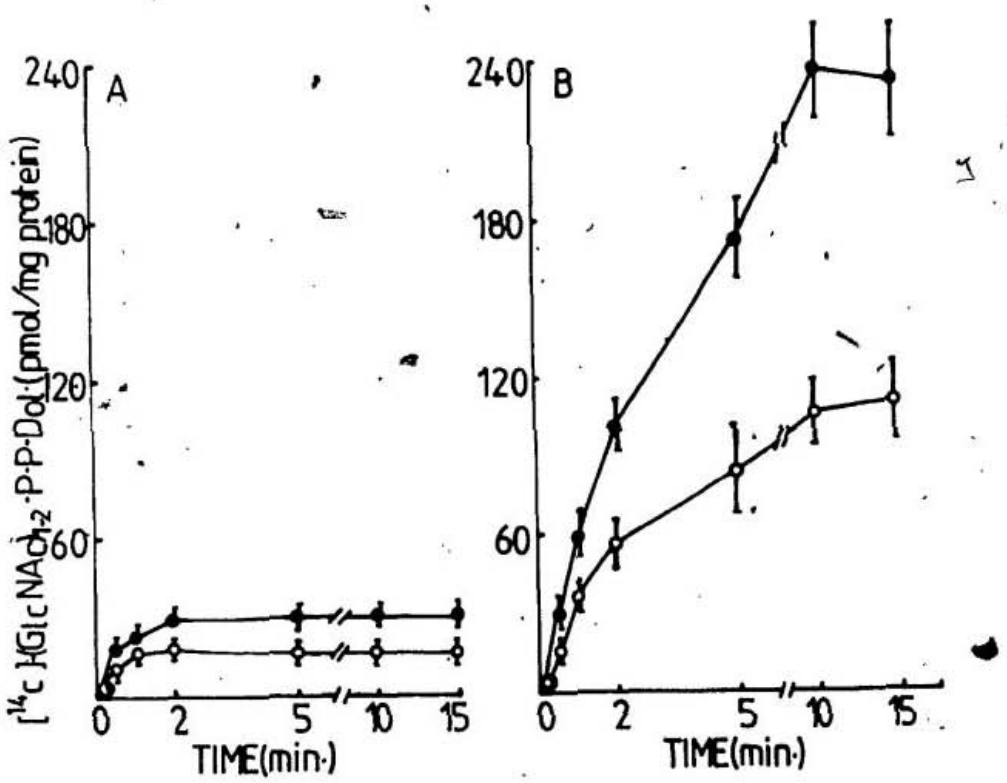

Fig. 44. Effect of dexamethasone on N-acetylglucosaminyl transferase activity either in the presence or absence of dimercaptopropanol and ATP.

Hepatocytes isolated from control rats, were incubated with dexamethasone (1µM) in Waymouth's medium for 12 h. The reaction mixture consisted of "total membrane preparation" (0.3 mg); UDP-[^{14}C]-N-acetylglucosamine (1.6 nmol); 6.5 mM Tris/HCl buffer (pH 7.4); 6.5 mM $MnCl_2$; 1.5 mM EDTA; Triton X-100 (0.15% w/v) and dolichol phosphate (7.5 nmol) in a total volume of 310 µl. Incubations were performed at 30°C. A, No addition of inhibitors; B, Addition of 5 mM dimercaptopropanol and 100µM ATP. O, control; ●, dexamethasone-treated. Results are mean ± S.D. from three different experiments.

dimercaptopropanol and ATP. Dimercaptopropanol specifically-chelates Zn^{2+}, necessary for nucleotide sugar pyrophosphatase activity, but it does not bind the divalent cations required for glycosyltransferases (208). The initial rate of N-acetylglucosaminyltransferase activity was 1.4 fold higher (22 versus 16 pmol GlcNAc/min/mg protein) in hepatocytes treated with dexamethasone in the absence of dimercaptopropanol and ATP (Fig. 44A). Addition of these inhibitors did not markedly increase the initial rate of enzyme reaction (1.7 fold, 52 versus 30 pmol GlcNAc/min/mg protein) in dexamethasone treated cells, but did increase the capacity of the dexamethasone treated cells to a greater extent than control cells (Fig. 44B). In the absence of inhibitors, the total capacity of N-acetylglucosaminyltransferase capacity was 28 pmol of GlcNAc/10 min/mg protein in dexamethasone treated cells, compared to 20 pmol of GlcNAc/10 min/mg protein in control cells (1.4 fold). With inhibitors these values were increased to 235 and 105 pmol of GlcNAc/10 min/mg protein respectively (2.2 fold), (Fig. 44B).

ii) <u>Effect of nucleotide sugar pyrophosphatase inhibitors, dimercaptopropanol and ATP on mannosyltransferase activity</u>:

The initial rate of [^{14}C] mannose incorporation into Man-P-Dol was higher in membrane preparations from dexamethasone treated hepatocytes compared to control (1.4 fold, 216 pmol Man/min/mg protein versus 160 pmol Man/min/mg protein)

(Fig. 45A). Similar to the results shown for N-acetylglucosaminyltransferase, dimercaptopropanol and ATP did not markedly increase the initial rate of enzyme activity in hepatocytes treated with dexamethasone (1.8 fold, 900 pmol Man/min/mg protein versus 500 Pmol Man/min/mg protein) (Fig. 45B). The capacity of control cells for mannose incorporation without dimercaptopropanol and ATP was 165 pmol Man/5 min/mg protein compared to 250 pmol Man/5 min/mg protein (1.5 fold) in dexamethasone treated cells (Fig. 45A). In the presence of dimercaptopropanol and ATP, this capacity was increased to 850 pmol Man/5 min/mg protein in control cells whereas after dexamethasone treatment the capacity was increased to 1925 pmol Man/5 min/mg protein (2.3 fold) (Fig. 45B).

D. Effect of dimercaptopropanol and ATP on the incorporation of [^{14}C] mannose from GDP-[^{14}C] mannose into oligosaccharide lipid.

Table 21 summarizes the results of the effect of dimercaptopropanol and ATP on the synthesis of oligosaccharide lipid in membranes prepared from control, inflamed and dexamethasone treated hepatocytes. A 2.5 fold increase in the incorporation of [^{14}C] mannose into oligosaccharide lipid was observed in dexamethasone treated cells. Addition of pyrophosphatase inhibitors, dimercaptopropanol and ATP, to the assays, increased the synthesis of oligosaccharide lipid

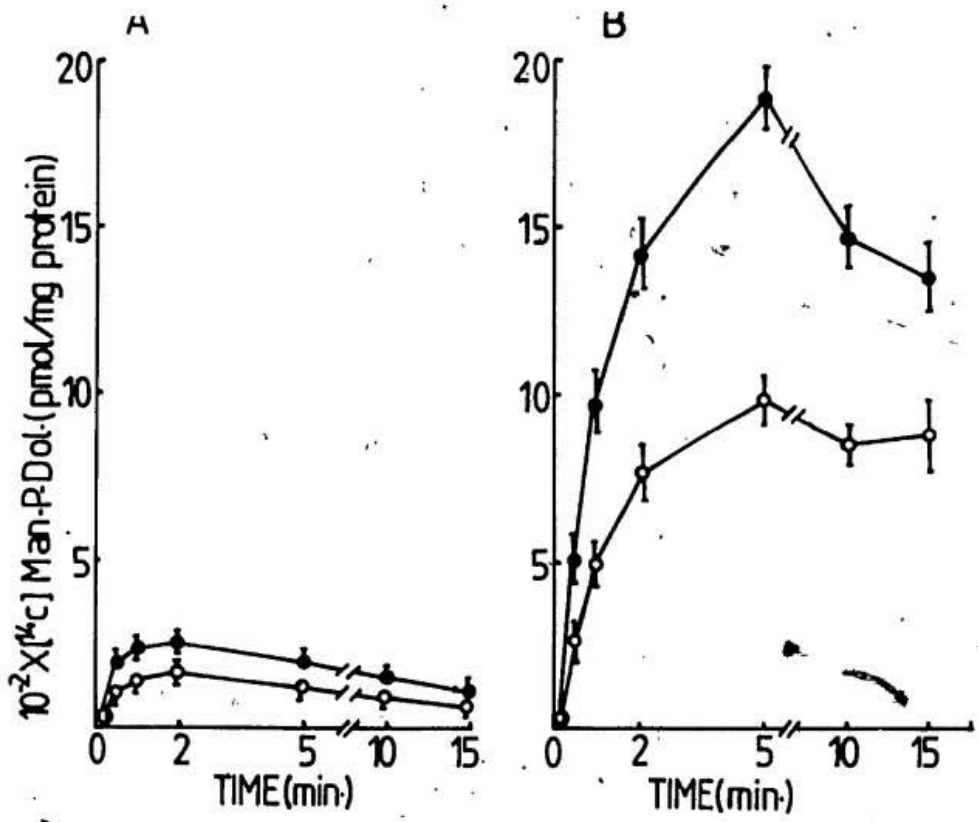

Fig. 45. Effect of dexamethasone on mannosyltransferase activity either in the presence or absence of dimercaptopropanol and ATP.
Reaction conditions were as described in Fig. 44 except that 0.28 nmol of GDP-[^{14}C] mannose was used instead of UDP-[^{14}C]-N-acetylglucosamine. A, No addition, B, Addition of dimercaptopropanol and ATP. O, control; ●, dexamethasone treated. Results are mean ± S.D. from three different experiments.

Table 21. Effect of dimercaptopropanol and ATP on the incorporation of [^{14}C] mannose into oligosaccharide lipid by membranes derived from control and dexamethasone treated hepatocytes.

'Total membranes preparation' was prepared as described in 'Materials and Methods' section and used for the enzyme assays. The reaction mixture consisted of 6.5 mM Tris/HCl, pH 7.4; 6.5 mM $MnCl_2$; 1.5 mM EDTA; GDP-[^{14}C] mannose (0.56 nmol); 0.32 mM unlabelled UDP-glucose and UDP-N-acetyl-glucosamine, dolichol phosphate (7.5 nmol); 0.15% (w/v) Triton X-100 and 0.3 mg protein in a total volume of 310µl. After 5 min of incubation at 30°C, the labelled oligosaccharide lipid was extracted following the procedure outlined in 'Materials and Methods' section. Results are expressed as mean dpm/mg protein/5 min from three experiments.

Treatment of hepatocytes	Assay Condition	Mannose[^{14}C]oligo-saccharide lipid synthesis in membrane
	100µM ATP + 5 mM Dimercaptopropanol	Incorporation in DPM
Control	-	547
Control	+	1168
Dexamethasone	-	1297
Dexamethasone	+	4788

in dexamethasone treated cells to a greater extent than in controls.

E. Determination of nucleotide sugar pools in hepatocyte culture:

The levels of three nucleotide sugars, UDP-N-acetyl glucosamine, GDP-mannose and UDP-glucose were measured in control, inflamed and dexamethasone treated cells. This study on the level of nucleotide sugar pools was expected to bear a correlation with nucleotide sugar pyrophosphatase activity. The levels of UDP-GlcNAc, GDP-mannose and UDP-glucose in hepatocytes under different conditions are shown in Table 22. The levels of these nucleotide sugars present in control, inflamed and dexamethasone treated cells were not changed, although we have observed changes in nucleotide sugar pyrophosphatase activities in dexamethasone treated conditions.

6.3. Discussion

The results presented in this section indicated that nucleotide sugar pyrophosphatase activity was increased in the hepatocytes by dexamethasone. Increased enzymatic activities were observed as early as 4 h after administration of dexamethasone. Similar findings have been described for a few other enzymes induced by certain glucocorticoids (Chapter 4 of this thesis, 201,209). In hepatocyte systems,

Table 22. Levels of nucleotide sugars in hepatocytes.

Hepatocytes isolated from control and inflamed rats were incubated in the presence of dexamethasone for 12 h. Nucleotide sugars were extracted from the hepatocytes by perchloric acid and were measured by a linear gradient of phosphate buffer by HPLC as described in 'Materials and Methods' section. The values are means ± S.D. from five different experiments.

	Nucleotide sugars (pmol/mg protein).		
Treatment	UDP-GlcNAc	GDP-Mannose	UDP-glucose
Control	340±7	32±3	535±11
Dexamethasone	373±14	34±5	577±22
Inflamed	326±7	37±4	565±22

it is thought that the mechanism of induction involves the binding of the steroid to a cytoplasmic receptor, which is then translocated to the nucleus. Once it reaches the nucleus, the hormone-receptor complex recognizes and interacts with specific regions of DNA to subsequently stimulate transcription of specific mRNA. The messages are then translated into protein in the cytoplasm and the modulation of enzymatic activity is observed. Results of actinomycin D and cycloheximide effects on the dexamethasone induced nucleotide sugar pyrophosphatase activities are consistent with the dependence of dexamethasone effect on RNA and protein synthesis.

Dexamethasone increases tyrosine aminotransferase and alkaline phosphodiesterase I in a rat hepatoma cell line (201). It is also known (201) that one enzyme is responsible for both alkaline phosphodiesterase I and nucleotide sugar pyrophosphatase activities. The similar competitive inhibition of phosphodiesterase activity with various nucleotide sugars and similar time course of induction with identical pH profiles for both activities, indicate that one enzyme catalyzed both reactions. A shared activity by alkaline phosphodiesterase and nucleotide sugar pyrophosphatase activity was also demonstrated in rat liver (199,200).

The exact physiological function of nucleotide sugar pyrophosphatase/alkaline phosphodiesterase remains unknown, although various authors have speculated a possible function

of its activity in the plasma membrane. A concomitant increase of nucleotide sugar pyrophosphatase activity with the increase in intracellular adhesion (210) has led to the speculation that enzyme induction and cellular adhesion may be functionally related.

The nucleotide sugar pyrophosphatase may provide an alternative control mechanism for regulating nucleotide sugars within the cell. Feedback inhibition of nucleotide sugar biosynthesis has been demonstrated in rat liver and bacteria (211,212). In these systems, the nucleotide sugars inhibit the first enzymatic step in their biosynthetic pathway. The enzymatic hydrolysis of the nucleotide sugars could be another mechanism for regulating nucleotide sugar concentrations within the cells.

Nucleotide sugar pyrophosphatase is known to be a membrane bound enzyme and the enzymatic activity has been found in the plasma membrane as well as in the endoplasmic reticulum (121,200). It seemed unlikely, therefore, that nucleotide pyrophosphatase could regulate nucleotide sugar levels in different cell compartments, since the membrane location of the enzyme would likely make it inaccessible to the cytoplasm which contains the major pools of nucleotide sugars. This is supported by our finding, that there are no differences in nucleotide sugar pool sizes (UDP-GlcNAc, GDP-Man and UDP-Glc) in control and dexamethasone-treated cells, despite a higher specific activity of nucleotide sugar

pyrophosphatase in dexamethasone treatment.

Our findings on the parallel increase of nucleotide sugar pyrophosphatase and glycosyltransferase by dexamethasone, and the effects of pyrophosphatase inhibitors on the expression of glycosyltransferase in dexamethasone treated cells, underline the importance of measuring the pyrophosphatase activity.. Welply et al (213) have reported the concomitant increase of nucleotide sugar pyrophosphatase and glycosyltransferase activities in conditions of enhanced N-linked glycoprotein synthesis in developing sea urchin embryos. In developing rabbit skeletal muscle, mannosyl and glucosyltransferases were elevated 6- and 5-fold respectively in neonatal rabbit skeletal muscle compared to adult. The neonate also exhibited 4-fold greater GDP-mannose pyrophosphatase activity than adults (214). These examples are similar to our observation of a parallel increase of nucleotide sugar pyrophosphatase and glycosyltransferase activities. However, a precise role of nucleotide sugar pyrophosphatase in overall glycoprotein synthesis, is not known. One may speculate that this enzyme controls the substrate levels for glycosylation by lowering their level at the site of synthesis of lipid linked saccharides and, thereby, triggering an increase of glycosyltransferase activity. Further investigations will be necessary to ascertain such regulatory role of nucleotide sugar

pyrophosphatase in glycosylation of protein by devising methods to measure the levels of nucleotide sugars in cellular compartments.

CHAPTER VII

DISCUSSION

Certain glycoprotein levels in serum increase in response to experimental inflammation by turpentine injection (126,215,216). These proteins are known as 'acute-phase proteins' and most of them are glycoproteins. The mechanism of induction of hepatic synthesis of acute-phase proteins in response to inflammatory stimuli is largely unknown. Macrophage factors (130) and glucocorticoids (128,129) have been implicated as mediators responsible for the induction of acute-phase proteins. α_1-Acid glycoprotein, one of the major acute-phase proteins and its mRNA, are elevated by administration of glucocorticoids in adrenalectomized and normal rats (133), and in hepatoma cells (HTC) in culture (217,218). Turpentine-induced acute-phase inflammation also induces α_1-acid glycoprotein synthesis and elevates α_1-acid glycoprotein mRNA levels in normal and adrenalectomized rats (133,219). This suggests independent regulatory processes for induction by glucocorticoid and by other mediators of the acute-phase response. It has been shown very recently that dexamethasone and turpentine induce several acute-phase proteins in adrenalectomized and normal rats (135). Combined treatment of the rats with dexamethasone and turpentine showed a synergistic increase for a few acute-phase proteins and an additive increase of a few others. Glucocorticoids may

influence the synthesis of acute-phase proteins by more than one mechanism. As in vitro, these hormones may exert their effects directly on the liver cells by affecting some of the subcellular processes involved in N-linked glycoprotein synthesis. Alternatively, the changes in the synthesis of these proteins could be induced indirectly, occurring secondarily to the effects of these hormones on the metabolic pathway of other organs or tissues. To elucidate the possibility that acute phase protein induction by glucocorticoid is due to a direct action of the hormone on the liver cell, we decided to use isolated hepatocytes in our studies. This system also reduces the complexity usually observed with studies in intact animals. Since inflammation and dexamethasone induce the synthesis of N-linked glycoproteins it was of special interest to determine the factors regulating the assembly of glycoproteins in liver. Such regulatory factors can include changes in the activities of relevant enzymes, the levels of nucleotide sugar and dolichol phosphate, as well as the expression of mRNA's for glycosylatable proteins.

7.1. Role of dolichol phosphate in glycoprotein synthesis:

It is now established that dolichol phosphate availability is a major regulatory factor for glycoprotein assembly in several systems (220,221). Indeed, Lucas and Levin (176) provided evidence that the increase in endogenous glycosyltransferase activities of estrogenized chick oviduct can be

attributed to increases in the availability of endogenous dolichol phosphate. It has also been shown that increased dolichol kinase activity accompanied these events (222). The results presented in Chapter IV support the possibility that the increase of dolichol linked intermediates during inflammation was due to increases in endogenous level of dolichol phosphate. It has also been shown by Rip et al (188) that dolichol phosphate levels are indeed increased in the liver during inflammation. Results presented in this thesis provided the evidence that synthesis of dolichol and dolichol phosphate from mevalonate was increased in inflammation.

7.2. Role of dolichol linked glycosyltransferase activities in regulating N-linked glycoprotein synthesis.

In addition to enzymes controlling the concentration of dolichol phosphate, there are a variety of other steps that could regulate the complex biosynthetic pathway of dolichol linked oligosaccharides (Fig. 9). These include the activity of glycosyltransferases in the sequential addition of sugar units to dolichol phosphate, ultimately resulting in the production of a mature oligosaccharide chain containing two N-acetylglucosamine units, nine mannose and three glucose residues. We have assessed the rate of formation of Man-P-Dol, $(GlcNAc)_{1-2}$-P-P-Dol and oligosaccharide lipid in hepatocytes from control and inflamed rats. Also the

effects of dexamethasone on the synthesis of the above products have been studied in hepatocyte cultures. The hepatocyte homogenates were incubated with GDP-[^{14}C] mannose and an increasing amount of exogenous dolichol phosphate. The higher rate of formation of these dolichol linked intermediates was observed even at saturating concentrations of dolichol phosphate in dexamethasone-treated hepatocytes. This result suggested that the increased rate of formation by dexamethasone was due to a true increase of glycosyltransferase activity. A similar finding of a true increase of enzyme activity was reported for developing sea urchin embryos (223) and in mice during estrogen treatment (224). An increase of dolichol linked glycosyltransferase activity has been reported in a number of cases during stimulation of N-linked glycoprotein synthesis. During development of rabbit skeletal muscle from neonate to the adult, when glycoprotein synthesis is reduced, there is a reduction in the level of activity of enzymes involved in the formation of Man-P-Dol and Glc-P-Dol (214), as well as in the level of a sialyltransferase activity involved in the synthesis of complex N-linked oligosaccharides (225). The formation of Man-P-Dol and $(GlcNAc)_{1-2}$-P-P-Dol was increased about 1.5 fold by dexamethasone in cell free preparations and intact hepatocytes concomitant with increased glycoprotein biosynthesis (Chapters IV, V).

Incorporation of [^{14}C] mannose into oligosaccharide

lipid was increased about 3 fold by dexamethasone. The increased incorporation of [^{14}C] mannose or [^{14}C] N-acetylglucosamine into dolichol-linked saccharides is not merely due to the increased levels of GDP-mannose, UDP-GlcNAC or alteration of the dolichol phosphate level. In this thesis, we have provided evidence that the levels of these precursors of dolichol linked saccharides are not changed by dexamethasone treatment (Chapters IV and VI). The results presented in the thesis suggest that dexamethasone probably activates some enzymatic steps for the formation of Man-P-Dol, $(GlcNAc)_{1-2}$-P-P-Dol and the elongation of sugar chains from $(GlcNAc)_{1-2}$-P-P-Dol to dolichol linked oligosaccharides.

7.3. Role of nucleotide sugars in N-linked glycoprotein synthesis.

As described in chapter I, acute-phase proteins are induced during inflammation and in dexamethasone treatment. Most of these acute-phase proteins are glycoproteins containing N-linked complex type carbohydrate chains. Complex type carbohydrate chains are synthesized through the dolichol pathway and by direct transfer of sugar from nucleotide sugars. Therefore, experiments were performed to investigate the level of nucleotide sugars in hepatocytes during enhanced glycoprotein synthesis. This may provide us with an insight into the role of the nucleotide sugar pools in the control

of dolichol-linked saccharide biosynthesis. The level of nucleotide sugars in the cell can be regulated by the alteration in their biosynthetic, or degradative enzyme activities. In this thesis we have studied the nucleotide sugar pyrophosphatase activities, which hydrolyze the GDP-mannose and UDP-GlcNAc in hepatocytes. The results have shown that dexamethasone increases both nucleotide sugar pyrophosphatase activities in cells obtained from control and inflamed rats (Chapter VI). However, there was no difference in the pyrophosphatase activity in the cells from inflamed rats compared to controls. Despite the increase of nucleotide sugar pyrophosphatase activities by dexamethasone, the levels of total cellular UDP-GlcNAc, GDP-mannose and UDP-glucose were not changed (Table 22).

Therefore, these results do not support the idea that the increased synthesis of saccharide lipid is due to changes in the level of nucleotide sugars. The synthesis of lipid linked saccharides and the transfer to protein occurs within the lumen of the endoplasmic reticulum (95,226). In contrast, the synthesis of the nucleotide sugars occurs in the cytoplasm of the cell (113,227). Therefore, the site of synthesis and the site of utilization of the nucleotide sugars for lipid linked saccharide synthesis are separated by the membrane of the endoplasmic reticulum. The exact mechanism responsible for the movement of the nucleotide sugar moieties across this membrane into the lumen of the endoplasmic reticulum is not

well understood, although recent work has indicated that some sort of transport mechanism may exist for certain nucleotide sugars (228). The membrane nucleotide sugar pyrophosphatase is probably located in the endoplasmic reticular membrane (229). Since the nucleotide sugars need to approach the surface of endoplasmic reticulum for their utilization in the lipid linked saccharide synthesis and protein glycosylation, nucleotide sugar pyrophosphatase enzymes localized there could have dramatic effects on the concentration of the nucleotide sugar within the endoplasmic reticulum. The enzyme may decrease levels of nucleotide sugars within the lumen of the endoplasmic reticulum without affecting cytoplasmic levels, and a reduction in cellular pool sizes may not be observed. Hickman et al (230) have shown increased nucleotide sugar pyrophosphatase activity coupled with a decrease in oligosaccharide lipid synthesis in plasmacytoma cells. The results presented in this thesis indicate that lipid linked saccharide synthesis is higher in dexamethasone treated cells even though nucleotide pyrophosphatases are also increased by dexamethasone. If it is true that nucleotide pyrophosphatase reduces the nucleotide sugar level at the site of synthesis of lipid-linked saccharides, the cells possibly compensate the loss due to lowering of the nucleotide sugars by triggering the increased enzyme activities in endoplasmic reticulum involved in synthesis of lipid linked saccharides. Similar increases of glycosyltransferases and

nucleotide sugar pyrophosphatases have been observed in developing sea urchin embryo (213) and in developing skeletal muscle (214), where the biosynthesis of glycoproteins are enhanced.

7.4. Role of the expression and translation of mRNAs encoding N-linked glycoproteins in glycoprotein synthesis as related to the effects of dexamethasone and inflammation on dolichol phosphate dependent glycosyltransferases.

Another important factor relating to the induction of glycosylation during enhanced acute-phase protein synthesis is the role of polypeptide acceptors. It is known from a number of studies that N-linked glycosylation is a cotranslational process that occurs in the rough endoplasmic reticulum. A number of studies have shown that formation of mRNAs and glycosylatable polypeptides are increased in the rough endoplasmic reticulum during inflammation and in dexamethasone treatment (133). These observations are supported by the fact that increased synthesis of acute phase-proteins are inhibited by actinomycin D (215,231) and puromycin (215,232). The results regarding the inhibition of synthesis of dolichol linked saccharide by actinomycin D and cycloheximide presented in this thesis indicate that glycosylatable polypeptides for acute phase proteins are essential for the formation of dolichol linked saccharides. However, limited results obtained with the effects of

actinomycin D and cycloheximide in the present work did not distinguish if the effects of dexamethasone stimulation are on the synthesis of polypeptides and/or on the glycosyltransferase enzymes.

7.5. Conclusions

In summary, the inflammatory process and the effect of dexamethasone on this process, provide a number of useful observations about the synthesis of N-linked glycoproteins in rat liver. In this organ, N-linked glycoprotein synthesis is increased during inflammation and by dexamethasone treatment. A series of prior events set the scene for the onset of glycoprotein synthesis. These include induction of dolichol kinase and synthesis of dolichol and dolichol phosphate during inflammation. The major new information in these studies suggest an induction of glycosyltransferase activities in hepatocytes particularly during dexamethasone treatment. However, no correlations were observed between the cellular levels of nucleotide sugars, activities of nucleotide sugar pyrophosphatases and induction of dolichol-linked saccharide synthesis. It also seems likely that the formation of dolichol linked saccharides is regulated by mRNAs, encoding the acute-phase proteins and the glycosylatable polypeptide formed from these mRNAs. It remains to be established, whether these processes are also accompanied by changes in the activity of the enzymes involved in the.

ultimate transfer of the fully formed oligosaccharide from dolichol pyrophosphate to the newly synthesized proteins.

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